

**PROTEOMIC INVESTIGATION OF THE HIV RECEPTORS  
CD4 AND DC-SIGN/CD209**

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**A THESIS SUBMITTED IN FULFILMENT OF THE REQUIREMENTS FOR  
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## **PREFACE**

The work presented in this thesis was performed by the author at the Centre for Virus Research, Westmead Millennium Institute, Westmead, NSW, Australia and the Department of Chemistry, University of Wollongong, Wollongong, NSW, Australia. The projects described herein were supervised by Prof. Tony Cunningham (Westmead) and Prof. Margaret Sheil (Wollongong). The work described in chapter three was also supervised by Dr Tim Hochgrebe (formerly of Westmead). The work was conducted between February 2000 and December 2003 and supported by a scholarship from the German Chemical Industry (“Chemfonds”) until February 2001. The author was then supported by an International Postgraduate Research Scholarship and an International Postgraduate Award from the Department of Education, Training and Youth Affairs and the University of Sydney respectively.

The author is entirely responsible for the work in this thesis unless otherwise indicated.

Signed \_\_\_\_\_

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## ABSTRACT

HIV infection and disease is a multistage process that involves a variety of cell types as the virus spreads through the body. Initially, dendritic cells (DCs) present at the mucosal site of infection bind and internalise HIV for degradation and presentation to T cells. As the DCs migrate to lymph nodes and mature, part of the internalised virions remains infective inside endosomal compartments. During formation of the immunological synapse between CD4 T cells and DCs, infective virions from dendritic cells are transferred to CD4 T cells leading to a strong infection of those cells allowing rapid virus dissemination throughout the body and establishment of the typical HIV infection. Various membrane receptors are involved in this process. Initial HIV binding to DCs is mediated by C-type lectin receptors such as the mannose receptor or DC-SIGN (DC specific intracellular adhesion molecule 3 grabbing non integrin) which is followed by virus internalisation and lysis albeit virus induced changes in endocytic routing prevents a proportion from degradation. Productive infection of DCs has also been observed allowing *trans* infection of CD4 T cells through a different mechanism. HIV infection of CD4 T cells, DCs and other cells is a multistep process initiated by binding of HIV envelope gp120 to the CD4 receptor, a 55 kDa transmembrane glycoprotein. Subsequent conformational changes in gp120 allow binding to a chemokine receptor, either CCR5 or CXCR4, followed by membrane fusion and infection.

The aim of this thesis was to investigate protein associations with the HIV receptors DC-SIGN and CD4 in order to elucidate the mechanism of complex formation, virus entry and/or defining target sites for antiretroviral drugs. This thesis used a proteomic approach for studying the receptors with mass spectrometry-based protein identification as its core technology. A range of different approaches were developed and compared for identification of protein interactions and characterisation of the identified protein associations. An affinity purification of the CD4 receptor complex from lymphoid cells was used as the basis for detecting novel CD4-binding proteins. For this approach a strategy based on mass spectrometry identification of CD4 associating proteins using affinity chromatography and affinity-tag mediated purification of tryptic peptides was developed. This method proved successful for the identification of CD4 interacting

proteins such as the strongly associated kinase p56lck, however a limited number of non-specifically bound proteins were also identified along the receptor complex. Using one-dimensional SDS-polyacrylamide gel electrophoresis followed by in-gel digests and mass spectrometry analysis, a large number of non-specifically binding proteins were identified along the CD4/lck complex. Evaluation of different lysis buffers in several independent experiments demonstrated that there was a large and inconsistent array of proteins that were obviously non-specifically bound to the receptor. No further specific binding partners were detected. These data suggested that protein interactions of CD4 on this cell type are of weak and/or transient nature. It also demonstrated a need for careful interpretation of proteomic data in the light of the propensity of non-specific binding under these conditions.

To overcome dissociation of weak protein interactions, a method was developed using chemical cross-linking to preserve weak protein interactions on lymphoid cells. Affinity purification was used to purify CD4 along with cross-linked associated proteins and mass spectrometry analysis identified an interaction with the transferrin receptor CD71 and the tyrosine phosphatase CD45. The CD45-CD4 interaction is well known. The CD4-CD71 interaction was demonstrated to be a result from colocalization of the two molecules during formation of endocytic vesicles. Flow cytometry-based fluorescence resonance energy transfer (FRET) measurements were applied to confirm colocalization. A similar interaction was suspected for CD4 and DC-SIGN on the plasma membrane of DCs as *cis* infection of DCs has been demonstrated i.e. initial binding to DC-SIGN then to CD4/CCR5 on the same cell. Therefore, protein associations of DC-SIGN were investigated using the developed techniques. Using cross-linking, DC-SIGN was shown to assemble in large complexes on the surface of immature monocyte-derived DCs. Mass spectrometry analysis of the purified complexes identified them as homooligomers of DC-SIGN. The absence of CD4 suggested that the fraction interacting with CD4 at any one time must be small. The complexes of DC-SIGN were further characterised to be tetramers and successfully co-immunoprecipitated with HIV gp120 and mannan. DC-SIGN monomers were not evident demonstrating that the assembly of DC-SIGN into tetramers is required for high affinity binding of its natural and viral

ligands. Thus potential antiviral agents aimed at blocking the early stage of HIV binding to DCs must simulate tetramers in order to neutralise the virus efficiently.

Overall the thesis provides new information on protein interactions of CD4 and DC-SIGN, a careful investigation of “proteomics” techniques for identifying the proteins in affinity-purified samples and demonstrates the need for multifaceted analytical approaches to probe complex cellular systems.

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## **LIST OF PUBLICATIONS RESULTING FROM THIS WORK**

### **CHAPTER 3:**

Bernhard, O. K., Burgess, J. A., Hochgrebe, T., Sheil, M. M. and Cunningham, A. L. (2003): Mass spectrometry analysis of CD4-associating proteins using affinity chromatography and affinity-tag mediated purification of tryptic peptides. *Proteomics*, **3**, 139-146.

### **CHAPTER 4:**

Bernhard, O. K., Sheil, M. M. and Cunningham, A. L.: Mass spectrometry analysis of proteins copurifying with the CD4/lck complex using 1D PAGE-MS: Comparison with affinity-tag based protein detection and evaluation of different solubilisation methods. *J. Am. Soc. Mass Spectr.*, *in press*.

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### **CHAPTER 6:**

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## ABBREVIATIONS

AIDS	acquired immunodeficiency syndrome
AP	adaptor protein
APC	antigen presenting cell
BIAM	<i>N</i> -(biotinoyl)- <i>N'</i> -(iodoacetyl)-ethylenediamine
BSA	bovine serum albumin
CCR5	“CC”-type chemokine receptor 5
CD	circular dichroism
CDK	cyclin dependent kinase
cDNA	copy DNA
CHO	chinese hamster ovary
CID	collision induced-decay
CLB	cross-link buffer
CLR	calcium dependent lectin receptor
CMV	cytomegalovirus
CNS	central nervous system
CTL	cytolytic T-lymphocyte
CXCR4	“CXC”-type chemokine receptor 4
DC	dendritic cell
DC-SIGN	dendritic cell specific ICAM3-grabbing non-integrin
DIG	detergent insoluble glycolipid-enriched complexes
DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid
DOC	sodium deoxycholate
DSP	dithiobis(succinimidylpropionate)
DSS	disuccinimidylsuberate
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetate
EGTA	ethylene glycol- <i>O</i> ,- <i>O'</i> -bis(2-amino-ethyl)- <i>N</i> , <i>N</i> , <i>N'</i> , <i>N'</i> , -tetraacetic acid

ER	endoplasmatic reticulum
ESI	electrospray ionisation
FACS	fluorescence activated cell sorting
FITC	fluorescein isothiocyanate
FRET	fluorescence resonance energy transfer
gp	glycoprotein
GPI	glycosylphosphatidylinositol
GTP	guanosine triphosphate
HAART	highly active antiretroviral therapy
HBS	hepes-buffered saline
HIV	human immunodeficiency virus
HLA	human leukocyte antigen
HPLC	high performance liquid chromatography
HRP	horseradish peroxidase
HSPG	heparan sulfate proteoglycans
ICAM	intracellular adhesion molecule
ICAT	isotope-coded affinity tag
Ig	immunoglobulin
IgSF	immunoglobulin superfamily
IL	interleukine
ITAM	immune receptor tyrosine-based activation motifs
kDa	kilo Dalton
LAT	linker for activation in T cells
LC	liquid chromatography
LTR	long terminal repeat
MA	matrix, HIV structural protein
MALDI	matrix-assisted laser desorption ionisation
MAPK	mitogen associated protein kinase
MBP	mannose-binding protein
MCP	multichannel plate (ion detector)
MDDC	monocyte-derived dendritic cell

MHC	major histocompatibility complex
MR	mannose receptor
mRNA	messenger RNA
MS	mass spectrometry
MS/MS	tandem mass spectrometry
MW	molecular weight
NK	natural killer
NMR	nuclear magnetic resonance
PAGE	polyacrylamide gel electrophoresis
PBMC	peripheral blood mononuclear cell
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PEG	polyethyleneglycol
PI-3K	phosphatidylinositol-3 kinase
PIC	preintegration complex
PKC	protein kinase C
PLC	phospholipase C
PMA	phorbol 12-myristate 13-acetate
PMSF	phenylmethylsulfonylfluoride
PR	protease, HIV enzyme
RF-10	RPMI cell culture medium with 2.05 mM L-glutamine and 10 % fetal calf serum
RNA	ribonucleic acid
RNase	ribonuclease
RP	reverse phase
RT	reverse transcriptase, HIV enzyme
SBTI	soybean trypsin inhibitor
SDS	sodiumdodecyl sulfate
SH2/3	src homology domain 2/3
SIV	simian immunodeficiency virus
TAR	<i>tat</i> responsive element

TBS	tris buffered saline
TCEP	tris(2-carboxyethyl)phosphine
TCR	T cell receptor
TFR	transferrin receptor
TGN	trans golgi network
TOF	time-of-flight
tRNA	transfer RNA
V <sub>H</sub>	immunoglobulin variable region heavy
V <sub>L</sub>	immunoglobulin variable region light
ZAP	zeta associated protein

## AMINO ACID ABBREVIATIONS AND RESIDUE MASS

<b>Amino acid</b>	<b>Single Letter code</b>	<b>Three letter code</b>	<b>Residue average mass</b>	<b>Residue monoisotopic mass</b>
Glycine	G	Gly	57.052	57.021
Alanine	A	Ala	71.079	71.037
Serine	S	Ser	87.078	87.032
Proline	P	Pro	97.117	97.053
Valine	V	Val	99.133	99.068
Threonine	T	Thr	101.105	101.048
Cysteine	C	Cys	103.145	103.009
Isoleucine	I	Ile	113.159	113.084
Leucine	L	Leu	113.159	113.084
Asparagine	N	Asn	114.104	114.043
Aspartic acid	D	Asp	115.089	115.027
Glutamine	Q	Gln	128.131	128.059
Lysine	K	Lys	128.174	128.095
Glutamic acid	E	Glu	129.116	129.043
Methionine	M	Met	131.199	131.040
Histidine	H	His	137.141	137.059
Phenylalanine	F	Phe	147.177	147.068
Arginine	R	Arg	156.188	156.101
Tyrosine	Y	Tyr	163.176	163.063
Tryptophan	W	Trp	186.213	186.079

# **CHAPTER ONE:**

## **LITERATURE REVIEW**

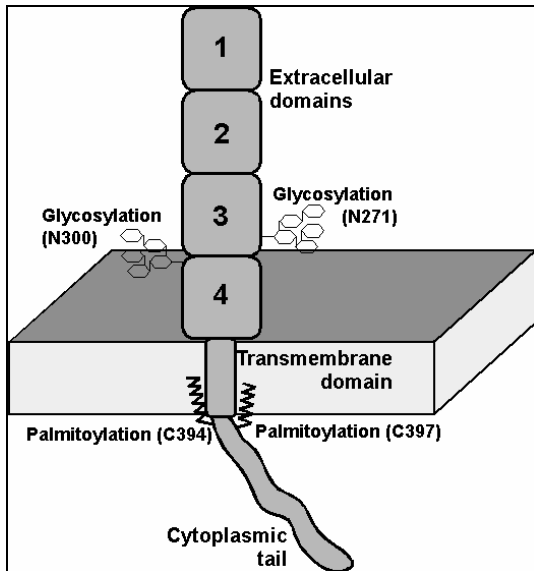
## 1.1 The CD4 receptor – structure and function

The CD4 molecule plays an important role in the immune response and also serves as a receptor for the human immunodeficiency virus (HIV). The structure and associations of CD4 are of crucial importance for the understanding of both CD4 functions and HIV entry. This review is aimed to give an overview on CD4 structure, function and its protein associations that have been reported so far.

### 1.1.1 The CD4 molecule

The CD4 protein is a 55 kDa transmembrane glycoprotein that belongs to the immunoglobulin superfamily (IgSF) of cell surface molecules. It is expressed on T helper lymphocytes, monocytes, macrophages, dendritic cells and specialised cells of the central nervous system (CNS) (Wood et al. 1983; Tourvieille et al. 1986). The 435 amino acid long CD4 polypeptide comprises of a 371 amino acid long extracellular region, a 26 amino acid long transmembrane domain and a 38 amino acid long cytoplasmic tail (Maddon et al. 1987). The extracellular part is folded into four IgSF like domains named D1 to D4 (Maddon et al. 1985).

The domains, one, two and four, each contain an intramolecular disulfide bond between C16 and C84 in domain one, C130 and C159 in domain two and C303 and C345 in domain four (Bour and Strebel, 1995). The molecule contains two N-linked glycosylation sites at N271 and N300 (Carr et al. 1989) and two palmitoylation sites on C394 and C397 (Crise and Rose, 1992). The cytoplasmic tail is tightly associated with the tyrosine kinase p56<sup>lck</sup> through a CQC motif (Veillette et al. 1988). Figure 1.1 schematically illustrates the structure of CD4 and the location of its protein modifications.



**Figure 1.1:**

Schematic illustration of CD4. Shown is the extracellular part consisting of four immunoglobulin superfamily like domains, the transmembrane domain and the cytoplasmic tail. N-linked glycosylation sites and palmitoylation sites are indicated.

### 1.1.2 Crystal structure of the CD4 molecule

Initial studies investigating the crystal structure of the whole extracellular fragment of CD4 were hindered by the low diffraction owing to the large size of the unit cells in crystals of those fragments (Brady et al. 1993). Nevertheless, Kwong et al. (1990) deduced some structural information from the obtained crystals (Kwong et al. 1990). They reported that CD4 was an elongated flexible molecule that crystallises as a dimer or as a tetramer. Proteolytic cleavage showed a flexible hinge between the D1/D2 and D3/D4 fragments.

#### 1.1.2.1 Structure of the extracellular D1/D2 fragment

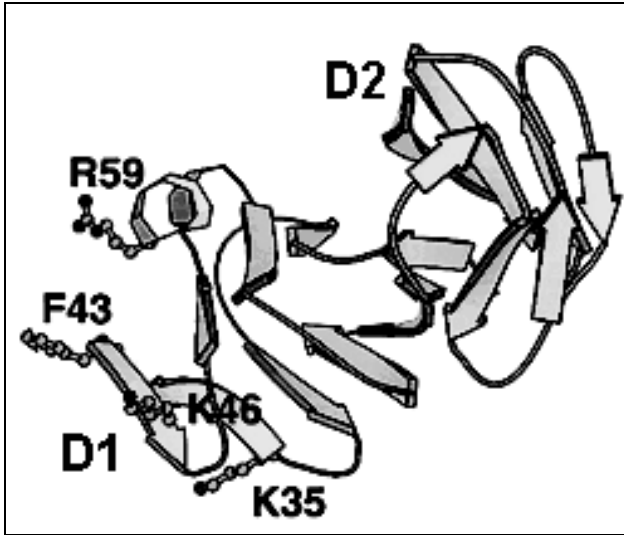
In 1990, two independent publications in Nature reported the resolution of the X-ray crystal structure from a fragment comprising of domains D1 and D2 of the extracellular part of CD4 (Ryu et al. 1990; Wang, J. H. et al. 1990). Wang and co-workers studied recombinant CD4 D1/D2 fragments produced in CHO cells. The overall structure of this fragment was described as rod-like, consisting of two adjacent domains each of which has an IgSF-like fold. Whereas D1 is homologous to the IgSF variable domains, D2 shows similarity to the constant domains. The entire fragment is characterised by little flexibility between the domains D1 and D2 with the last  $\beta$ -strand of D1 running directly into the first strand of D2. Figure 1.2 is an illustration of the CD4 domain D1/D2

fragment from a crystal structure analysis performed in the context of its function in T cell activation.

IgSF-like molecules have a structure consisting of two antiparallel  $\beta$ -sheets made up from nine strands labelled ABCC'C''DEFG. ABDE form one sheet and CC'C''FG the other sheet. According to the crystal structure analysis the CD4 domain D1 is similarly built except that one sheet is made up of strands BDE and the other of strands ACC'C''FG (Wang, J. H. et al. 1990). Despite the overall similarity between CD4 domain D1 and IgSF variable domain structure, several differences can be distinguished. These include that the CC' loop is shortened by 4 residues and the FG loop is shortened by 4 to 6 residues. In immunoglobulin molecules those loops mediate the association between the V<sub>H</sub> and V<sub>L</sub> chain. CD4 is not involved in such an association explaining the reduction of the CC' and the FG loop (Ryu et al. 1990). The C'C'' loop, however, is 3 residues longer than in normal IgSF molecules.

Domain D2 contains only seven  $\beta$ -strands and also forms an IgSF like fold with one sheet consisting of strands ABD and the other of CC'FG. Although the immunoglobulin-typical hydrophobic core is preserved in domain D2, the overall size of only 75 amino acids (compared to 100 for an IgSF constant domain) is relatively small. The disulfide bridge is also differentially located bridging two cysteine residues on one single  $\beta$ -strand rather than connecting two  $\beta$ -strands from the two different  $\beta$ -sheets as in other IgSF-like molecules (Ryu et al. 1990). The detailed structure of Domain D1 is also characterised by a typical IgSF hydrophobic core made up of the disulfide bridge and several hydrophobic residues. A salt bridge between an arginine and an aspartic acid further enhances the fold. Domain D2 contains a modification in the salt bridge (Wang, J. H. et al. 1990).

A refined X-ray diffraction analysis of a D1/D2 fragment later confirmed that domains D1/D2 form a rather rigid unit with flexibility left at the D2/D3 interface (Ryu et al. 1994). The data presented are consistent with the earlier reports, describing the folding pattern of both domains as IgSF-like. The residues forming the hydrophobic core of IgSF domains, one disulfide bridge, a tryptophan and a tyrosine are present in D1 and modified in D2. A prominent feature is the phenylalanine at position 47, that is highly solvent exposed, which is rather unusual for such a hydrophobic residue.



**Figure 1.2:**

The structure of the extracellular D1D2 fragment of CD4:

The two domains D1 and D2 can be clearly distinguished with D1 made up of 9 and D2 of 7 beta strands. Some residues critical for interactions are also shown.

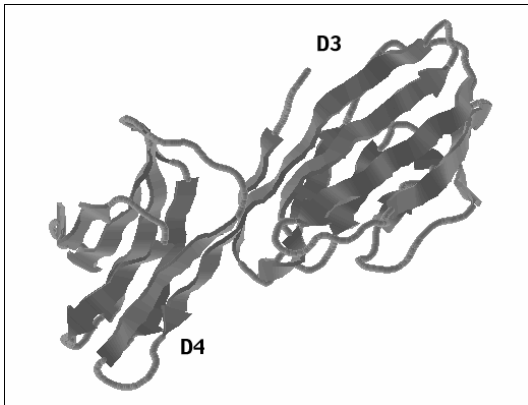
Taken from Wang et al. (2001).

#### 1.1.2.2 Structure of the extracellular D3/D4 fragment.

Whereas the D3/D4 fragment of the extracellular part of human CD4 has not yet been crystallised, 1993 the resolution of the crystal structure of recombinant rat CD4 D3/D4 was reported (Brady et al. 1993). This is representative for the human D3/D4 fragment as rat CD4 shares a significant homology to human CD4). Analogous to the structure of the D1/D2 fragment, the D3/D4 fragment also forms a compact rod shaped unit and D3 and D4 both form IgSF-like domains (Brady et al. 1993). This supports the initial description of the structure of CD4 as a linear array of four IgSF like domains (Clark et al. 1987). Figure 1.3 shows the crystal structure of the D3/D4 fragment as described by Brady and co-workers.

The same report demonstrated that the domain D3 is similar to domain D1 (and other IgSF variable domains), comprising nine  $\beta$ -Strands forming two antiparallel  $\beta$ -sheets. However, the segments involved in the gp120 binding site (discussed later) in domain D1 have a different conformation in D3 and the disulfide bridge is not present in D3. Together with the increased presence of bulky leucine residues, this leads to an increased separation of the two  $\beta$ -sheets in D3 compared with domain D1 (Brady et al. 1993). These alterations are not uncommon for IgSF-like domains. CD4 domain D2 is also characterised by an uncommon disulfide bridge (Ryu et al. 1990) and an IgSF like domain of CD2 that also lacks a disulfide bridge is not characterised by an expanded structure (Driscoll et al. 1991).

Similar to domain D2, D4 only has seven  $\beta$ -strands that make up the IgSF like structure. Both domains can be superimposed easily even though there are differences in the structure between D2 and D4. Both key hydrophobic residues and the location of the disulfide bridge are altered and the structure is characterised by the two prominent loops, CD and FG, that protrude to the side (Brady et al. 1993).



**Figure 1.3:**

The crystal structure of the D3D4 fragment of rat CD4. The homology to the structure of the human D1D2 fragment is obvious.

As in Figure 2, D3 consists of nine  $\beta$ -strands that make up two  $\beta$ -sheets whereas D4 consists of only seven  $\beta$ -strands which also align to form two  $\beta$ -sheets.

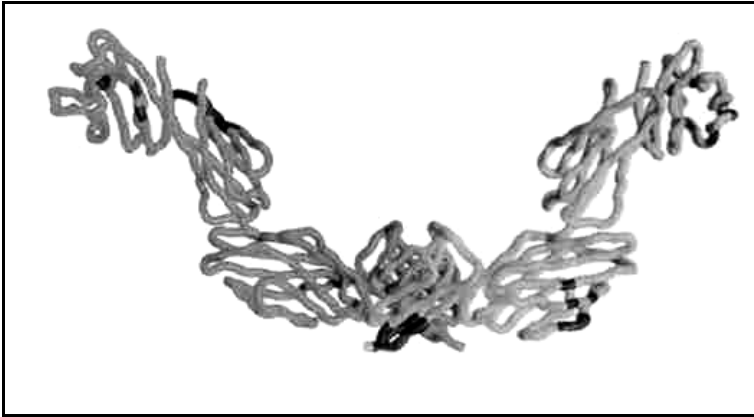
Taken from Brady et al. (1993).

### 1.1.2.3 Crystal structure of the whole extracellular region

The attempts to define the structure of the whole extracellular region of CD4 finally lead to the successful determination of the crystal structure of soluble CD4 (Wu, H. et al. 1997). The crystal structure confirms the previous findings that each the D1/D2 domains and the D3/D4 domains form an entity that is connected through a flexible hinge at the residues L177 and A178. The transmembrane proximal region of amino acids 364 to 369 was also shown to be unordered conferring more mobility to the molecule on the cell surface (Wu, H. et al. 1997).

Besides defining the crystal structure of CD4, the authors also observed formation of CD4 dimers that are butterfly shaped with Domains D1 to D3 as “the wings” and D4 as “the torso”. The interaction between the two monomers is reportedly weak with a  $K_D$  of about 1 mM but could be confirmed in experiments with soluble CD4 (Wu, H. et al. 1997). The dimer interface is exclusively located at domain D4 with rather small surface areas involved. Neither the glycosylation sites, nor the protruding loops CC’ and FG, interfere with the dimerisation. Absolutely conserved glutamine residues at position 344, which are separated by a hydrogen bond distance are at the core of the dimerisation

interface (Wu, H. et al. 1997), albeit the exact distance of the glutamine residues is not reported. Figure 1.4 shows the crystal structure and dimerisation of CD4 at domain D4.



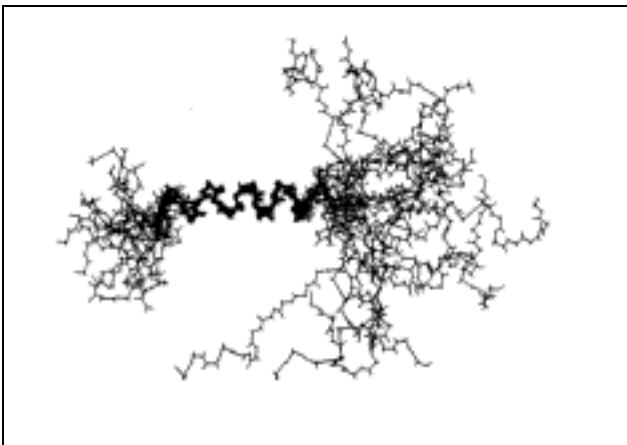
**Figure 1.4:**  
Crystal structure of the extracellular part of CD4. Front view showing the butterfly shape of two CD4 molecules forming a dimer. The two D4 domains are superimposed in this view. Taken from Wu et al. (1997).

Apart from the dimerisation indicated from the CD4 crystal structure (Wu, H. et al. 1997), disulfide bridged CD4 dimers have also been observed (Lynch et al. 1999). The role of CD4 dimerisation has been controversial and it has been reported to be required for binding of MHC class II molecules (Sakihama et al. 1995; Li et al. 1998) but not for binding of HIV gp120 (Sakihama et al. 1995). Recent studies have shown that the domain D2 disulfide bond is redox active and thus free thiols can be detected on the extracellular part of CD4 (Matthias et al. 2002). Those free thiols may be involved in the formation of disulfide bridged dimers (Lynch et al. 1999). These authors also reported evidence that disulfide exchange involving the domain D2 cysteines is required for HIV-1 entry (Matthias et al. 2002), but no further data are currently available supporting this, neither does the report on the crystal structure of the whole extracellular region of CD4 characterise the CD4 D2 disulfide bridge.

#### **1.1.2.4 Structure of the cytoplasmic tail**

With the main focus regarding the CD4 structure being on the extracellular part, relatively little attention has been paid to the cytoplasmic domain. Though the intracellular part of CD4 is rather small, it is absolutely required for function, for example, it mediates the association with the protein kinase lck (Veillette et al. 1988). Wray et al. (1998) performed NMR and CD spectroscopic investigations with a synthetic peptide comprising the whole intracellular domain of CD4. Chemical shift data on the

C<sub>α</sub>-hydrogen atoms indicate an  $\alpha$ -helical structure between residues 399 to 419 with the centre of the helix stretching from Q403 to S415. This helix is flanked by a short mobile linker adjacent to the transmembrane region and the unstructured C-terminal part starting with the CQC motif required for interaction with Ick. Figure 1.5 illustrates the structure of the cytoplasmic tail. The five cysteine residues of the cytoplasmic tail are not involved in disulfide bonds but are susceptible to oxidation. Thus the experiments had to be carried out in an oxygen-free environment and in presence of a reducing agent, (i.e. DTT). It was also shown that the cytoplasmic tail has an intrinsic tendency to associate with membranes. About 70% of the PKC phosphorylated peptides were shown to be associated with microsomal membranes which demonstrates that the CD4 intracellular domain has some common features of membrane-associated proteins (Wray et al. 1998).



**Figure 1.5:** Structure of the CD4 cytoplasmic tail. Superimposition of the 20 best final restrained structures of the whole cytoplasmic peptide of CD4. The proposed  $\alpha$ -Helical structure can be clearly distinguished whereas the adjoining regions at the N-terminus and C-terminus are highly unstructured. Taken from Wray et al. (1998).

### 1.1.3 CD4 trafficking

The CD4 gene is made up of nine exons. The first exon codes for the leader sequence, followed by five exons coding for the extracellular part, one exon coding for the transmembrane domain and two exons encoding the cytoplasmic tail (Maddon et al. 1987). During the synthesis, the leader sequence directs the nascent polypeptide into the endoplasmatic reticulum where the leader is cleaved away. CD4 is then transported rapidly and efficiently from the ER through the Golgi apparatus (where it is glycosylated) and then to the cell surface (Crise et al. 1990).

### 1.1.3.1 CD4 endocytosis

Cell surface proteins are normally internalised through endocytosis, which allows degradation of the protein (Pearse and Robinson, 1990) or mediates dissociation of ligands bound to a membrane protein (Gruenberg and Maxfield, 1995). Endocytosis of a receptor can occur passively via “bulk flow” (e.g. macrophages are known to internalise 200 % of their surface area every hour (Mellman 1996; Steinman et al. 1983)) or actively if the protein carries an endocytosis signal on its cytoplasmic tail. Signal sequences for endocytosis that have been described are tyrosine-based motifs eg in the mannose receptor (MR) (East and Isacke, 2002) or dihydrophobic sequences e.g. in CD4 or DC-SIGN (Pitcher et al. 1999; Engering et al. 2002). They function via binding of the clathrin adaptor proteins AP-1 and AP-2, which then target the receptor for endocytosis (Mellman 1996; Rodionov and Bakke, 1998). After internalisation, the proteins first reside in vesicular compartments named early endosomes. The early endosomes form part of a complicated vesicular network responsible for endocytosis, recycling and degradation of membrane proteins (Mellman 1996). From early endosomes, proteins can be targeted back to recycling vesicles that mediate their transport back to the cell surface. Some proteins carry a signal on their cytoplasmic tail that promotes the return to the cell surface via the recycling pathway. For example, the mannose receptor possesses a diaromatic motif (Schweizer et al. 2000). If not recycled, receptor molecules are targeted from early endosomes to late endosomes and then lysosomes. The lysosomes are characterised by a low pH and acid proteolytic activity. In this acidic environment, ligands can be dissociated from their receptor as, for example, transferrin dissociates from the transferrin receptor (TFR) (Baker and Morgan, 1994). The ligands then can be degraded and their cargo utilised and the receptor return to the cell surface. Alternatively, the receptor can also be degraded. The signals which govern sorting towards the lytic pathway are not clear, however it is thought that a triacidic motif found in some receptors (Dendritic cell specific intracellular adhesion molecule 3 grabbing non-integrin, DC-SIGN/CD209) promotes sorting to more acidic vesicles (Engering et al. 2002).

CD4 contains a dileucine motif that mediates its endocytosis via clathrin-coated pits (Pitcher et al. 1999), however, the mechanism of CD4 endocytosis is more complicated and dependent on other proteins as well. Studies on the endocytosis of cell

surface CD4 have shown that CD4 transfected into non-lymphoid cells such as HeLa cells is rapidly internalised and subsequently recycled to the cell surface. About 30 % to 40 % of the total CD4 was internalised at any time (Pelchen-Matthews et al. 1989). However, a fundamental difference between the CD4 internalisation on lymphocytic and non-lymphocytic cells was observed later (Pelchen-Matthews et al. 1991). Whereas CD4 endocytosis through coated pits in non-lymphoid cells such as the promyelocytic HL-60 cell line resembled the dynamics observed in HeLa cells, the lymphocytic cells showed little internalisation of CD4. The authors suggested a role for the lymphocyte specific kinase p56lck ie cell surface retention could be mediated through lck binding to the CD4 cytoplasmic tail (Pelchen-Matthews et al. 1991). This suggestion was confirmed one year later when the same research group reported that p56lck inhibits CD4 internalisation by preventing its entry into coated pits. The expression of an intact cytoplasmic tail of CD4 was reported necessary for endocytosis and this was not prevented when a chimera of lck is expressed that lacks the CD4 binding domain (Pelchen-Matthews et al. 1992). The differential internalisation of CD4 in lymphocytic and myelocytic cell lines could be confirmed for primary lymphoid and myeloid cells. P56lck-positive primary lymphocytes showed little endocytosis whereas monocytes and macrophages rapidly internalised CD4 (Pelchen-Matthews et al. 1998).

### **1.1.3.2 The effect of PMA on CD4 endocytosis**

Phorbol esters such as Phorbol 12-myristate 13-acetate (PMA) are known to activate cellular mechanisms associated with cell growth through their ability to activate protein kinase C (Smith, A. D. 2000). Stimulation of lymphocytes with PMA has been shown to lead to the downregulation of cell surface CD4 (Acres et al. 1986), phosphorylation of serine residues in the CD4 cytoplasmic tail (Acres et al. 1986) and dissociation of CD4-p56lck (Hurley et al. 1989). The phorbol ester-induced downregulation of CD4 was later described to be a multistep process, which is attributed to several effects of PMA (Pelchen-Matthews et al. 1993). In lymphocytes, PMA leads to the phosphorylation by protein kinase C of the key serine residues S408 and S415 flanking the dileucine motif mediating endocytosis. This was proposed to lead to the dissociation of p56lck (Pelchen-Matthews et al. 1993) and enhanced the affinity of CD4 for the clathrin adaptor proteins

AP-1 and AP-2 up to 700 fold (Pitcher et al. 1999). PMA also directs the sorting of endocytosed receptor molecules towards late endosomal compartments instead of the recycling pathway (Pelchen-Matthews et al. 1993).

#### **1.1.4 CD4 associating molecules**

Before proceeding to the discussion of the various functions which the CD4 molecule plays in immunology, it is necessary to know and understand the different molecules that can associate with CD4. The following part of this review will discuss the molecules that naturally associate with CD4 in lymphoid cells starting with the molecules associating with the CD4 cytoplasmic tail, then the extracellular ligands MHC class II molecules and the soluble ligands IL-16 and gp17. It will finish with an overview of the molecules associating with CD4 in non-lymphoid cells. The HIV proteins gp120, vpu and nef, which can also associate with CD4, are discussed later in this review, in the section on the human immunodeficiency virus (sections 1.2.3.2 and 1.2.5.3).

##### **1.1.4.1 Proto-oncogene protein tyrosine kinase lck (p56lck)**

The expression of p56lck was first reported in 1986 in the human T-cell leukemia Jurkat cell line and the gene was described as homologous but distinct from the src, yes and fgr oncogenes (Trevillyan et al. 1986). Similar cDNA could not be isolated from B cell lymphoma lines or myeloid cell lines, showing the expression of the protein was T cell specific (Trevillyan et al. 1986). The homology of 70 % to 80 % between a putative protein tyrosine kinase catalytic domain of the deduced protein sequence and sequences of catalytic domains of other protein kinases suggested kinase activity of the protein (Koga et al. 1986). Later the association of lck with the cytoplasmic tail of the CD4 and CD8 molecules in T-lymphocytes was described (Veillette et al. 1988). The gene organisation reflects the homology of lck with other protein kinases of the src family. Except for the unique N-terminal sequence, the organisation of the exons is the same as in other protein tyrosine kinases of the src family (Rouer et al. 1989).

Protein tyrosine kinases of the src family were first discovered in the “rous sarcoma virus” and comprise of a family of tyrosine kinases that are characterised by not only a protein kinase domain but also a src homology 2 domain (SH 2) and src homology

3 domain (SH 3) following a unique N-terminal domain. Prominent members apart from lck are src, yes, fyn, lyn, blk, hck, fgr and yrk (Smith, A. D. 2000). The SH 2 domain binds phosphorylated tyrosine residues in peptides/proteins and also mediates autoinactivation of the kinase (Smith A. D. 2000). The SH 3 domain mediates protein-protein interactions with specific protein sequences containing hydrophobic and proline residues (Smith, A. D. 2000). In lck, as in most other src-family kinases, the unique N-terminal domain is followed by the SH 3 and then SH 2 homology domain. The protein kinase domain is located at the C-terminus followed by a short regulatory domain (Eck et al. 1994). A residue important in lck function is Y394, the phosphorylation of which is required for activation of kinase function (Veillette and Farnel, 1990). Lck can phosphorylate its own Y394 residue leading to lck autoactivation (Caron et al. 1992). It also possesses an inhibitory residue, Y505. Phosphorylation of this residue leads to a change in lck structure via binding of the Y505 containing regulatory stretch to the SH2 homology domain. This structural change inactivates the kinase domain (Yamaguchi and Hendrickson, 1996). The phosphorylation states of both Y394 and Y505 thus control lck kinase activity.

Crystal structure analyses on individual parts of lck have been reported, focussing on the SH2 homology domain, which binds to tyrosine, phosphorylated molecules. The resolution of the crystal structure of the lck SH 2 homology domain complexed with an 11 residue phosphopeptide revealed involvement of R134 in the recognition of the phosphotyrosine. On the phosphopeptide, the isoleucine residue at the +3 position with respect to the phosphotyrosine (Tong et al. 1996) was also shown to be involved in the binding. Lck possesses an intrinsic ability to localise with the plasma membrane, which is mediated through its unique N-terminal domain (Bijlmakers et al. 1997).

### **Association between CD4 and lck and the cytoskeleton**

The connection between CD4 and lck was first discovered via co-immunoprecipitation of both molecules from a lymphoid CD4<sup>+</sup>CD8<sup>-</sup> cell line (Veillette et al. 1988). The association was soon mapped to the CD4 cytoplasmic tail where the CQC motif was shown to be critical for the interaction (Shaw et al. 1990; Turner et al. 1990). The interaction is mediated via a Zn<sup>2+</sup> ion between the two “CQC”-cysteines of CD4 and

another pair of cysteines in the amino terminal domain of lck (Huse et al. 1998). Studies using lymphoid cell lines as well as fibroblasts transfected with lck have shown association of lck with the detergent insoluble matrix (Louie et al. 1988). Further studies observed that lck expression can also render CD4 resistant to detergent solubilisation and that lck can mediate reorganisation of the cell surface into structures resembling adhesion junctions upon CD4 activation (Kinch et al. 1994). The authors conclude that there is a linkage between lck and the cytoskeleton that is involved in the modelling of cell adhesion but the nature of which remains elusive. It was reported that the cytoskeletal protein ezrin is phosphorylated upon stimulation of T lymphocytes with CD3 antigen in a manner suggesting the involvement of lck (Thuillier et al. 1994). Further evidence arised from colocalization studies between CD4 and ezrin (T. Hope, unpublished data). Analysis of the partitioning of CD4 and lck between the detergent-soluble and insoluble fractions (containing the cytoskeleton) after CD4 cross-linking with antibodies was shown to increase insolubility of those two proteins in nonionic detergent, which was interpreted as an association with the cytoskeleton (Ha-Lee et al. 2000). Another study investigated location and movement of CD4 on lymphoid and myeloid cells. The authors reported that lck binds CD4 and targets it to microvilli independent of kinase activity. They also observed reduced mobility of CD4 on the cell surface and interpreted it as an association of CD4 with the cytoskeleton (Foti et al. 2002). However, those observations have to be interpreted in the context of lipid rafts (discussed later in section 1.1.6), which can also render proteins insoluble in non-ionic detergents. Taken together, those findings suggest that there is a possible connection between CD4/lck and the cytoskeleton which might be mediated via adaptor proteins or via phosphorylation of proteins through lck which then bind to either CD4 or lck (Kinch et al. 1994). Direct evidence for binding of CD4 and/or lck to a cytoskeletal protein has yet to be reported. However, the recent finding that  $\beta$ -adducin, an actin filament associated protein, can interact with the SH2-homology domain of the src kinase fyn after phosphorylation suggests the possibility of such a link (Shima et al. 2001).

#### **1.1.4.2 Other proteins associating with the CD4 cytoplasmic tail**

The following proteins have been described to associate with CD4 but will only be discussed briefly as either the association is controversial or has not been confirmed by independent experiments.

In 2001 the identification of a novel intracellular CD4 binding protein was reported and named ACP33 (Zeitlmann et al. 2001). The protein was identified in an attempt to screen for interactors of the cytoplasmic tail using the yeast two-hybrid system. The interaction was confirmed with co-immunoprecipitation and could be mapped to the hydrophobic C-terminal part of the cytoplasmic tail. The authors suggested a role of this molecule in the transduction of “negative signals” through the CD4 receptor in contrast to the activating signals transmitted by p56lck (Zeitlmann et al. 2001).

The linker for activation of T cells (LAT) is a protein involved in the signalling pathway triggered upon TCR stimulation (Zhang, W. et al. 1998a). In 1999, Bosselut and co-workers reported that it can associate with CD4 providing a mechanism through which it is recruited to the TCR signalling machinery. The association was detected via immunoprecipitations and was said to be dependent on the same cysteine motif required for the CD4-lck association even though the interaction mechanism is different (Bosselut et al. 1999). The authors suggest that LAT-1 phosphorylation participates in the signal transduction event during T cell activation (Bosselut et al. 1999).

The association of the CD4/lck complex with both a GTP binding protein p32 and a raf related p110 polypeptide was reported in 1992 (Prasad and Rudd, 1992). The p110 protein bound preferentially to the assembled CD4/lck complex and was shown to be serine-phosphorylated upon treatment of cells with phorbol esters (Prasad and Rudd, 1992). The protein was thought to act as a bridge between the CD4/lck receptor complex and serine/threonine kinase pathways during T cell activation (Prasad and Rudd, 1992).

#### **1.1.4.3 Laterally associated membrane proteins**

Being a membrane protein, CD4 can not only bind intracellular and extracellular proteins, but also engage in lateral associations with other membrane proteins. Proteins residing in proximity of CD4 can highly influence its function and the two lateral associations, the T cell receptor (TCR) and CD45 will be discussed here.

### **CD45 and its association with the CD4 receptor complex:**

CD45 is a transmembrane glycoprotein of a mass ranging from 180 kDa to 220 kDa (Trowbridge and Thomas, 1994; Thomas 1995). Alternative splicing of exons 4, 5 and 6 creates up to 8 different isoforms of the CD45 molecule depending on whether those exons are included or not. The shortest isoform, termed CD45R0, consists of 1143 amino acids whereas the longest isoform, CD45RABC, is 1304 amino acids long (Streuli et al. 1987; Hall et al. 1988). CD45 is expressed on all nucleated haematopoietic cells (Trowbridge and Thomas, 1994; Thomas 1995) and constitutes 10 % of all cell surface glycoproteins on T cells (Veillette et al. 1999). Of the 8 different isoforms of CD45, five are expressed at significant levels in T cells (Rogers et al. 1992). All T cells express more than one CD45 isoform and the expression of the different isoforms is tightly regulated during T cell development and activation (Alexander 1997). Though CD45 is expressed at very high levels on the cell surface, it is not incorporated into HIV virions (Esser et al., 2001).

CD45 is thought to exert its function through its tyrosine phosphatase activity, targeting the CD4 associated kinase lck (Koretzky et al. 1990). To exert its effect on lck, CD45 needs to interact with the CD4/lck complex. Investigating noncovalent associations of cell surface molecules using a mild detergent (Brij-58), two large complexes of membrane proteins one of which contained CD45 along with the TCR proteins and CD4 were found (Cerny et al. 1996). A different study, using co-capping and fluorescent microscopy reported high association of CD4 with CD45RC, CD3 and other molecules but only weak association with CD45RB and no association with CD45R0 and CD45RA (Dianzani et al. 1995). However, a report in 2002 employing FRET technology showed that the CD45R0 isoform preferentially associated with CD4 (Dornan et al. 2002). The authors also provide evidence for homodimers of CD45, which have a potentially inhibitory function on T cell receptor activation. Besides the abovementioned, other reports have also demonstrated an association of CD4 and CD45 so that it is now a generally accepted concept (Leitenberg et al. 1996; Bonnard et al. 1997).

CD45 can act on lck by dephosphorylating either the activating tyrosine Y394 (Yamaguchi and Hendrickson, 1996) or the inhibitory tyrosine Y505 (Ostergaard et al. 1989). Thus it can either increase or decrease lck activity. It is now thought to maintain

lck in a partially activated stage preventing autoactivation but facilitating full activation after stimulation of the T cell with antigens (Holdorf et al. 2002).

### **The T cell receptor complex (TCR)**

Given the fact that during recognition of a MHC class II molecule carrying an antigenic peptide both CD4 and the T cell receptor make contact with the MHC molecule, it has been suggested that there is a preformed association between the TCR and CD4 before antigen recognition in order to facilitate the complex formation. This suggestion, however, has been subject to long and rather controversial research. Already during the discovery of the CD4-lck association, Vieillette and co-workers found a protein co-precipitating with the CD4-lck complex that they described as “TCR-like” but the authors failed to precipitate CD4 and/or lck together with the TCR (Veillette et al. 1988). In the following years, several publications have described an association between CD4 and some or all of the TCR components. Using fluorescence resonance energy transfer (FRET) technology, the CD3 complex was found to associate with the CD4 molecule dependent on the interaction of CD4 with lck (Mittler et al. 1989). This study, however, was conducted on T cells that were treated via CD3 receptor ligation and thus is not representative for resting T cells. In 1991, the successful co-immunoprecipitation (CoIP) of the CD3  $\epsilon$ - and  $\zeta$ -chain with the CD4/lck complex was reported (Burgess et al. 1991). The authors, however, could only identify those complexes using a T lymphoblastoid line but could not detect any CD4-TCR association in resting peripheral blood lymphocytes (Burgess et al. 1991). Similar results were obtained by Beyers et al., who conducted immunoprecipitation studies to detect associations between CD4/CD8, the TCR, the CD2 and CD5 marker as well as the kinases lck and fyn. The authors report association of all those molecules in a loose complex and explain different results obtained by other groups with the loose nature of the association and the use of different detergents (Beyers et al. 1992). A paper on CD45 and the CD45 associating protein (CD45AP) also reported successful CiP of CD4 and the TCR complex. CD4, the TCR and CD45 and CD45AP were found to be associated in a membrane complex with the TCR providing the binding of CD45 and CD45AP (Veillette et al. 1999). Using mutational analysis creating 61 different mutants of CD4, the research group of Vignali and co-workers aimed to identify

epitopes in CD4 that bind to the TCR complex. Studying T cell function in dependence of CD4 structure, the authors identified several residues on CD4, the mutation of which, reduced or abrogated the T cell response that was investigated. Given the location of those mutations on the CD4 domains D3 and D4, this part of the molecule was suggested to be involved in a CD4/TCR association (Vignali et al. 1996; Vignali and Vignali, 1999).

Recent kinetic and structural studies, however, have questioned the proposed association of CD4 and the TCR. Using BIAcore™ technology, the affinity constants for CD4/MHC class II/TCR associations were determined to be 6-8  $\mu\text{M}$  for the TCR-MHC class II association. The MHC class II-CD4 association was reported to be 200  $\mu\text{M}$  but a TCR-CD4 association could not be detected (Xiong et al. 2001). This is in accordance with the structural investigations of the MHC class II-CD4 interaction, which provides a ternary model of the MHC class II complex with CD4 and the T cell receptor. The structure of this complex describes CD4 and the TCR as rather distant from each other and makes an association of both molecules during MHC recognition unlikely (Wang, J. H. et al. 2001). However, this is contradictory to the model that CD4 functions to bring TCR in proximity to the TCR cytoplasmic domains (Konig et al. 1995). These different views of the CD4-TCR associations have yet to be reconciled, leading to definitive conclusions about the CD4-TCR interaction.

#### **1.1.4.4 Class II major histocompatibility complex**

The human major histocompatibility complex (MHC) was identified as the genes that are responsible for tissue rejection reactions in humans (Bouvier, M. 2003). The reason for this is the polymorphism among the MHC genes among humans, which lead to the recognition of graft MHC molecules as “foreign”, thus inducing an immune response. The function of MHC molecules is to present peptides derived from foreign organisms to T lymphocytes to stimulate an immune response (Abbas et al. 2000). The MHC molecules are distinguished into two different classes: Class I molecules present peptides derived from cytoplasmic proteins to  $\text{CD8}^+$  cytolytic T-lymphocytes (CTLs), assisting in the destruction of intracellular microbes like viruses. Class II molecules present peptides derived from endocytosed extracellular proteins to  $\text{CD4}^+$  helper T-lymphocytes assisting

in the eradication of extracellular organisms such as bacteria. For details on antigen processing see reviews (Germain 1994) or standard books on immunology (Abbas et al. 2000).

Cells presenting MHC class II-peptide complexes to T cells are mainly dendritic cells, macrophages and B cells. Dendritic cells function as professional antigen presenting cells (APCs) sampling antigen from all areas of the body and after a process of maturation and migration to lymph nodes there they present MHC-peptide complexes to naïve T cells. T cells specific for the antigen are then activated and after clonal expansion form a large number of effector T cells which migrate to the sites of inflammation. If the T cells then encounter macrophages or B cells, presenting peptides specific for the TCR, they activate those macrophages to increase their lytic activity or they stimulate the B cells towards antibody secretion (Abbas et al. 2000).

The first step of T cell activation is the interaction of peptide-MHC class II complexes with the T cell receptor complex, which accounts for the specificity of the peptide. The MHC class II molecule also binds to CD4 which increases the binding affinity and accounts for the MHC class II restriction of CD4<sup>+</sup> T cells (Marrack and Kappler, 1986; Gay et al. 1987). The structure of class II molecules is perfectly refined to its function in binding and presenting peptides. Each MHC class II molecule is a heterodimer of an  $\alpha$ - and a  $\beta$ - chain which are non-covalently associated. Each chain consists of a membrane proximal domain, ( $\alpha$ 2 and  $\beta$ 2) which has an IgSF-like fold and a membrane distal domain ( $\alpha$ 1 and  $\beta$ 1). The membrane distal domains form a flat surface consisting of eight  $\beta$ -strands (four from each polypeptide) on top of which are 2  $\alpha$ -helices (one from each polypeptide) forming a peptide binding cleft occupied by the antigenic peptide (Brown, J. H. et al. 1993). During the antigen recognition, CD4 binds to nonpolymorphic regions located on the membrane proximal domains of the MHC molecule (Gay et al. 1987). The interaction between CD4 and MHC class II molecules is rather weak, a  $K_D$  of 200  $\mu$ M has been reported (Xiong et al. 2001), which is still stronger than the  $K_D$  determined for the CD4 self association of 1 mM (Wu, H. et al. 1997).

Attempts to define the segments that are involved in the binding of MHC class II molecules on CD4 first described the area of MHC binding as a rather broad area involving residues from both domain D1 and D2 (Clayton et al. 1989). Key residues for

the interaction were found to be K35, K46, R59 and especially the exposed hydrophobic residue F43. These residues are all located in the C'C'' ridge suggesting the importance of this stretch for the binding which is further confirmed by the involvement of the buried residues W62 and S49 that support the top and the bottom of the C'C'' loop (Moebius et al. 1992). Further studies have shown the area on CD4 involved in MHC class II recognition extends to residues on all lateral surfaces of D1 and neighbouring parts of domain D2 (Moebius et al. 1993). The size of the CD4 surface involved requires that there is more than one MHC class II molecule involved in binding. Thus the authors hypothesised that CD4 can bind more than one MHC class II molecule or that part of CD4 domain D1 is involved in a CD4-CD4 association which is necessary for the MHC class II recognition (Moebius et al. 1993).

A more detailed view of the CD4-MHC class II interaction arose from the resolution of the crystal structure of a MHC class II-peptide complex with the D1/D2 fragment of CD4 (Wang, J. H. et al. 2001). In contrast to the previous reports of an extended binding area containing CD4 domains D1 and D2, the authors found only D1 involved in MHC II binding, occupying a groove between the two membrane proximal domains of the two MHC II chains. The main binding interaction is mediated through the hydrophobic residue F43 on the CD4 C'' strand, which is surrounded by a pocket of five hydrophobic amino acids from the  $\alpha 2$  and  $\beta 2$  domain. A second contact site is formed by the CD4 C'' strand and the  $\beta 2$  D strand which supposedly supports the F43 mediated interaction. Furthermore, a third association site is made up of salt bridges between the DE loop  $\alpha$ -helical segment and residues of the MHC  $\alpha 2$  domain. The majority of the strength for the interaction is, however, derived from the binding through F43. Regarding the extensive contact area reported via mutagenesis studies, the authors conclude that their results are in accordance with the majority of the residues reported to be important through mutagenesis. However, the residues that lie outside the interaction site determined via X-ray diffraction are supposedly involved in a hydrogen-bonding pattern, the abrogation of which, interferes with MHC class II binding (Wang, J. H. et al. 2001).

#### **1.1.4.5 Extracellular soluble ligands**

##### **Lymphocyte chemoattractant factor IL-16**

The discovery that the effect of a lymphocyte chemoattractant factor (now interleukin-16) is dependent on surface expression of CD4, showed that there is a different role for CD4 as a receptor for IL-16 (Cruikshank, W. W. et al. 1994). IL-16 was first discovered in 1982 as a lymphocyte attractant factor, which is secreted from stimulated PBMCs (Cruikshank and Center, 1982). Interestingly, IL-16 has also been found to inhibit HIV mRNA synthesis (Baier et al. 1995) underlining its importance. This will be discussed in section 1.2.5.4.

IL-16 is synthesised in the form of a 631 amino acid precursor (Baier et al. 1997) that is cleaved by caspase-3 to form the 121 amino acid bioactive molecule which is secreted (Zhang, Y. et al. 1998c). The level of IL-16 production upon stimulation is not regulated on the level of transcription or translation (Laberge et al. 1995) but through activation of caspase-3 followed by processing of the constitutively expressed precursor (Wu, D. M. et al. 1999). After release, IL-16 assembles into tetramers (Cruikshank, W. W. et al. 1994), but the importance of IL-16-oligomerisation has been subject to some controversy. While one study reported that all the constructs they investigated showed bioactivity in the monomeric state (Muhlhahn et al. 1998), other papers report that its bioactivity is dependent on formation of tetramers (Cruikshank, W. W. et al. 1994; Amiel et al. 1999).

The amino acid sequence of the secreted part of IL-16 does not share any significant similarity with known cytokines and chemokines but primary structure analysis as well as comparison of NMR and X-Ray crystal structure data predicted the presence of a PDZ domain (Muhlhahn et al. 1998). PDZ domains are usually found intracellularly where they mediate the protein associations required for the formation of large protein complexes such as ion channels or for signal transduction (Ponting and Phillips, 1995). IL-16 might therefore be the first secreted protein containing such a domain. NMR structural analysis of the PDZ domain of IL-16 together with sequence comparison to other PDZ proteins showed only low homology between the PDZ domain of IL-16 and that of other proteins (Center et al. 2000). Additionally, it has been found

that the IL-16 PDZ domain does not possess the usual peptide binding properties of a PDZ domain which is partly due to a bulky tryptophan residue located at the centre of the peptide binding cleft (Muhlhahn et al. 1998). These findings suggest that the bioactivity of the molecule is not mediated via the PDZ domain.

The sequence homology between the IL-16 molecules of different species suggests that the CD4 binding site is also conserved. Peptide inhibitory studies together with mutational analysis have revealed two short sequence stretches on CD4 domain D4 as critical with the residues V334 and V336 (murine counting) being most important (Liu et al. 1999). The proposed IL-16 contact site lies in close proximity to the CD4 dimer interface reported by Wu et al. (1997) and this might have physiological consequences.

The concept that CD4 is the receptor for IL-16 has been challenged by one study where PBMCs from CD4 knockout mice were shown to be similarly responsive to IL-16 as PBMCs from CD4 positive mice (Mathy et al. 2000). This, however, leaves the possibility that IL-16 is recognised by receptors other than CD4, especially in the absence of the receptor, a feature that is common among cytokines and called redundancy (Abbas et al. 2000). IL-16 is synthesised upon stimulation by an array of cells. Among the cells that have been found to secrete IL-16 are epithelial cells, mast cells, eosinophils, fibroblasts, neuronal cells and CD4<sup>+</sup> and CD8<sup>+</sup> T cells. Similarly striking is the list of target cells for IL-16, which includes T cells, eosinophils, monocytes, dendritic cells, neuronal cells and pro B cells (Center et al. 2000; Cruikshank, W. W. et al. 2000). The function of CD4 as a chemotactic receptor for IL-16 provides an explanation for its presence on a large number of cells where it does not function as a co-receptor for MHC class II molecules. CD4 has been shown to mediate IL-16-induced migration of eosinophils (Bandeira-Melo et al. 2002), activated CD8<sup>+</sup> T cells expressing CD4 (Kitchen et al. 2002) and, at least in part, Langerhans cells (Stoitzner et al. 2001). The mechanism of IL-16 induced signalling through CD4 will be addressed later. For more detailed information on IL-16 see the recent reviews cited (Center et al. 2000; Cruikshank, W. W. et al. 2000).

## **Gp17**

In 1991, the identification of a CD4 binding glycoprotein in human seminal plasma named gp120, was reported which was found to bind CD4 in Domains D1 and D2 (Autiero et al. 1991). Two gp17 regions involved in CD4-binding are located at the amino- and carboxyterminal part of gp17 with amino acids D87, R90 and E91 most likely representing contact residues (Basmaciogullari et al. 2000). The affinity of the gp17-CD4 interaction is high with a  $K_D$  of 9.1 and 38 nM (Autiero et al. 1997). Gp17 was shown to inhibit T cell apoptosis induced by CD4 cross linking and TCR/CD3 activation in monocyte-depleted PBMCs (Gaubin et al. 1999) further confirming an immunomodulatory role of CD4 distant from its role in MHC class II recognition.

### **1.1.4.6 CD4 associating proteins in non-lymphoid cells**

Despite evidence that CD4 functions as a signalling molecule on cells other than CD4<sup>+</sup> T cells (Parada et al. 1996; Krautwald 1998), no CD4 associating proteins that could initiate signalling events have been identified yet. However, on monocytoïd Thp-1 cells, CD4 was also shown to be active as a signalling molecule, and two proteins of 45 and 55 kDa were reported to bind to the CD4 cytoplasmic tail. Mass spectrometry analysis indicated that both proteins exhibit novel sequences (Graziani-Bowering et al. 2002).

## **1.1.5 CD4 function**

### **1.1.5.1 T Cell activation through MHC class II molecules.**

Binding of MHC class II molecules presenting non-self peptides located on the surface of antigen presenting cells (APCs) leads to full T cell activation, as reviewed by (Germain and Stefanova, 1999). T cell activation is initiated by a low-affinity binding of the antigen specific TCR to the MHC class II molecule presenting the antigenic peptide (Germain 1994). Subsequent binding of CD4 to nonpolymorphic regions on the MHC class II molecule (Doyle and Strominger, 1987) stabilises the TCR-MHC association but also recruits the src homology kinase p56lck to the intracellular part of the TCR complex through the association of CD4 with lck (Konig et al. 1995). Before T cell activation, lck

is being kept in a semi-activated stage through association of the CD4/lck complex with the tyrosine phosphatase CD45 (Holdorf et al. 2002).

Activated lck then phosphorylates immune receptor tyrosine-based activation motifs (ITAMs) located on the cytoplasmic site of the TCR complex CD3 and  $\zeta$ -chains. Subsequently, the  $\zeta$ -associated protein (ZAP-70) is recruited to the phosphorylated ITAMs and activated by lck which leads to the assembly and activation of further molecules like p36, vav and SLP-76 (Kane et al. 2000). Those activated molecules initiate signalling cascades such as the phospholipase C- $\gamma$ 1 (PLC- $\gamma$ 1)-dependent cascade (Noh et al. 1995). Other signalling pathways activated are the p21<sup>ras</sup> triggered MAPK pathway and PI-3K pathway (Franklin et al. 1994; Rudd et al. 1994). These signalling processes finally result in a change of gene expression characteristic for T cell activation (Kane et al. 2000).

Whereas this view might indicate that the signalling entities consist of one MHC class II heterodimer, one TCR heterodimer associated with the CD3 and  $\zeta$ -chains and one CD4 molecule, recent reports suggested that dimerisation or oligomerization of some or all of the components is required for efficient signalling. Sakihama et al. (1995) report that dimerisation of CD4 is required for MHC class II binding by investigating the effects of a dominant negative mutation of CD4 that inhibits binding of CD4 to the MHC class II molecule. Introduction of this mutation into CD4<sup>+</sup> cells led to inhibition of MHC class II molecule binding even though wt CD4 is still present on the cell surface. Furthermore, studies with CD4 chimeras where domains 3 and 4 were replaced by different domains that were unable to dimerise indicated that CD4 molecules lacking this part were not able to bind MHC class II molecules presented by B cells (Sakihama et al. 1995). These findings are consistent with the later resolution of the CD4 crystal structure (Wu, H. et al. 1997) which suggests a formation of CD4 dimers through domain D4. Li et al. (1998) discuss the recent evidence for a CD4 dimerisation site in domain D4 and follow up with the discussion of a putative second dimerisation site in domain D1. This site was investigated by molecular modelling studies, which showed a possible D1-D1 interaction through positively and negatively charged patches on the domain. The authors suggest that the CD4 dimerisation through domain D1 is much weaker than the one mediated through domain D4 and thus cannot be seen in the crystal structure or in solution.

According to the authors the function of this interaction might be in the formation of a network of signalling molecules on the cell surface (Li et al. 1998). A model proposed is that initial binding of 2 TCR molecules to a proposed MHC class II “dimer of dimers” leads to the binding of 2 CD4 molecules. The dimerisation of the MHC class II complex, which is a heterodimer of an  $\alpha$  and a  $\beta$  chain was first described with the resolution of the crystal structure of the HLA-DR1 molecule (Brown, J. H. et al. 1993). The CD4 molecules then dimerise through their D4 domain and further oligomerise through their D1 domains leading to a “supercluster” of signalling molecules on the cell surface. This is supported by a study which conducted mutational analysis on both the MHC class II  $\alpha$  and  $\beta$  chain and reported that, in addition to the CD4 binding site on the  $\beta$  chain, another binding site exists on the  $\alpha$  chain (Konig et al. 1995). The possible formation of a MHC class II molecule “dimer of dimers” is further supported by a study employing double amino acid mutations in the putative dimerisation site on MHC class II molecules. Those mutations lead to a reduced T cell response. Single amino acid substitutions, however, did not affect T cell activation (Lindstedt et al. 2001).

Those findings have to be seen in the context of the formation of the immunological synapse, that is the area of close contact between the APC and the T-cell which is characterised by a very tight association through a very ordered pattern of the molecules involved in MHC class II recognition, signal transduction and adhesion. Formation of the immunological synapse is a prerequisite for successful T cell activation (Dustin et al. 2000). The process of synapse formation starts with a ring of TCR-pMHC II interactions surrounding a central region of integrin-mediated adhesion termed the “nascent immunological synapse” (Lee, S. J. et al. 2002b). Within five minutes, the nascent synapse develops into a “mature synapse” with an inverted pattern of TCR-pMHC II interactions in the centre surrounded by a ring of integrin-mediated adhesion (Lee, S. J. et al. 2002b). Research on the immunological synapse is moving rapidly and the interested reader is referred to recent reviews (Dustin et al. 2000; Lee, S. J. et al. 2002b; Sumen et al. 2002).

### **1.1.5.2 Partial T cell activation**

As described above the full activation of T cells, as seen in peripheral T cell activation and thymic selection, requires the involvement of the T cell receptor and CD4 for efficient signalling. However, activation of a T cell is not an on-off response as partial T cell activation has been described. Full T cell activation requires a sustained signal over an extended period of time (>2 h) eventually leading to IL-2 synthesis, which serves as a marker for full T cell activation (Iwashima 2003). Ligands that lead to partial activation of a T cell are divided into partial agonists if they lead to incomplete activation of T-cells and partial antagonist ligands if they inhibit agonist-induced activation. There are several reviews on partial T cell activation through the T cell receptor (Kersh and Allen, 1996; Madrenas and Germain, 1996; Iwashima 2003).

The early events of partial activation are similar among different partial agonists and are mainly characterised by phosphorylation patterns that are suboptimal compared to those in full T-cell activation, especially if the partial T cell activation is independent of CD4 (Chau et al. 1998). To explain those findings a model was proposed suggesting a threshold of signals that are required to accumulate during T cell activation to finally trigger full activation and IL-2 production (Iwashima 2003). Failure to accumulate the required quantity of signals does not result in IL-2 production, however, some changes in the T cell phenotype have been observed. An increase in cell size, together with a decrease in IL-2 secretion after subsequent activation of the T cell receptor with manipulated ligands has been reported (Racioppi et al. 1993). Other responses that have been observed are acidification of the extracellular environment (Beeson et al. 1996), transient and/or partial calcium signals (Wulfing et al. 1997), modulation of TCR expression (Valitutti et al. 1995) and the upregulation of certain cell surface molecules like the IL-2 receptor or LFA-1 (Kersh and Allen, 1996; Madrenas and Germain, 1996). The downstream responses after these effects are inhibition of agonist induced cell proliferation and/or cytokine production, split cytokine production and induction of T cell anergy (Racioppi et al. 1993) that are described as partial T cell activation.

Taken together, these findings suggest that activation of T-lymphocytes is more than just a single mechanism operating in an on-off matter, but rather a delicately

balanced response to various different stimuli that can trigger signals through some or all of the components of the T cell receptor and/or CD4.

### **1.1.5.3 CD4 mediated signalling**

CD4 has been described to function as a chemotactic receptor for IL-16 on T cells and other cells (Cruikshank, W. W. et al. 1987) but little was known about the mechanism of chemotaxis induced through CD4 engagement. Signals that were observed after CD4 stimulation with IL-16 are an increase in intracellular  $\text{Ca}^{2+}$ ,  $\text{IP}_3$  and CD4 phosphorylation (Cruikshank, W. W. et al. 1991). Additionally, in lymphocytes, autophosphorylation of lck occurs (Ryan et al. 1995) as well as desensitisation of the CXCR4 receptor (Van Drenth et al. 2000). In eosinophils, IL-16 treatment induced release of IL-4 but not IL-12 and primed cells for enhanced calcium ionophore induced secretion of leukotriene (C4) in a manner that was dose dependent and also dependent on the chemokine receptor CCR-3 (Bandeira-Melo et al. 2002). Signalling events such as PKC translocation and pathway activation have also been observed in monocytes/macrophages (Parada et al. 1996; Krautwald 1998) or on the monocytoid cell line Thp-1 (Graziani-Bowering et al. 2002).

The phenotypic changes in T cells upon IL-16 activation are described as an “inflammatory phenotype” (Center et al. 2000) to discriminate them from the T cell response that is triggered upon MHC class II interaction with the T cell receptor and CD4. The suggested physiological role of this T cell phenotype is to increase  $\text{CD4}^+$  T cell accumulation while preserving future antigen responsiveness which is in accordance with the transient nature of the “inflammatory phenotype” (Center et al. 2000).

Interestingly, CD4 ligands other than IL-16, such as anti CD4 antibodies, also trigger a cellular response as CD4 signalling induced by antibody-mediated ligation modulates subsequent TCR signals (Newell et al. 1990). Because this antibody stimulation of CD4 altered the TCR induced signalling similar to the effects of IL-16 but fails to switch on the “inflammatory phenotype” (Center et al. 2000) it was suggested that the CD4 receptor can discriminate between different ligands. Taken together, those findings show a different, but yet ill-defined role of CD4 as a chemotactic receptor on T lymphocytes as well as other CD4 expression cells.

### **1.1.6 Lipid rafts**

The fluid mosaic model described by Singer and Nicholson in 1972 has been the standard model for the description of cellular membranes for decades (Singer and Nicholson, 1972). However, in the last ten years, more and more evidence has demonstrated that there are different compartments in membranes distinct from the fluid phase that are enriched in special lipids which are called lipid rafts. “Lipid rafts” is being used according to the nomenclature proposed earlier (Simons and Toomre, 2000) for all liquid-ordered membrane microdomains.

Lipid rafts were first discovered during investigations of vesicle trafficking in epithelial cells (Simons and van Meer, 1988). Epithelial cells have two distinct membranous compartments, with different lipid constitutions. These are the apical membrane facing the lumen and the basolateral membrane facing the connective tissue. Vesicles budding from the trans Golgi network (TGN) trafficking towards the membrane have been found to be organised into two different circuits. One pathway is targeted to the basolateral membrane whereas other vesicles traffic towards the apical membrane. Different lipids are sorted into lipid rafts before budding from the TGN along these two pathways, resulting in different sets of lipids being transported to the apical and basolateral membranes (van Meer and Simons, 1988).

The concept of lipid rafts was initially attacked as detergent-induced artefacts (Mayor and Maxfield, 1995). Later studies however have shown that artefactual creation of protein-enriched microdomains does not occur (Ostermeyer et al. 1999). Recent technological advances also overcame the initial difficulties in observing of such dynamic structures on the cell membrane. Techniques like photonic force microscopy (Pralle et al. 2000) have made the observation of those structures possible and led to the acceptance of the raft hypothesis by the scientific community. Lipid rafts are covered in this review because Ick and possibly CD4 are raft associated (Ilangumaran et al. 1999; Percherancier et al. 2003). T cell receptor mediated signalling has also been shown to occur in rafts (Xavier et al. 1998). Moreover, the human immunodeficiency virus, which is the subject of the second part of this review, buds from lipid rafts in the cell membrane of T cells and probably also enters these cells similarly (Manes et al. 2000; Campbell et al. 2001).

### **1.1.6.1 Structure and investigation of lipid rafts**

Lipid rafts are dynamic regions on the plasma membrane that are rich in cholesterol and sphingolipids. They are less fluid than the surrounding membrane owing to the different composition in fatty acids and cholesterol (Schroeder et al. 1994). Rafts are found in all types of mammalian cells and even in yeast. Estimates of the percentage of cell-surface area occupied by rafts range from 5 % to 50 % (Horejsi 2003). They are characterised by insolubility in non-ionic detergents like Triton X-100 (but soluble in other detergents like n-octylglucosid or SDS (Horejsi 2003)) leading to the description of rafts as “detergent insoluble glycolipid-enriched complexes” (DIG). However, when investigating rafts, care has to be taken as, depending on the type of detergent and the extraction conditions, varying results can be obtained towards association of compounds with DIGs (Simons and Toomre, 2000).

Caveolae are a different type of raft that is characterised by the incorporation of caveolin. They are morphologically characterised as deep invaginations in the plasma membrane. As T lymphocytes do not contain Caveolae (Simons and Toomre, 2000), they will not be reviewed here.

As mentioned above, the existence of rafts has been controversial for a rather long time until defined by recent technological advances such as fluorescence resonance energy transfer (FRET) (Varma and Mayor, 1998), Chemical cross-linking (Friedrichson and Kurzchalia, 1998), immunofluorescence microscopy (Harder et al. 1998), photonic force microscopy (Pralle et al. 2000), single dye tracking (Schutz et al. 2000) and electron microscopy (Wilson et al. 2000). Further, studies of rafts included extraction with different detergents (Hooper 1999), gradient centrifugation (Brown and Rose, 1992) or altering the lipid composition of membranes (Liao et al. 2001). For example, incubation with beta-cyclodextrin depleted membranes of cholesterol and renders the raft-associated proteins detergent soluble. However, care must be taken when using such compounds as they might have other effects on the cellular metabolism than mere cholesterol depletion (Ikonen 2001; Horejsi 2003).

### **1.1.6.2 Raft associated proteins**

The most important property of rafts is that they can specially include or exclude proteins and thus lead to enrichment of proteins in special domains. Proteins can reside in rafts transiently, for up to several minutes (Pralle et al. 2000) and clusters of up to 15 identical proteins have been observed in the same raft (Friedrichson and Kurzchalia, 1998; Varma and Mayor, 1998). Classes of proteins that are targeted to lipid rafts include GPI-anchored proteins (Hooper 1999), N-terminally myristoylated and palmitoylated proteins like src family kinases (Wolven et al. 1997; Webb et al. 2000) or  $\alpha$ -subunits of heterotrimeric G-proteins (Moffett et al. 2000).

Whereas the GPI-anchor seems to function as a raft-targeting signal for extracellular proteins (Sharom and Lehto, 2002) and myristoylation for intracellular proteins, no direct raft-targeting signal has been identified for transmembrane proteins. Some proteins like CD45 are known to be non-raft resident (Horejsi 2003) whereas others like CD44 were described as raft associated (Neame et al. 1995). It has been suggested that transmembrane proteins are packed into lipid rafts owing to the interaction of their hydrophobic membrane anchor and adjacent lipid modifications with the raft lipids. Nevertheless, several transmembrane proteins that do not contain lipid modifications can partition in lipid rafts. This has been attributed to amino acids at the exoplasmic part of their transmembrane domain (Scheiffele et al. 1997; Simons and Toomre, 2000). Marker proteins for rafts are mainly GPI-anchored proteins and caveolin for Caveolae. Other raft markers reported are flotillins and ganglioside GM1 and GM3 (Zajchowski and Robbins, 2002).

For further reading on lipid rafts see reviews on rafts and signal transduction (Simons and Toomre, 2000), rafts and membrane transport (Ikonen 2001), general concept of rafts (Simons and Ikonen, 1997; Zajchowski and Robbins, 2002), rafts and T cell activation (Horejsi 2003) or lipid modifications of proteins (Casey 1995).

### **1.1.6.3 CD4 partitioning in lipid rafts**

Several findings support the notion that CD4 resides inside lipid rafts. First, it contains two palmitoylation sites which can increase the affinity of proteins to lipid rafts (Crise and Rose, 1992; Melkonian et al. 1999). Second, CD4 functions in T cell activation and T

cell signalling has been reported to originate from rafts (Janes and Peterlin, 2000). Third, it associates with p56lck, a kinase that has been shown to be located in lipid rafts as a result of its lipid modifications (Ilangumaran et al. 1999) and the CD4-lck interaction has been shown to be responsible for targeting of CD4 into lipid rafts (Foti et al. 2002).

Lipid rafts can also mediate a connection between the cellular membrane and the cytoskeleton (Babiychuk and Draeger, 2000; Rozelle et al. 2000). The reported associations between CD4/lck and the cytoskeleton, as discussed in part 1.1.4.1, could be a result of the location of CD4 in the lipid raft microdomains and the partitioning of CD4 in detergent-insoluble compartments. It could well be that CD4 residing in rafts is connected to the cytoskeleton via lck and adaptor molecules. However no such molecules have been identified yet. It is also worth noting that the controversial results obtained in experiments aimed at co-immunoprecipitating TCR components with CD4 (part 1.1.4.3) could be a product of the colocalization of these proteins and CD4 in the same raft, although not physically associated with the TCR components.

#### **1.1.6.4 Functions of lipid rafts**

The two main roles that have been assigned to lipid rafts are signal transduction and transport. The role of rafts in transport led to their initial discovery when they were found to support a distinct traffic pathway from the TGN to special membrane compartments (van Meer and Simons, 1988). Moreover, rafts have been shown to be involved in secretion as cholesterol depletion blocked the formation of regulated, constitutive secretory vesicles in neuroendocrine ArT-20 cells (Wang, Y. et al. 2000b). The described link between rafts and the actin cytoskeleton (Babiychuk and Draeger, 2000; Rozelle et al. 2000) could provide the mechanism for raft-mediated transport.

Rafts can also play a role in endocytosis and could provide an alternative mechanism for protein endocytosis in comparison to clathrin-coated pits (Deckert et al. 1996). The most important role that rafts play on the cell surface is in signal transduction. Rafts provide platforms for signalling by specifically recruiting the requisite molecules and by excluding molecules that could inhibit signalling, such as phosphatases. Therefore, these lipid domains create a new microenvironment for the signalling molecules leading to an amplification of the signalling (Simons and Toomre, 2000).

Signalling processes that have been shown mediated by lipid rafts are immunoglobulin E signalling (Sheets et al. 1999), GDNF (glial cell line-derived neurotropic factor) signalling (Tansey et al. 2000), ras signalling (Roy et al. 1999), hedgehog signalling (Incardona et al. 2000), other growth factor signalling processes and GPI-protein signalling (Zajchowski and Robbins, 2002) and TCR signalling (Janes and Peterlin, 2000). The latter is of particular interest here. For a detailed review on the role of rafts in signalling see recent publications (Simons and Toomre, 2000; Zajchowski and Robbins, 2002).

Most of the molecules that are involved in TCR signalling are initially associated with lipid rafts or become associated during TCR activation. The molecules involved in TCR signalling that are constitutively associated with rafts include LAT, lck, fyn, PIP<sub>2</sub> and ras (Liu et al. 1998; Ilangumaran et al. 1999; Roy et al. 1999). The association of the TCR complex with lipid rafts itself has been more controversial. The components of the complex have been found to be detergent soluble as well as detergent insoluble, as reviewed by Janes et al. (2000). Those discrepancies are likely to originate in differential detergents and differential methods when the extraction was performed and the TCR in the non-activated stage is likely to have only a moderate affinity for lipid rafts (Janes and Peterlin, 2000). Upon activation, however, the amount of TCR associated with lipid rafts increases (Montixi et al. 1998; Xavier et al. 1998) and other proteins are also recruited to lipid rafts, including PLC- $\gamma$ 1, Sos and Grb2 (Zhang, W. et al. 1998a; Zhang, W. et al. 1998b). Indeed treatment of cells with agents that disrupt lipid rafts inhibits signalling through the TCR complex (Xavier et al. 1998). This supports the model of the role of lipid rafts in TCR signalling described by Janes et al. (2000) i.e. the cell surface contains small isolated patches of lipid microdomains each containing only a few molecules involved in the TCR response. Upon TCR activation, those patches cluster together to form a large signalling platform, which specially includes the molecules, involved in signalling and excludes inhibitory molecules. This model is also consistent with the concept of the immunological synapse (see 1.1.5.1) where the interface between the APC and the T cell has a very ordered structure of molecules involved in signal transduction and adhesion. The clustering of lipid rafts could therefore be the key step for the formation of the immunological synapse (Janes and Peterlin, 2000). This model has been

subsequently refined. The first steps in TCR signalling seem to occur independently of lipid rafts but rafts are required for later stages of signalling (Horejsi 2003). This is consistent with the recent finding that the early TCR signalling events are also independent of immunological synapse formation (Lee, K. H. et al. 2002a).

## 1.2 The Human Immunodeficiency Virus



**Figure 1.6** Worldwide prevalence of HIV. Adults and children to be living with HIV/AIDS as of end of 2003. Figure and number obtained from UNAIDS.

### 1.2.1 Introduction to HIV

The Human Immunodeficiency Virus HIV-1 is the etiologic agent of AIDS, the “acquired immunodeficiency syndrome”, a disease to which more than 20 million people have succumbed by the year 2001. Alone in 2001, three million people died of AIDS, there are 14 million orphans because of it and another 40 million infected people (Stover et al. 2002). Predictions estimate another 45 million infections between 2002 and 2010 if adequate prevention mechanisms are not developed and/or implemented (Stover et al. 2002). Figure 1.6 shows the worldwide prevalence of HIV as estimated for the end of 2003. So far no vaccine is available to prevent HIV infection and no cure has been found to eradicate the virus from body tissue. The ongoing spread of HIV in the world therefore highly requires effective and cheap ways to prevent and treat HIV infection (See (Weiss 2001) or (Stover et al. 2002) for more information). This review aims to give an overview over the structure and pathogenesis of HIV with the ultimate aim of identifying possible research targets for the improvement of our understanding of HIV.

### **1.2.1.1 History and global impact of HIV/AIDS**

AIDS was first discovered in 1981 (Centres of Disease Control and Prevention, 1981) and HIV was identified as its etiological agent in 1983 (Barre-Sinoussi et al. 1983). The virus is transmitted through sexual contact (sexual transmission), from mother to child (vertical transmission) or through horizontal transmission via needles and blood transfusions. The latter has become very rare owing to screening of blood products for HIV from 1985 leaving the sharing of syringes between intravenous drug users as the main way for horizontal transmission (Levy 1998). HIV has spread widely throughout the world and the area that is worst affected by the epidemic is sub-saharan Africa. Recent spread of the virus in southern and south east Asia and eastern Europe/Russia is of great concern. In developed countries, the spread of HIV/AIDS has been reduced because of promotion of prevention measurements such as the use of condoms and through the introduction of efficient treatment for HIV-positive patients. In developing countries, the lack of education and funds for expensive therapies has lead to a spread of HIV that is threatening the stability of some countries. With up to 36 % of the population between 15 and 49 infected with HIV in some African countries, the disease is already severely affecting the economic and social structure of those countries by depleting the population of young adults (Piot et al. 2001). A second, though less discussed danger accompanied by the HIV epidemic is the possibility that microbes, previously not adapted for humans, can adapt to the human environment in AIDS immunocompromised patients and evolve as pathogens that could “settle” into the healthy population (Piot et al. 2001; Weiss 2001). In developed countries the most successful control measure for HIV has been education. An increased awareness for HIV together with the use of condoms and screening of blood products for HIV as well as syringe exchange programs have reduced the risk for an infection in western countries and some countries in the developing world (Force for Change: World AIDS campaign with young people, Joint United Nations Programme on HIV/AIDS 1998; Bluthenthal et al. 2000). Additionally “Highly active antiretroviral therapy” (HAART) has had a positive impact on HIV disease progression and life expectancy in western countries (Richman 2001) as well as reducing viral load in blood and genital secretions. However, in 2003 a rise in HIV infections was reported for New South Wales for the first time in eight years (Jones, Cindy 2003). Because of the

persistent lack of a vaccine and cure against HIV further research on this highly pathogenic virus is of the utmost importance.

### **1.2.1.2 HIV and SIV**

HIV-1 and HIV-2, which causes a similar disease belong to the Retroviridae family of viruses and within those to the genus of lentiviruses. Lentiviruses share several characteristics including long incubation period, host species specificity, latent and persistent infections of target cells as well as having a highly polymorphic genome. Other lentiviruses apart from HIV-1 are Equine infectious anaemia virus, Bovine, Feline and Simian Immunodeficiency Virus See (Levy 1998) for more details on lentiviruses. The origin of HIV-1 is thought to be a cross-species transfer of Simian Immunodeficiency Virus (SIV) to humans probably from chimpanzees. The time when the cross-species transfer occurred is now estimated to be the 1930s (Korber et al. 2000). The first evidence for HIV-1 infection in humans came from a sample taken in 1959 (Nahmias et al. 1986). The virus remained silent/hidden until it began to spread around the globe assisted by increasing mobility of individuals some 25 years ago.

The simian hosts of SIV do not develop AIDS indicating that their immune system is capable of controlling the virus. Crossing the species to humans, however, has enabled the virus to acquire new terrain and spread extensively. The high mutational rate of HIV-1 together with selective pressure allowed for the development of a high genomic diversity of the virus around the world and, to a lesser extent, in single infected individuals. HIV-1 can create more genetic diversity in one single individual than the influenza A virus creates worldwide during an epidemic. According to its genetic evolution, HIV-1 is divided into two groups main (M) or outlier (O) with group M being responsible for the worldwide expansion. The M group is further divided into clades, which differ between geographic regions. So clade B predominates in North America and western Europe, clade E in some Asian Countries and clade C predominates in Southern Africa. Recombination between different clades further complicates the genealogy of HIV-1 especially the definition of these clades (Weiss 2001). In newly infected regions often two or more clades are introduced.

### 1.2.1.3 HIV-1 Disease Progression

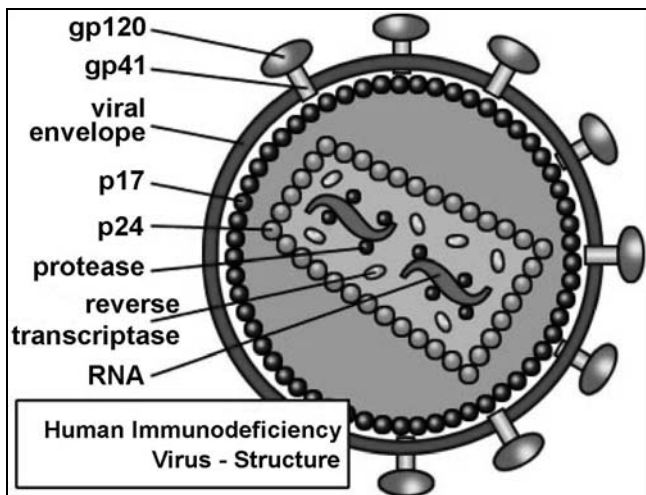
The progression of HIV-1 in individual humans from infection to AIDS can be divided into three principal stages (see (Levy 1998) for a more detailed description). The first stage is the acute infection occurring in 30-50 % of infected patients and characterised by symptoms that are common to many viral infections like fever, headaches, a sore throat and rashes (Levy 1998; Anderson 2003). In this stage, the virions have travelled from the site of entry to the lymph nodes where they infect CD4<sup>+</sup> cells. The host immune system responds to the primary infection with high levels of HIV-1 specific antibodies (mainly against the viral envelope or the matrix protein p24) and HIV-1 specific cytolytic T lymphocytes. This latter response correlates with a quick decline in the load of viral particles in the plasma and a recovery of the patient (Niu et al. 1993). However, the immune system is not able to totally eradicate the virus. Following the primary infection is a long asymptomatic period that is characterised by very high levels of HIV replication in CD4<sup>+</sup> T cells together with the decline of those blood cells (Ho, D. D. et al. 1995). The level of CD4<sup>+</sup> T cells, normally around about 1000 cells/ $\mu$ l ranges from around 700 cells/ $\mu$ l to around 300 cells/ $\mu$ l in this phase (Abbas et al. 2000).

Some infected individuals have survived for almost 20 years after being infected with HIV-1 but without developing AIDS (longterm non-progressors). Usually, after months to years the immune system is no longer able to control the HIV-1 replication, probably due to the reduction of CD4<sup>+</sup> T cells and destruction of lymphoid tissue (McCune 2001). In this last phase of the disease, the virus increases replication, becomes more aggressive towards CD4<sup>+</sup> T cells and opens the gate for opportunistic infections that eventually kill the host (Abbas et al. 2000). When the CD4<sup>+</sup> T cell count falls below 200 cells/ $\mu$ l, the immune system is no longer capable of controlling otherwise harmless pathogens. AIDS patients thus succumb to microbes that are usually not fatal such as Protozoa, Bacteria, Fungi or other viruses (“opportunistic infections”). Additionally, patients may develop tumours, encephalopathy and wasting syndromes (Levy 1998; Abbas et al. 2000). The use of antibiotics and antiretroviral therapy has eased the symptoms and reduced the disease progression, but still, HIV-1 eventually leads to death and especially in countries where the economic situation does not allow therapy, many people die in inhuman circumstances. The search for a vaccine/cure is therefore of high

priority and understanding of the structure and replication of the virus and its immune control is the key to accomplish this.

## 1.2.2 Structure of the HIV-1 virus

### 1.2.2.1 Structure of the virion

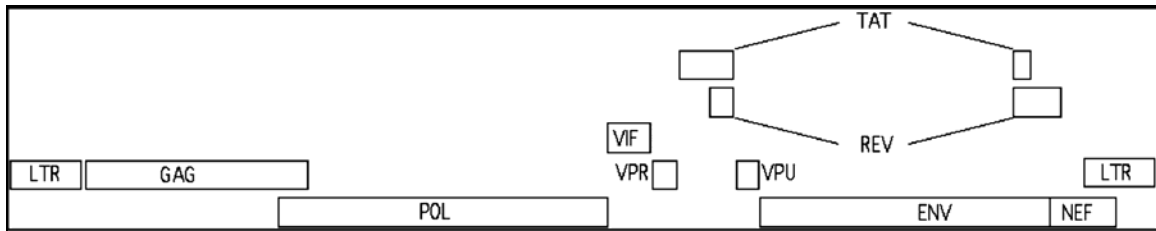


**Figure 1.7**

Structure of the HIV-1 virion

Figure 1.7 shows the simplified structure of a typical HIV-1 virion. The cone-shaped core structure contains a dimer of the viral RNA with which the HIV-1 proteins reverse transcriptase, protease and integrase are associated. It is surrounded by a layer of the capsid protein p24 and also contains the nucleocapsid proteins p6 and p9. This nucleocapsid is surrounded by the matrix, which is made up of the matrix protein p17. This structure is enveloped by a cholesterol-rich host cell-derived lipid bilayer which also contains host cell-derived proteins (Aloia et al. 1988; Graham et al. 2003). The matrix is attached to the membrane through myristoylation of the p17 protein. The nucleocapsid also contains the accessory proteins Vpr, Vif and Nef but their location is ill defined (Frankel and Young, 1998; Levy 1998). On the surface of the membrane in the shape of 72 spikes is the viral envelope protein. Each spike consists of a trimer of the transmembrane protein gp41 and a trimer of the surface protein gp120 (Lu et al. 1995). The overall shape of the submembraneous matrix is icosahedral and the size of the virion is about 100 to 120 nm (Collier et al. 1998).

### 1.2.2.2 Structure of the HIV-1 genome:



**Figure 1.8:** Structure of the HIV-1 genome

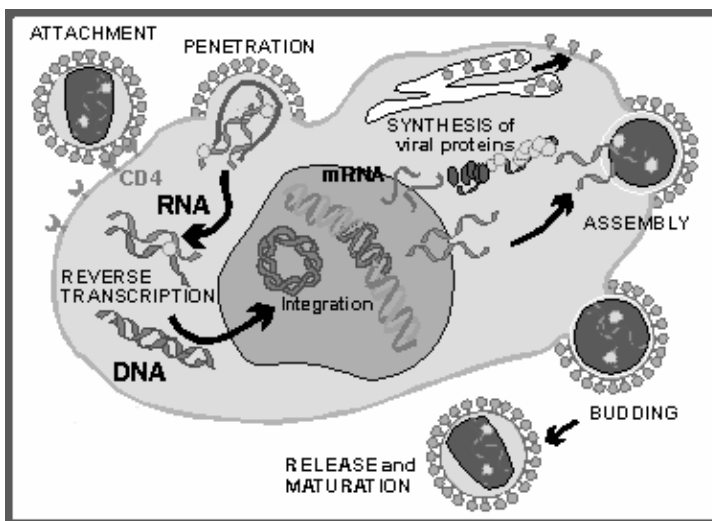
Figure 1.8 shows the HIV-1 genome. It has approximately 9 kb, is flanked by two long terminal repeats (LTR) and codes for a total of 15 proteins in nine open reading frames. Besides the three major viral components gag, pol and env, six accessory proteins are encoded (Frankel and Young, 1998). Gag, pol and env are each synthesised as large precursors which are then processed into smaller components. The gag precursor (p55) is cleaved to give rise to the matrix protein (MA, p17), the Capsid protein (CA, p24) and the nucleocapsid proteins NC (p7) and p6. The pol protein yields the necessary viral enzymes reverse transcriptase (RT), integrase (IN) and protease (PR). The env protein is synthesised as a 160 kDa precursor (gp160) and cleaved by cellular proteases that are located in the Golgi, forming the transmembrane protein (TM, gp41) and the surface protein (SU, gp120). The accessory proteins consist of two regulatory proteins (tat and rev) which are spliced from the primary transcript and four other proteins (vif, nef, vpu and vpr). The functions of those proteins will be discussed later. Three of them, vif, vpr and nef are part of the virion (Frankel and Young, 1998; Levy 1998). Apart from coding for the viral proteins, the viral RNA contains several regulatory elements that are required for virus function namely (Frankel and Young, 1998):

- (i) nucleotides 1 to 55 form the TAR loop, which is recognized by the regulatory protein tat;
- (ii) the primer-binding site from nucleotide 182 to 199 recruits a tRNA<sup>Lys</sup> from the host cell to become the site of initiation for the reverse transcription;
- (iii) the packing signal  $\Psi$  is required for assembly of the viral RNA into the budding virion. It comprises of the nucleotides 240 to 350;

- (iv) the sequence between nucleotide 248 and 271 is called the kissing loop: it is involved in dimerisation of the RNA leading to the assembly of two copies of the viral RNA in a budding virion;
- (v) the dominant donor splice site at nucleotide 290 is required for the formation of the subgenomic RNAs;
- (vi) a gag-pol frameshift region is necessary for the pol proteins to be translated correctly. It includes nucleotides 1631 to 1673, which form a secondary structure that leads to the reading of eight nucleotides as three codons thus shifting the reading frame by  $-1$ ;
- (vii) the rev response element (RRE), including nucleotides 7362 to 7596, binds the regulatory protein rev and increases the production of structural proteins and full length RNA by preventing splicing of the primary transcript; and
- (viii) many donor and acceptor splice sites are present which provide multiply spliced transcripts encoding various combinations of the regulatory proteins. A poly A signal at nucleotides 9205 to 9210 is also present.

### 1.2.3 The HIV-1 Life Cycle

#### 1.2.3.1 Overview

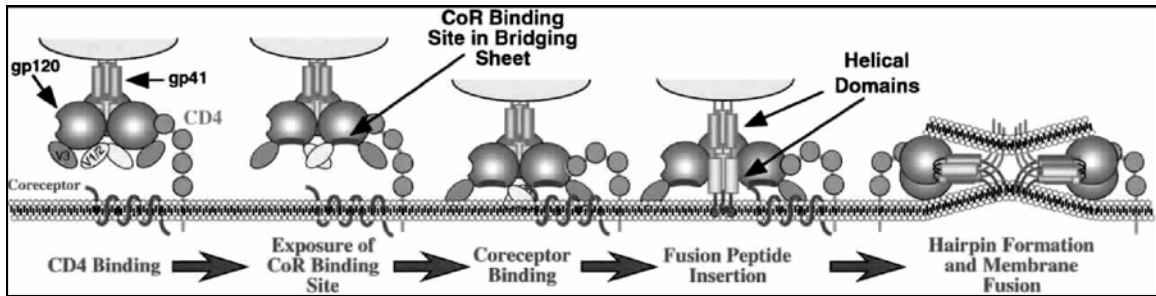


**Figure 1.9**  
Schematic diagram of the HIV-1 life cycle seen from within an infected cell

Figure 1.9 shows an overview of the HIV-1 life cycle from within an infected cell, starting with the entry and ending with the release and maturation of new virions. The HIV-1 life cycle has also been described from the perspective of the virion starting with the transcription of the viral RNA and finishing with the integration of the viral DNA into the host cell genome (Frankel and Young, 1998). The HIV-1 life cycle can be divided into up to 15 discrete steps (Frankel and Young, 1998), but in this review, it will be divided into only four major steps. The first step is the entry of the infectious virion starting with the virus attachment, receptor and coreceptor engagement and finally membrane fusion. The second step describes the post entry events including transport to the nucleus, reverse transcription and integration of the viral DNA into the genome, which in the case of infected lymphocytes can be followed by a period of cellular latency. The third step is the transcription of the viral genes, starting with the transcription of early genes and ending in the production of full-length viral RNA and structural proteins. The last step starts with the assembly of viral particles at the membrane, the budding of the virus followed by its maturation to become infectious. Each of these steps harbours several potential target sites for interference with HIV-1 replication.

### **1.2.3.2 HIV-1 entry**

HIV-1 entry into its target cells is a multistep process starting with the attachment of the virus to the target cells via the envelope glycoprotein gp120 binding to the virus receptor CD4. This is followed by the exposure of a coreceptor-binding site in gp120 and binding of a coreceptor, usually CXCR-4 or CCR-5. Further changes in the gp120 structure finally lead to the fusion of the viral and cellular membrane. This has been reviewed by Doms and Trono (2000). Figure 1.10 illustrates the major steps of HIV entry that are understood so far.



**Figure 1.10:** The entry of the human immunodeficiency virus into a target cell. Taken from (Doms and Trono, 2000)

### Virus attachment

CD4 was identified early as the receptor of HIV (Klatzmann et al. 1984; Maddon et al. 1986) which gains entry into target cells via binding of the viral envelope protein gp120 to CD4 (Lasky et al. 1987). Binding of gp120 to CD4 was initially thought to be the only mechanism for initial attachment of the virus, which was supported by the high binding constant of 1 to 10 nM (Moore and Binley, 1998). Recent studies, however, have shown that other receptors may assist initial binding, particularly in certain cell types eg. anti-CD4 antibodies blocked HIV-1 attachment to T cells but not to HeLa cells expressing CD4 (Mondor et al. 1998). Interestingly, though HIV-1 does attach to HeLa cells even in the presence of anti-CD4 antibodies, it does not attach to CD4 negative T cells (Mondor et al. 1998) suggesting that HeLa cells express surface molecules capable of serving as HIV receptors. This suggests that CD4 is the major receptor for HIV attachment on T cells but that on other cells other receptors can mediate virus attachment as well (Ugolini et al. 1999). Those data have to be interpreted with caution, though, as virus from different sources may exhibit different binding characteristics.

It is now known that a series of other molecules serve as accessory HIV-1 attachment receptors on cells where CD4 expression is low. Macrophages express a high level of heparan sulfate proteoglycans (HSPG) that have been shown to mediate initial binding of gp120 to the cells as well as CD4 which is still a major CD4 binding receptor on those cells (Saphire et al. 2001). HIV-HSPG binding is due to an interaction of basic residues in the gp120 V3 loop and the negatively charged HSPG proteins explaining also the HIV-1 attachment to CD4 negative HeLa cells which are HSPG<sup>+</sup> cells (Saphire et al. 2001).

Macrophages and dendritic cells (DCs) express a series of C-type (Calcium dependent) lectin receptors (CLRs) that normally serve to trap soluble glycosylated antigens consistent with the function of the cells as professional antigen presenting cells (Figdor et al. 2002). Those CLRs are the mannose receptor (MR, expressed on macrophages and DCs), dendritic cell specific ICAM-3 grabbing non-integrin (DC-SIGN, expressed on monocyte-derived DCs) and Langerin (expressed only on langerhans cells, a sub-set of DCs in the epidermis) (Figdor et al. 2002). Those molecules can efficiently bind HIV-1 and concentrate it on the cell surface (Turville et al. 2002). Interestingly, whereas macrophages can be easily infected with HIV-1 (Cunningham et al. 2000) immature DCs are infected at low levels and mature DCs hardly at all. Nevertheless, both can mediate the infection of CD4 lymphocytes at virus concentrations that are not sufficient to infect the CD4 lymphocytes in the absence of DCs (Cameron et al. 1992; McDonald et al. 2003), (reviewed by (Pope and Haase, 2003)). This mechanism of *trans* infection is partly mediated by DCs endocytosing HIV followed by diversion to the T cell contact zone, away from the normal endolysosomal degradation of the virus. Dendritic cells also present HIV antigens to these contacting T cells (as reviewed in the part on CD4). Indeed specific activation of responding CD4 lymphocytes at the same time as infection may lead to explosive HIV replication in DC-T cell clusters. Immature DCs can also be infected by HIV at low levels and this virus transferred to contacting T cells. Work in our institute has demonstrated that there are two sequential phases of transfer from DCs to CD4 lymphocytes, leading to the theory that early phase transfer may occur while the DC is still in the mucosa whereas later stage transfer probably occurs after the DC has migrated to local lymph nodes (Turville et al. 2003).

Apart from HPSGs and CLRs, virus attachment can be increased by an array of host cell derived membrane proteins that become part of the viral envelope during budding and then recognize their ligand on the target cell. CD44 is an example for one of those molecules (Guo and Hildreth, 1995). See also Baribaud et al. (2001) or (Ugolini et al. (1999) for more details on host cell derived factors that increase virus attachment.

### **Binding to the CD4 receptor**

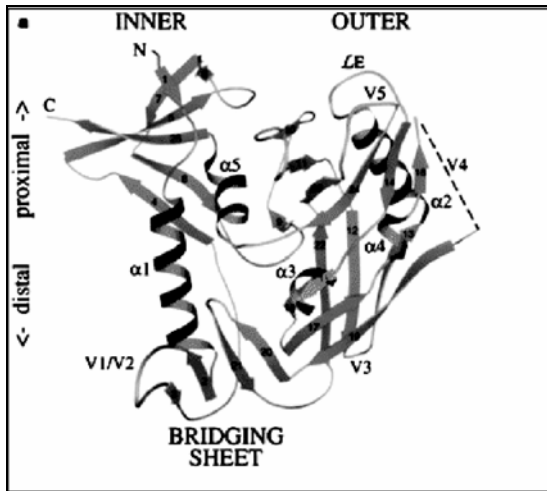
The CD4 receptor was established early on to be the principal receptor for HIV-1 entry into its target cells. This was based upon the findings that CD4 is expressed on all cells that are susceptible to HIV-1 and that cells can be infected with HIV-1 after transfection of the CD4 gene (Maddon et al. 1986). Additionally, anti CD4 antibodies blocked HIV-1 infection of target cells (Klatzmann et al. 1984). Soon thereafter, a viral protein named gp110 (now gp120) was identified as responsible for binding of HIV to target cells (McDougal et al. 1986). Understandably, intense research was then aimed at investigating the epitopes involved in this binding reaction, especially with the aim to develop structure-based drugs. Lasky et al. (1987) reported the determination of an epitope on gp120 responsible for CD4 binding. This epitope comprised the amino acids 397 to 439. Additionally the binding constant was measured to 4 nM indicating a very strong binding reaction (Lasky et al. 1987). Mutational analysis carried out with the CD4 molecule showed that the gp120 binding is located at domain D1 with the residues 40 to 48 in the CDR2 loop playing the most important role. Again, the unusually protruding hydrophobic residue F43 was reported to be absolutely essential for binding, with other residues being less important (Clayton et al. 1988; Mizukami et al. 1988; Peterson and Seed, 1988; Arthos et al. 1989).

### **Structure of gp120**

As gp120 is the viral protein responsible for binding and entry into the target cells, understandably intense research was conducted towards determining the structure of this molecule. Initial studies focussed on the amino acid sequence and showed that gp120 was characterised by five variable regions (named V1 to V5) located between more conserved regions (Starcich et al. 1986). The four variable regions V1 to V4 form loops protruding from the surface which contain a disulfide bridge at their base (Leonard et al. 1990). The same report also described the extensively glycosylated nature of gp120. A major breakthrough came in 1998, when Hendrickson and Sodroski published the resolution of the crystal structure of gp120 complexed with CD4 and a neutralising human antibody. They also reported on the antigenic structure of gp120 (Kwong et al. 1998; Wyatt et al. 1998a).

In order to obtain structural information, the researchers had to extensively reduce the structure of the gp120 molecule. Both termini were truncated, the V1/2 and V3 loops were substituted by short amino acid stretches and the molecule was extensively deglycosylated. Still, the gp120 molecule did bind CD4 and was recognized by the antibody indicating that large regions of the molecule are not required for CD4 engagement. Figure 1.11 shows the structure of gp120 determined in that study. The overall shape of the gp120 molecule is a heart-shaped ellipsoid with the approximate dimensions 50Å x 50Å x 25Å. It comprises of 25 β-sheets, 5 α-helices and 10 loop segments. The molecule can be divided into two major parts: an inner domain and an outer domain (referring to the relative location of the gp120/gp41 trimer axis) from which the loop segments protrude. The inner domain is a two-helix two-strand bundle with a five-stranded β-sandwich at its termini proximal end and a projecting V1/2 stem. The outer domain is a stacked double barrel that runs parallel to the inner domain. The two barrels form one hydrophobic core. The V1/V2 loops are located at the inner domain on the opposite site as the N and C termini. The V3 loop emanates from the distal end of the outer domain and the V4/V5 loops are located at its proximal end.

The gp120 structure is unique sharing only some similarities with FabA dehydrase and dUTP pyrophosphatase. Assigning conserved and variable regions to the gp120 structure reveals that the inner domain is more conserved than the outer domain, that the most variable parts are the solvent exposed ones whereas the hydrophobic core is more conserved. HIV-1 gp120 shares 35 % identity with HIV-2 gp120. The identity between clade M and O is 51 % and between M and C is 77 % revealing a certain level of conservation despite the high variability in HIV (Kwong et al. 1998).



**Figure 1.11:**  
Structure of HIV-1 gp120. The division of the molecule into inner and outer domain can be seen as well as the makeup of the domains from the different  $\beta$ -sheets and  $\alpha$ -helices. Also the location at the variable loops that have been cut are shown. Taken from (Kwong et al. 1998).

### CD4-gp120 interaction

The crystal structure of gp120 engaged with CD4 and an antibody showed that CD4 occupies a cavity between the inner and outer domain of gp120. The structure of CD4 is not altered by binding as the structure of free and bound CD4 Domains D1/D2 do not show any striking changes. The binding site on gp120 is free of carbohydrate residues and the residues of CD4 at the binding site were similar to the residues that were identified by mutational analysis. The important residues are R59 and F43 with F43 alone accounting for about one quarter of all interatomic contacts. Interestingly, 60 % of the contacts to CD4 are made by main-chain atoms of gp120. This has consequences for the ability of antibodies to neutralise gp120. As antibodies are predominantly directed towards side chain atoms, a mutation in gp120 that changes the side chains will lead to the loss of binding ability of the antibody, however, as the contacts to CD4 are made by the main chain, this interaction will not be influenced. This is one example for the strategies of HIV-1 to escape immune surveillance (Kwong et al. 1998; Wyatt et al. 1998a). The gp120-CD4 binding is of very high affinity compared to the majority of protein-protein interactions. Myszka et al. (2000) report an extremely high binding enthalpy (-63 kcal/mol) balanced by a substantial loss in the degrees of freedom of both molecules upon binding which still results in a very strong binding constant of 5 nM (Myszka et al. 2000).

### **Structural changes triggered by the CD4-gp120 interaction**

Binding of gp120 to CD4 has been reported to cause rearrangements in the CD4 domain D1 (Denisova et al. 1997). This has been probed using various monoclonal antibodies directed against CD4. Similar to the binding of the various ligands of CD4, binding of gp120 also causes signalling events through CD4 that can lead to functional changes such as T cell apoptosis (Laurent-Crawford et al. 1993). (The role of gp120 induced signalling will be discussed later). Interestingly, this observed structural change is contradictory to the observation of Kwong et al (1998) that CD4 structure is not significantly altered upon gp120 binding (Kwong et al. 1998). More important in the context of HIV-1 entry is the conformational change in gp120 structure upon CD4 binding. One early observation pointing towards a structural change in gp120 taking place after CD4 binding was the shedding of gp120 from virions after binding of sCD4 (Moore et al. 1990). This rearrangement was found to be concentration, temperature and time dependent (Hart et al. 1991) and was then (not now) thought to be an integral event in the virus entry. Further evidence came from the reported increased sensitivity of the gp120 V3 loop to protease cleavage (Sattentau and Moore, 1991) and from studies with monoclonal antibodies indicating the exposition of previously masked regions on gp120 upon CD4 binding (Sattentau and Moore, 1991; Thali et al. 1993). The main rearrangements that are thought to occur are a movement of the V1/V2 loop structure together with a rearrangement of the gp120 hydrophobic core structure bringing together previously loosely associated regions (Wyatt et al. 1995; Zhang, W. et al. 1999; Myszka et al. 2000). The gp120-CD4 crystal structure suggests that the observed structure with normally hidden sites exposed, is unlikely to exist without CD4 bound to gp120. That is this structure developed after binding of CD4 through rearrangements (Kwong et al. 1998). The accompanied increase of order accounts for the unfavourable entropy of the reaction which is still efficiently driven by the high binding enthalpy (Myszka et al. 2000) (See standard textbooks on thermodynamics for explanations of enthalpy and entropy). After rearrangement, the coreceptor binding site becomes exposed on gp120. The mechanism of masking the coreceptor binding site is thought to contribute to HIV-1 immune evasion. The coreceptor binding site is conserved and also susceptible to neutralising antibodies. Therefore, hiding this area from immune surveillance as long as possible helps the virus to escape

the attack of neutralising antibodies. Thus the use of CD4 as a receptor followed by exposure of the coreceptor binding site is thought to be an evolution from a primordial system where only the coreceptor was used for binding. In such a system, the coreceptor binding site would be permanently exposed to the host immune system and the virus more likely to be eradicated by neutralising antibodies (Wyatt and Sodroski, 1998; Doms and Trono, 2000).

### **Binding of the Coreceptor**

The fact that CD4 alone is not sufficient for full entry of HIV-1 into target cells (Chesebro et al. 1990) but triggers conformational changes (discussed above) finally led to the discovery of coreceptors which are bound after the CD4-induced rearrangement of gp120. The two major coreceptors that are used by HIV-1 are “CC”-type chemokine receptor 5 (CCR-5) (Trkola et al. 1996; Wu, L. et al. 1996) and “CXC”-type chemokine receptor 4 (CXCR4) (Lapham et al. 1996). Several other potential coreceptors besides these have also been described (Berger et al. 1999).

The use of different coreceptors finally elucidated the mystery of virus tropism. It was found that most primary HIV-1 isolates, as well as the HIV-1 strains mediating transmission, are M-tropic, which means that they infect only macrophages and monocytes (and also activated primary T cells but not T cell lines). Conversely, HIV-1 strains that are associated with disease progression predominantly infect T-lymphoblastoid cell lines and primary T-lymphocytes and are thus called T-tropic. The finding that M-tropic strains enter their target cells predominantly via CD4 and CCR5, whereas T-tropic strains enter T-lymphoblastoid cells and primary T-lymphocytes via CD4 and CXCR4 could provide an explanation for the viral tropism (Berger 1997). According to the coreceptor usage, strains are now termed R5 (if they use CCR5) or X4 if they use CXCR4. Strains responsible for transmission are usually R5 viruses whereas X4 viruses evolve later during disease progression. The emergence of dual tropic R5X4 strains prior to X4 strains supports the concept of virus evolution (Berson and Doms, 1998). Mutations in coreceptors can confer high level resistance to HIV-1 infection as for example observed for a 32 bp deletion mutant in CCR5 that leads to a non-functional

receptor. Homozygous carriers of this mutation are highly resistant to HIV-1 infection (Samson et al. 1996).

The coreceptor binding site on gp120 is the region exposed during the rearrangement of gp120 (mainly V1 & V2 loops) that follows CD4 binding as well as part of the V3 loop (Kwong et al. 1998). Important residues that make contact with the coreceptor on gp120 are the basic and polar residues K121, R419, K421 and Q422 (Kwong et al. 1998). A later report describes the residues I420, K421, Q422, P438 and G441 as important (Rizzuto and Sodroski, 2000). Those basic residues are likely to interact with the acidic N-terminus of CCR-5 (Kwong et al. 1998). This mechanism for the gp120-CCR5 interaction is confirmed by the interaction of tyrosine sulfated peptides comprising the CCR5 N-terminus with gp120 (Cormier et al. 2000) and the inhibition of HIV-1 entry by a similar peptide (Farzan et al. 2000). Those findings point towards an importance of the tyrosine residues in the CCR5 N-terminus for HIV-1 entry. Residues on CXCR4 that are critical for HIV-1 entry are Y7, Y12, D193, D262 and D97. Again, the critical residues are either tyrosines or acidic residues pointing towards a similar mechanism for both CCR5 and CXCR4 binding through gp120 (Brelot et al. 2000). The evolution from R5 to X4 viruses is thought to be an accumulation of positively charged residues in the V3 loop that enable gp120 to interact with a second negatively charged surface of CXCR4 (Berson and Doms, 1998).

### **Membrane fusion**

The final stage of the virus entry (and the target for the newly developed drug T20) is the fusion of viral and host membrane. It is not fully understood how the recruitment of the coreceptor finally triggers the steps leading to the membrane fusion, but several of the structural changes in gp41 that lead to fusion have been described (Doms and Trono, 2000). The fusion is mediated by insertion of the fusion peptide, a trimer of helical bundles, into the host cell membrane (Blair et al. 2000). Conformational changes in gp41 lead to the formation of a six-helix bundled gp41 ectodomain core structure which presumably occurs concomitantly with fusion (Melikyan et al. 2000). The opening of the fusion pore then enables the entry of the viral capsid (Melikyan et al. 2000). For more

information on membrane fusion see the reviews of Doms et al. (2000), Blair et al. (2000) and especially Gallo et al. (2003).

### **Preformed complexes of CD4 with the chemokine receptors**

Some evidence has been reported on complexes of CD4 with the chemokine receptors in the absence of gp120. Though Ugolini et al. could only detect an increased colocalization of CD4 and CXCR4 upon incubation with gp120 (Ugolini et al. 1997), Xiao et al. (1999) report a constitutive cell surface association between CD4 and CCR5 even without treatment with gp120. Further studies have shown colocalization of CD4, CXCR4 and CCR5 in microvilli on macrophages and T cells facilitating interaction of HIV-1 gp120 with the chemokine receptors upon CD4 interaction (Singer, II et al. 2001). However, more evidence is needed to prove whether direct protein-protein interactions between CD4 and the coreceptors really exist.

### **1.2.3.3 Reverse transcription and integration**

After the naked viral nucleocapsid has entered the cell, it adheres to the internal surface of the cell membrane and is reorganised into a preintegration complex (PIC) (T. Cunningham, *pers. communication*). This process is facilitated by cellular kinases contained within the virion, assisted by cyclophilins and sensitive to cyclosporin A (Luban et al. 1993; Levy 1998). Subsequently, a double stranded DNA copy of the viral RNA is synthesised. This process is catalysed by the enzyme reverse transcriptase and initiated through binding of a cellular tRNA<sup>Lys</sup> to the primer-binding site on the viral RNA. The synthesis of the DNA is a complex process of RNA-dependent DNA synthesis, DNA-dependent DNA synthesis, degradation of viral RNA by RNase H and strand transfer reactions (Hsu and Wainberg, 2000). The nucleocapsid protein p7 is involved in this process. Reverse transcription proceeds within the preintegration complex consisting of MA, vpr and integrase proteins while being transported to the nucleus. This transport occurs along microtubules and mediated by cytoplasmic motor proteins (McDonald et al. 2002) though the exact mechanism of transportation is still subject to research. After transport to the nucleus vpr and MA trigger the entry of the complex through the intact nuclear envelope. (Bukrinsky et al. 1993; Heinzinger et al.

1994). Then the viral DNA (provirus) is integrated into the host genome. This reaction is catalysed by the viral integrase enzyme (Brown, P. O. 1997). This reaction completes the infection of a cell with HIV-1 and can be followed by a period of cellular latency, where the provirus is present in the genome but no proviral RNA and viral proteins are synthesised (Levy 1998).

#### **1.2.3.4 Transcription of viral DNA and synthesis of viral proteins**

The first mRNA species synthesised after integration is a doubly spliced mRNA coding for the regulatory proteins *tat*, *rev* and *nef* (Frankel and Young, 1998). The promoter that is responsible for the transcription is located in the 5' LTR region and susceptible to the cellular molecules NF- $\kappa$ B, SP-1 and TBP (Jones, K. A. et al. 1994). However, transcription is very inefficient and results in premature termination until the viral protein *tat* is synthesised. This accessory protein interacts both with RNA polymerase II and the TAR element of the synthesised viral RNA and other cofactors eg cyclin T1 and cdk 9 leading to an increase in the transcription of viral RNA by about 100 fold (Brigati et al. 2003). After establishing high level RNA transcription and the synthesis of the regulatory (early) proteins, full length viral RNA must be synthesised for both the production of the *gag*, *pol* and *env* proteins as well as for assembly of new virions which require full length RNA. This switch from production of spliced RNA to unspliced RNA is accomplished by the *rev* protein. This protein binds to the *rev* response element (RRE) on nascent RNA and leads to the export of unprocessed full length RNA into the cytosol that can then be translated into *gag*, *pol* or *env* proteins or incorporated into new virions (Fischer et al. 1995). *Gag* and *pol* are synthesised in the cytosol whereas *env* is translated at the ER (Frankel and Young, 1998). See the review from Peterlin and Trono (2003) for more details on HIV-1 transcription.

#### **1.2.3.5 Virus assembly and budding**

The release of new virions from infected cells requires the assembly of all particles that make up the new virion in a defined order inside the cell membrane. This process is governed by intrinsic features of the *gag* and *gag-pol* polypeptides and assisted by some of the accessory proteins. *Gag* and *gag-pol* are assembled into the virion unprocessed and

are only cleaved into the individual components by the viral protease during or after the budding (Frankel and Young, 1998). Nevertheless, the individual parts of gag play different roles in the assembly process:

The matrix protein MA, which forms the N-terminal part of gag is myristoylated and assembles into trimers. Both trimerisation and membrane location, which is mediated by the acyl group and a stretch of basic amino acid residues is required for formation of new virions. The MA trimer also contains pockets to accommodate the cytoplasmic tails of the env proteins (Hill et al. 1996). The second component of gag, CA, also functions in viral assembly through its ability to dimerise the domain for which is located at the C terminus (Gamble et al. 1997). The NC protein, also part of the gag polypeptide, interacts with the  $\Psi$ -signal on the RNA through two zinc finger domains. Together with the ability of the RNA to dimerise via its kissing loop, this leads to the incorporation of exactly two copies of viral RNA (Schmalzbauer et al. 1996; Clever and Parslow, 1997). The C-terminal part of gag, p6 recruits vpr to the assembling particle (Kondo and Gottlinger, 1996). Other proteins that become incorporated into the budding virion except for vpr are nef (about 70 molecules) and vif, which is necessary for the production of infectious viruses in some cell types (non-permissive, discussed later in this review).

The env protein, which was synthesised in the ER and cleaved into gp120 and gp41 by cellular proteases (Moulard et al. 2000) is transported to the surface where the bud forms with the aid of nef and vpu. Vpu degrades preformed CD4-gp120 complexes and nef assists in the downregulation of CD4 to prevent the formation of such complexes on the cell surface (Bour et al. 1995b; Mangasarian and Trono, 1997). Once all parts are assembled the capsid is completely surrounded by the host cell-derived lipid bilayer and is released from the cell. This virion is not yet infectious but needs to undergo a final maturation step. The protease enzyme, which cleaves the gag and gag-pol polypeptides into the individual components MA, CA, NC, p6, IN, PR and RT is responsible for this maturation which is completed by structural rearrangements inside the capsid leading to a fully infectious virus (Kaplan et al. 1994; Frankel and Young, 1998). Reports have shown that trafficking of HIV-1 particles is dependent on lipid rafts (see part one of this review) and virus budding occurs through rafts supporting the importance of those domains in viral pathogenesis (reviewed by (Campbell et al. 2001)). Apart from rafts, some cellular

proteins have also been shown to be required for correct virus assembly (Garrus et al. 2001; Zimmerman et al. 2002).

#### **1.2.4 HIV-1 target cells and the formation of viral reservoirs**

Although CD4<sup>+</sup> T lymphocytes are the main target for HIV-1 a broad range of other cells in various tissues can be productively infected with HIV-1. Many other cells have been shown to contain HIV-1-DNA through sensitive PCR techniques (Levy 1998). The main target tissue types for HIV-1 are haematopoietic cells, lymphoid tissue, the brain and the gastrointestinal system. Other haematopoietic cells, apart from helper and memory T cells, that can be infected with HIV-1 are macrophages, monocytes or dendritic cells. (Cameron et al. 1992; Blauvelt et al. 1997; Levy 1998). HIV-1 has been detected in other blood cells like CD8<sup>+</sup> cells or NK cells but without any apparent physiological significance. More important is the infection of stromal fibroblasts and endothelial cells in the bone marrow (Scadden et al. 1990; Moses et al. 1996; Levy 1998).

Lymphoid tissue is another prime target of HIV-1 and the destruction of this tissue is associated with ongoing disease progression. Besides CD4<sup>+</sup> T cells residing in the lymph nodes, interdigitary dendritic cells can be infected by the virus (Levy 1998). The follicular dendritic cells (FDCs) of the lymphoid tissue, however are not infected by HIV-1, but can trap infectious viruses and present them to CD4<sup>+</sup> cells. Through this mechanism, FDCs lead to an extremely long infectivity of trapped HIV-1 virions (Smith, B. A. et al. 2001a). The level of HIV-1 in the lymph nodes is very high but still, only a fraction of the cells that are infected actively produces infectious virus (Chun et al. 1997a). Cells from the brain have also been shown to be susceptible to HIV-1. Productive infection occurs in resident macrophages and microglia cells and latent or nonproductive infection in astrocytes whereas neurons do not become infected. The exact mechanism of HIV-1 infection of brain cells in the cause of HIV-1 associated dementia has yet to be determined (Levy 1998; Kaul et al. 2001) although cellular toxins released from macrophages and acting on neurons appear to be important (Smith, D. G. et al. 2001b). Further tissues that are susceptible to HIV-1 are the gastrointestinal tract, where endothelial cells can be infected (Nelson et al. 1988) or the skin with a possible infection

of fibroblasts or langerhans cells (Kawamura et al. 2001). See (Levy 1998) for a more detailed overview of HIV-1 susceptible cells and tissues.

Though the development of the highly active antiretroviral therapy (HAART) can efficiently control HIV-1 replication, the virus is not totally eradicated from the body (Furtado et al. 1999). Monitoring the viral load after the start of the therapy shows a rapid decline during the first day representing the block of HIV-1 replication in CD4<sup>+</sup> T cells. After one day the viral load starts to fall more slowly, and the decline of this second phase has a half-life of about 2 weeks. This decline may be due to the eradication of HIV from cells more resistant to HIV cytopathic effects such as macrophages. Still the virus can be detected in body tissue and a third phase of decline with an extremely slow reduction of viral load shows that it would require more than 60 years on average of treatment to eradicate HIV-1.

This behaviour is due to the ability of HIV-1 to remain quiescent in viral reservoirs. As mentioned above, HIV-1 can infect a broad range of cell types, and depending on the latency of the infection and the average life span of the infected cell, survive very long without signs of replication. The most important viral reservoirs responsible for the three-phase decline of the viral load in response to therapy are first the short-lived CD4<sup>+</sup> T lymphocytes, which are replicating rapidly but are very sensitive to antiviral therapy. The block of HIV-1 replication in this cell type accounts for the steep decline of viral load during the first day of therapy (Abbas et al. 2000). HIV-1 infection of macrophages is probably responsible for the slower decline of HIV-1 load in the second phase (Abbas et al. 2000; Igarashi et al. 2001). However, drug therapy is not able to totally eradicate the virus from the body. This is due to the virus infecting microglial cells in the CNS, an immunological sanctuary where cell-mediated responses are reduced (Blankson et al. 2002). The third phase is provided by resting T cells where HIV remains for a long time in a state called proviral latency. Those cells can harbour the virus for a long period of time and then produce infectious viruses upon activation (Chun et al. 1997b; Blankson et al. 2002). Other cells in which HIV-1 enters a stage of proviral latency might include DCs, monocytes, microglia and seminal cells (Peterlin and Trono, 2003).

### **1.2.5 Viral and host factors in HIV-1 progression**

After discussing structure, life cycle and cellular targets for HIV-1, it is important to know how this virus interacts with the host system. What are the immune responses launched against HIV-1 by the host and how is it able to escape them? How does HIV-1 eventually destroy the host immune system? What are the roles of the various accessory proteins in the interaction with the host and why is HIV-1 replication susceptible to interleukin-16? Those questions will be discussed in the following sections.

#### **1.2.5.1 Immune response to HIV-1 and immune evasion**

The immune system, once confronted with HIV-1, launches both a cell-mediated and a humoral response that are capable of controlling the acute HIV-1 infection (Abbas et al. 2000). This first phase of control is accompanied by immune evasion of the virus. A cell-mediated immunity is the first response that can be detected after infection and consists of killing of HIV-1 infected cells by natural killer cells (NK cells) and CD8<sup>+</sup> CTLs. Indeed, there is a massive expansion of CD8<sup>+</sup> CTLs in the initial response and those cells account for the partial control of HIV infection during the early and late stage (Levy 1998; Abbas et al. 2000; McMichael and Rowland-Jones, 2001). Antibodies against HIV-1 can be detected from 6 to 9 weeks post infection and are mainly directed against the HIV-1 env protein, but antibodies against p24, gag, pol or RT have also been found (Abbas et al. 2000). However, the majority of those antibodies fail to have any effect on HIV-1. In contrast they can lead to enhanced effectiveness of HIV-1 through Fc receptor-mediated endocytosis of antibody coated virions (Levy 1998). A small portion of the antibodies, however have neutralising ability against HIV-1, those antibodies predominantly target epitopes on gp120 and some epitopes on gp41. The principal neutralising domain targeted by the antibodies is the V3 loop of gp120, a second domain the CD4 binding site. However, the overall role of neutralising antibodies in host defence is thought to be rather small. See Levy (1998) for a very detailed discussion of cellular and humoral immune response against HIV-1 or McMichael and Rowland-Jones (2001) for a review on the cellular immune response to HIV-1.

As mentioned above, the host immune system is not able to totally eradicate an HIV-1 infection. Several reasons account for this HIV-1 immune evasion. First, HIV-1 creates proviral latency in some cells and infects cells in immunological sanctuaries (discussed in chapter 4). Those cells provide an ongoing source of new virions while they are very hard to control by the immune system (see (McMichael and Rowland-Jones, 2001) and (Peterlin and Trono, 2003) for more details). Secondly, the high mutation rate during the HIV-1 reverse transcription leads to mutations in the most antigenic epitopes of gp120. Those mutated epitopes escape both the surveillance of neutralising antibodies and CD8<sup>+</sup> CTLs. It is thought that those mutations lead to the development of highly resistant virus quasispecies under the selective pressure of the immune system (McMichael and Rowland-Jones, 2001). Additionally, like so many other viruses, HIV-1 downregulates MHC class I molecules on the surface of infected cells. Those molecules normally present peptides derived from intracellular proteins (including viral proteins in infected cells) to CD8<sup>+</sup> CTLs helping in the killing of infected cells. Downregulation of MHC I molecules helps infected cells to escape recognition by CTLs (Schwartz et al. 1996). Interestingly, only the MHC I molecules HLA-A and HLA-B are downregulated, which prevents infected cells from being killed by natural killer cells that recognize cells that do not express any MHC I molecules (Lichtor et al. 1999). It has been found that HIV-1 specific CD8 CTLs have a reduced expression of perforin. That renders them less efficient in target cell lysis (Appay et al. 2000).

### **1.2.5.2 HIV-1 immune suppression**

One of the main features of HIV-1 infection is the eventual destruction of the host immune system. Several mechanisms induced by HIV-1 directly or indirectly contribute to the loss of host defence. The main contribution is the destruction of the CD4<sup>+</sup> T cell reservoir. Several mechanisms have been proposed for this as reviewed by McCune (2001). The loss of CD4<sup>+</sup> T cell function is due to anergy early after infection with HIV and later because of killing of the CD4<sup>+</sup> T cells. This increased destruction of mature CD4<sup>+</sup> T cells in blood and lymphoid tissue is accompanied by an HIV-1 induced impaired production of those cells from the thymus. The causes for the destruction of mature CD4<sup>+</sup> T cells are direct destruction of infected cells as a consequence of the

expression of viral gene products like env as well as indirect induction of death in bystander cells that are not infected. The death of peripheral CD4<sup>+</sup> T cells can also be induced by HIV specific CTLs (but only 10-20 % of the CD4 death is due to CTLs), through autoimmune reactions and via fusion of infected cells with uninfected cells by gp120-CD4 reactions (syncytia formation). Death can also be induced through action of unbound gp120 on uninfected cells, as a consequence of contact with an HIV infected antigen presenting cell or as a consequence of increased Fas expression of infected cells induced by nef. See McCune (2001) and Peterlin and Trono (2003) for a detailed discussion of that matter.

Much research has been devoted to the apoptotic properties of viral proteins mainly focussing on the apoptotic effects of gp120. Thus binding of gp120 to CXCR4 has been proposed to trigger T cell apoptosis (Roggero et al. 2001). Conversely, binding of gp120 to CD4 has been reported to prime T cells for activation-induced apoptosis (Banda et al. 1992), see (Badley et al. 2000) for a review on HIV-1 associated lymphocyte apoptosis. A decrease in the overall T cell count could be enhanced owing to a different migration pattern of CD4<sup>+</sup> T cells upon HIV-1 infection, additionally, reduced CD4<sup>+</sup> T cell production contributes to the loss of those cells. The mechanisms for reduced production of CD4<sup>+</sup> T cells may be direct consequences of HIV-1 infection of progenitor cells in the thymus or destruction of the supporting tissue needed for T cell maturation by the virus. Indirect mechanisms leading to decreased CD4<sup>+</sup> T cell production are cytokine dysfunctions, opportunistic infections, HIV-1 induced apoptosis, infiltrating malignancies, effects of drug therapy or vitamin deficiency (McCune 2001). Another contribution may be the exhaustion of the reservoir of memory T cells during the infection.

In summary, HIV-1 infection leads to a chronic infection of immune cells by virus mutants that escape the initial infection. This chronic infection leads to a persistently activated immune system through permanent exposure to the virus. HIV-1 induced T cell destruction together with reduced production of CD4<sup>+</sup> T cells leads to a final exhaustion of the immune system and to its collapse resulting in the establishing of opportunistic infections (AIDS) that eventually kill the host.

### **1.2.5.3 Functions of the HIV-1 accessory proteins**

Besides gag, env and pol, the HIV-1 genome codes for the six accessory proteins tat, rev, vpr, vif, vpu and nef which play various roles in HIV-1 infection. This section summarises the function that is known of the individual proteins.

#### **Tat**

It was discovered early that the HIV-1 tat protein is required for efficient replication of HIV-1. This was demonstrated as tat-defective viruses fail to productively replicate which is compensated by introduction of the tat gene that then positively regulates the expression of viral proteins (Dayton et al. 1986). It is now known that tat functions both in transcriptional activation and elongation of viral transcripts. In the absence of tat, not only is the transcriptional activation of the HIV-1 promoter poor, but also the RNA polymerase II prematurely terminates the transcription. The transcriptional activation of tat is via binding to the TAR sequence on the nascent RNA and subsequent recruitment of numerous transcriptional enhancers such as CDK 9 or cyclin T1. Those factors not only bridge transcriptional activators with the basal transcription machinery, but they also function as histone acetyl transferases which make chromatin more accessible to transcription (Brigati et al. 2003).

Interestingly, tat can be secreted from HIV-1 infected cells via a leaderless secretory pathway (Chang et al. 1997) reviewed by (Brigati et al. 2003). Extracellular tat can then bind to receptors on the cell membrane triggering signal transduction events (Brigati et al. 2003). Other reports on tat include the induction of apoptosis in endothelial cells (Park et al. 2001), cross-talk with p53 in an indirect way (Ariumi et al. 2001), chemotaxis (Lokensgard et al. 2001) and influence on proviral latency and MHC I gene expression as reviewed by (Peterlin and Trono, 2003).

#### **Rev**

As mentioned in section 1.2.3.4, the rev protein is required for the export of full length RNA for the production of the structural proteins or the assembly of new virions. Rev thereby overcomes the default splicing pathway by actively promoting RNA export rather than by inhibiting the splicing pathway (Fischer et al. 1995; Frankel and Young, 1998).

Rev is a nucleocytoplasmic shuttle protein that functions via binding to the rev response element (RRE) that is present on unspliced and incompletely spliced mRNAs. Upon multimerisation, rev binds to the export receptor CRM1/exportin1 via its nuclear export signal (NES) that constitutes of a short stretch of hydrophobic amino acids, mainly leucine (Fischer et al. 1995; Frankel and Young, 1998; Hofmann et al. 2001). Other factors that are required for the export process are eIF-5A and RanGTP (Elfgang et al. 1999; Hofmann et al. 2001). However, some reports suggest that entry into the splicing process is required for rev function and that rev can also inhibit nuclear splicing as reviewed by Frankel and Young (1998). See this report also for a structural discussion of the association between rev and the HIV-1 mRNA.

### **Vpr**

Vpr is a 96 amino acid highly conserved protein the structure of which is characterised by the formation of two alpha helical domains. Vpr is incorporated into the virus and it exerts several different functions: transcriptional activation; cell cycle arrest and nuclear localisation are the main functions that are known (Frankel and Young, 1998; Sherman et al. 2002).

The effect of HIV-1 Vpr on the cell cycle has long been known, however, the mechanism through which this effect is performed is not completely clear. It is assumed that vpr interferes with upstream phosphorylation mechanisms ultimately leading to the inactivation of cdc2 that then causes the cell cycle arrest in the G2 phase (Bour et al. 2000). Interestingly, virus associated vpr is sufficient to induce cell cycle arrest so that unproductively infected cells are locked in the G2 phase without viral proteins being synthesised (Poon et al. 1998). The cell cycle arrest by itself could already contribute to the transcriptional activation of vpr as the G2 phase is characterised by increased transcriptional activity (Goh et al. 1998). However, an intrinsic transcriptional-activating feature of vpr has been reported and is assumed to be associated with the leucine-rich region and mediated via binding to the p300 transcriptional activator (Felzien et al. 1998).

Another important role of vpr is the promotion of nuclear import of the preintegration complex into the nucleus. This active transport mechanism allows

infection of nondividing cells like macrophages as it overcomes the need for cell division and concomitant dissolution of the nuclear envelope for integration of the DNA into the nucleus. The role of vpr in nuclear import is not precisely defined. It is supposed to stabilise the interaction of the preintegration complex with the importin system (Popov et al. 1998). A different group has found recently that vpr actually contains two different nuclear localisation signals that are distinct and can use different receptors for their function (Jenkins et al. 1998).

Besides those functions, vpr has been shown to have apoptosis-modulating effects. It has the ability to induce host-cell apoptosis through direct binding to the mitochondrial membrane (Muthumani et al. 2003).

### **Vif**

Vif is a 23 kDa/192 amino acid basic protein that is incorporated in the virion and the function of which is associated with the production of infectious virus. This has been shown by evidence that cells infected with vif mutants produced viruses that are not infectious. Interestingly, only certain cell types show this observation. Whereas production of viruses in “permissive” cell types does not require a functional vif protein, virions produced by “nonpermissive” cells infected with vif mutated viruses are generally not infectious (Frankel and Young, 1998; Bour et al. 2000).

The mechanism of this phenomenon has been elusive until recently a human gene was isolated which inhibited HIV infection and was suppressed by vif (Sheehy et al. 2002). The gene was named Cem15 (now APOBEC3G) and has been shown to act as a cytidine deaminase inducing G to A hypermutation in newly synthesised viral DNA. This negative effect on the virus is counteracted by vif (Mangeat et al. 2003; Zhang, H. et al. 2003). The remaining effect of hypermutation on viral reverse transcription, however, contributes to the genetic variability of the virus (Harris et al. 2003).

Additionally, it has been shown that vif does associate with the cytoskeleton, precisely with the component of the intermediate filament, vimentin. However neither the function of this association is known, nor whether it is an independent role for vif or whether it is related to its role in viral infectivity (Bour et al. 2000).

## **Vpu**

Vpu is an 81 amino acid/17 kDa phosphoprotein that consists of a transmembrane helical domain and a cytoplasmic domain made up of two amphipathic helices that are separated by a linker region. It has several different functions, one of which is the formation of discrete ion-conducting channels in the lipid bilayer. This function is associated with the transmembrane domain of the protein (Marassi et al. 1999).

More important is the role of vpu in degrading newly synthesised CD4 in the ER. CD4 and env often form complexes in the ER preventing env from migrating to the cell surface. Vpu has been shown to physically interact with CD4 in the ER and this interaction is necessary to promote degradation of CD4 (Bour and Strebel, 1995). This interaction is mediated either directly or indirectly through the cytoplasmic tails of both molecules and regulated by the key serine residues S52 and S56 in the vpu molecule (Paul and Jabber, 1997). The mechanism of this degradation is thought to be the interaction of vpu with components of cellular proteasomes, however the exact way of CD4 degradation is not clear (Bour et al. 2001).

Additionally, vpu promotes increased particle release, which is thought to be a distinct function from the CD4 degradation and is dependent of the proliferation state of the cells. Vpu is therefore required for the efficient particle release from quiescent cells (Deora and Ratner, 2001). Recent data shows that vpu promotes the targeting of gag to the plasma membrane which is a possible way the protein exerts its function (Deora et al. 2000). Interestingly, CD4 has been found to inhibit the efficient release of viral particles (Bour et al. 1999).

## **Nef**

HIV-1 nef is a 206 amino acid protein, which is myristoylated at its N-terminus. A functional nef protein is essential for a productive HIV-1 infection as the absence of an intact nef gene was linked with long term nonprogressive HIV-1 infection (Kirchhoff et al. 1995). Like the other HIV-1 auxiliary proteins, nef exerts multiple functions that are important for HIV-1 pathogenesis. The most important roles for nef are downregulation of both MHC class I molecules and CD4 molecules (Mangasarian et al. 1999) but nef has

also been shown to increase viral infectivity (Chowers et al. 1994), to induce apoptosis in bystander cells but to protect the infected cell from apoptosis (Geleziunas et al. 2001).

The mechanism through which nef increases viral infectivity includes nef-mediated T cell activation which is caused by the interaction of nef with parts of the T cell receptor complex (Fackler and Baur, 2002). Another effect of this is the upregulation of Fas ligand on virally infected cells leading to the death of uninfected bystander cells via the Fas pathway (Geleziunas et al. 2001; Fackler and Baur, 2002). Interestingly, nef also promotes the expression of Fas on infected cells (Debatin et al. 1994) but protects those cells from apoptotic death through interfering with the necessary signalling cascades. Nef has hereby been shown to inhibit the ASK1 molecule which plays a key step in both Fas and TNF $\alpha$ -induced apoptosis and thus confers resistance to apoptosis to infected cells (Geleziunas et al. 2001).

The downmodulation of MHC class I molecules through nef protects HIV-1 infected cells from death induced from CTLs (Collins et al. 1998). The mechanism through which nef downregulates the HLA-A and HLA-B molecules is not fully understood but it has been shown that nef induces the diverting of cell-surface MHC I molecules to endosomal compartments. This is supposedly done through bridging of MHC I molecules to complexes of the endocytosis adaptor proteins by nef but direct binding of nef to MHC I molecules has not yet been shown (reviewed by (Peterlin and Trono, 2003)).

The way in which nef induces CD4 downregulation is better understood and is a sequential pathway of different events modulated by nef (Doms and Trono, 2000). Nef directly binds to the CD4 cytoplasmic tail (Preusser et al. 2001) as well as to the adaptor protein complex of clathrin coated pits leading to the formation of CD4 containing endocytic vesicles (Aiken et al. 1994; Mangasarian et al. 1997a). This interaction is probably mediated through a dileucine motif in nef. After formation of early endosomes, nef binds to the COP-I coatomer by a diacidic sequence triggering the sorting of the endosome towards the lytic pathway where CD4 is finally degraded (Piguet et al. 1999).

Other studies report the induction of cytoskeletal changes through nef (Fackler et al. 1999), the association of nef with lipid rafts (Wang, J. K. et al. 2000a) and the

interference with other signalling pathways (Renkema and Saksela, 2000; Wolf et al. 2001).

#### **1.2.5.4 IL-16 mediated inhibition of HIV-1 replication**

In 1995 Baier et al described that IL-16 isolated from either African green monkeys or humans has an inhibitory effect on HIV-1 replication in lymphocytes (Baier et al. 1995). Several assays designed to determine inhibiting effects of ligands of HIV-1 receptors ruled out any interference of IL-16 with the HIV-1 entry (Center et al. 2000). Zhou et al. (1997) reported that the inhibition of HIV-1 replication occurs at the level of mRNA synthesis and not at viral entry or reverse transcription (Zhou et al. 1997). Additionally, it was found that pretreatment of CD4<sup>+</sup> lymphoid cells with rIL-16 repressed the HIV-1 promoter activity up to 60 fold and thus prevented both tat and PMA activation (Maciaszek et al. 1997). Transfection of T cells with IL-16 also inhibits HIV-1 replication and that can be enhanced by cotransfection of an anti gp41 antibody (Devadas et al. 2003). The mechanism by which IL-16 induces CD4 cross-linking in T cells and how it modulates the transcription of the HIV-1 DNA is not clear but in monocytic cells the repressive effect of IL-16 seems to occur at the entry stage (see (Kornfeld and Cruikshank, 2001) for more details).

### **1.2.6 HIV-1 Therapy and vaccine development**

#### **Drug therapy**

The first drug developed that was successful in inhibiting HIV-1 replication was AZT (zidovudine). This nucleoside analogue interferes with the reverse transcription of viral RNA probably by terminating DNA polymerisation and competing with nucleosides. The detailed mechanism of its action however, is not yet understood. Following that, other nucleoside analogues and non-nucleoside inhibitors of RT (NNRTI) have been developed including didanosine, zalcitabine and stavudine (NRTIs) and nevirapine and efavirenz (NNRTIs). Apart from RT, the protease molecule is the second major target for antiretroviral drugs. Protease inhibitors interfere with the correct processing of the HIV-1 polypeptides into its functional components. Effective protease inhibitors used in HIV-1

therapy include saquinavir, ritonavir, indinavir or nelfinavir. Major problems with each of those drugs, apart from the side effects, are the evolution of mutated virus that are resistant to the drugs and the failure to eradicate viral reservoirs in macrophages or latently infected T lymphocytes (or more specifically in the brain). The most success has been achieved with a cocktail of two reverse transcriptase inhibitors in combination with a protease inhibitor and is termed “highly active antiretroviral therapy” (HAART). This therapy has led to the reduction of plasma RNA to undetectable levels in most patients for more than 3 years (Abbas et al. 2000). See also (Levy 1998) for a more information on HIV-1 drug therapy.

The first temporal target site in HIV replication for anti HIV-1 drugs is virus entry. After CD4 was established to be the receptor for HIV-1 entry, studies were conducted using sCD4 to inhibit the entry process. However, this approach failed during the clinical trials as the viral strains present *in vivo* were far less susceptible to sCD4 than the ones tested *in vitro* (Daar and Ho, 1991). Subsequent development of compounds interfering with the gp120-CD4 interaction that are currently investigated are PRO542, a fusion of CD4 with IgG2 or small molecule inhibitors like FP21399 or PRO2000. The discovery of the chemokine receptors as coreceptors for HIV-1 has introduced novel possibilities in the development of HIV-1 coreceptors. One strategy is to use the natural ligands of the chemokine receptors MIP-1 $\alpha$ , MIP-1 $\beta$ , RANTES (all CCR-5) and SDF-1 $\alpha$  (CXCR-4) as models for the development of components that block attachment of gp120/CD4 to the coreceptors. This is supported by the fact that the CCR-5 ligands are inhibitors of HIV-1 infection *in vitro* (Ross et al. 1999). A different strategy aims at blocking the epitope on gp120 responsible for attachment to CCR-5 or CXCR-4. Compounds developed towards blocking HIV-1 entry via this approach include a tyrosine sulfated peptide which is based upon the CCR-5 N-terminus peptide (Farzan et al. 2000). See also (Vermeire and Schols, 2003) for antiviral agents based on CD4 down-modulation.

A different target for antiviral drugs based on the HIV-1 entry is gp41. Several inhibitors of gp41 mediated membrane fusion have been developed, one of them, T20, a peptide based on the gp41 sequence has been approved as “Enfuvirtide” by most national authorities as a new antiretroviral drug and has been found to decrease viral load in

infected individuals (Lazzarin et al. 2003). However, treatment is very expensive and in the US the cost of a years supply is estimated to be just under \$20,000 (Steinbrook 2003). Despite the high cost, introduction of this new drug can be seen as an encouraging example of the clinically applied results of HIV-1 entry research. For further reading see (Steinbrook 2003).

### **HIV vaccine and post exposure prophylaxis (PEP)**

The development of an HIV-1 vaccine is still subject to intense research. A powerful vaccine probably needs to elicit both broadly neutralising antibodies and CD4<sup>+</sup>/CD8<sup>+</sup> memory T cells. To achieve that, viral antigens need to be effectively administered to trigger an immune response. Actual best strategies to achieve this are prime boost regiments, priming with DNA vaccines and boosting with recombinant vaccinia or recombinant pox viruses or using recombinant adenoviruses and/or recombinant oligomeric envelope protein. Unfortunately, there are still serious difficulties that need to be overcome to develop an effective HIV-1 vaccine. For example the heterogeneity of the virus makes it very difficult to generate broadly neutralising antibodies and effective T cells against all strains, clades and groups and the administration of life-attenuated viruses can still lead to pathogenesis induced by the defect virus. See (Tramont and Johnston, 2003) for more details and further reading.

Besides HIV-1 therapy and vaccine development, the existence of a post exposure prophylaxis (PEP) regiment should be mentioned that has been developed for immediate treatment of persons who were exposed to HIV-1 through situations such as sexual, needle sharing or contact of a child with milk from an HIV-1 infected woman. PEP consists of a short (28 days) retroviral treatment that needs to be started no longer than 72 h after exposure and consists of a combination of 2 to 3 retroviral drugs. PEP aims at stopping HIV-1 from disseminating through the body thus preventing the person from becoming HIV positive at all. See (Havens et al. 2003) for more information.

### **1.3 Summary**

The CD4 receptor is a multifunctional protein playing an essential role both in the immune response as in the pathogenesis of HIV-1. Additionally it may play a role in lymphotaxis but not much is known about that. The functions of CD4 are closely connected with its interactions. The interactions with CD45 and p56lck are well established and play a crucial role in the function of CD4 as a coreceptor in MHC class II recognition. However, much evidence points towards CD4 being associated with additional molecules. Reports have shown co-immunoprecipitations of various membrane proteins with CD4, novel intracellular ligands as well as the connection between CD4 and the cytoskeleton. Furthermore, CD4 is expressed on the surface of various cells that do not function in MHC class II recognition and the function of CD4 on those cells is not established. Those cells do not express p56lck and since CD4 itself cannot transduce signals, there are likely to be other associating partners in those cells. CD4-expressing cells are susceptible to infection with the HIV-1 virus. CD4 functions as a primary receptor to which the HIV-1 gp120 protein binds. Structural changes in gp120 lead to binding of the coreceptors and ultimately membrane fusion. Gp120 binding to CD4 and coreceptors is known to induce signalling and signalling through CD4 is likely to involve associated proteins. On lymphocytes, CD4 also functions as the receptor responsible for initial attachment of HIV. However, on other cell types other receptors can mediate the attachment of HIV. On dendritic cells, which are thought to be the first cells that HIV encounters after transmission, the virus attachment is mediated by a series of C-type lectin receptors one of which is DC-SIGN. Binding of DC-SIGN leads to internalisation of the virion, however, HIV induced changes in the endocytic routing of HIV prevents a proportion of it from degradation and allows virus transfer to T-cells. The quaternary structure of DC-SIGN has been suggested to be tetrameric and to mediate high affinity ligand binding. However, this has been done using synthetic ligands and recombinantly expressed extracellular DC-SIGN whereas no studies were conducted demonstrating quaternary structure of native DC-SIGN on dendritic cells and studying the influence of quaternary structure on binding affinity using its native ligands such as gp120.

## **1.4 Outline of thesis**

The work presented in this thesis bridges medical research with the relatively new discipline of proteomics. While this was a very difficult task, it has eventually proven to be extremely rewarding. Successful prosecution of this project required an understanding of the biology of the human immunodeficiency virus and its main receptor CD4 together with an evaluation and practical exposure to techniques broadly classified as “proteomics”. To reflect this knowledge in this thesis, the extensive literature review on the cell surface receptor CD4 and the human immunodeficiency virus presented above aims to provide a comprehensive introduction on the biology of this thesis. To address the knowledge required for this thesis on the use of mass spectrometry and “proteomics”, a brief introduction to mass spectrometry has been included into the chapter describing the methods that were used in this thesis. Each of the individual results chapters is designed to be “self-contained” with respect to the literature required for understanding the results and evaluation of the results within the available literature.

## **CHAPTER TWO:**

### **MATERIALS AND METHODS**

## 2.1 Cells

The human lymphoblastoid cell lines CemT4 and the CD4 negative lymphoblastoid derivative cell line A2.01 (both DC-SIGN negative) were obtained from the NIH AIDS Research and Reference Reagent Program (Rockville, MD, USA). The OKT4 hybridoma cell line was obtained from the American Type Culture Collection (ATCC) (Rockville, MD, USA). The Q4120 and the Q425 hybridoma cell lines were a generous gift from Dr Quentin Sattentau (Centre d'Immunologie de Marseille-Luminy, Marseille, France). WM82 hybridoma cells were generously donated by Dr Tony Hennicker (Westmead Hospital, Westmead, NSW, Australia). Monocyte-derived dendritic cells were obtained from Dr John Wilkinson, Dr Andrew Harman or Ms Kerrie Dunstan (all Centre for Virus Research, Westmead Millennium Institute, Westmead, NSW, Australia) and prepared from isolated blood monocytes as described elsewhere (Turville et al. 2002). Cells were normally maintained in RF-10 medium (CSL, Parkville, Vic, Australia) or in protein free hybridoma medium (CSL, Parkville, Vic, Australia) for antibody production.

## 2.2 Equipment

The following equipment was used for the experiments described in this thesis

**Table 2.1** Equipment used in this study:

Equipment	Source
<b>Chromatography</b>	
Biologic Low Pressure chromatography system	BioRad, Regents Park, NSW, Australia
Model 221 Fraction Collector	BioRad, Regents Park, NSW, Australia
<b>Electrophoresis and western blotting</b>	
Mini-PROTEAN II electrophoresis system	BioRad, Regents Park, NSW, Australia
Mini Trans-Blot Transfer Cell	BioRad, Regents Park, NSW, Australia
CP 1000 X-Ray processor	Agfa, Pymble, NSW, Australia

### **Mass spectrometry sample preparation and analysis**

Q-ToF2™	Micromass, Manchester, UK
Speed-vac™ vacuum centrifuge	Savant, Farmingdale, NY, USA

### **Spectroscopy and imaging**

Beckman DU520 UV/Vis Spectrometer	Beckman Coulter, Fullerton, CA, USA
Odyssey infrared imaging system	Li-Cor Biosciences, Lincoln, NE, USA
Fluor-S Multi-imager, Gel documentation System	BioRad, Regents Park, NSW, Australia

### **Flow cytometry and FRET measurements**

FACScalibur flow cytometer	Becton Dickinson, San Diego, CA, USA
FACSVantage SE DiVA	Becton Dickinson, San Diego, CA, USA

## **2.3 Buffer solutions**

The following buffers were used in the work described in this thesis

<i>Phosphate-buffered saline (PBS)</i>	<i>SDS-PAGE reservoir buffer</i>
50 mM Na <sub>2</sub> HPO <sub>4</sub>	25 mM Tris
140 mM NaCl	192 mM Glycine
pH 7.5	0.5 % (w/v) SDS
	no pH adjustment required
<i>HEPES-buffered saline (HBS)</i>	<i>Western blot transfer buffer</i>
10 mM HEPES	25 mM Tris
140 mM NaCl	192 mM Glycine
pH 7.5	0.025 % SDS
<i>TRIS-buffered saline (TBS)</i>	pH 8.3
25 mM TRIS	

140 mM NaCl  
3 mM KCl  
pH 8.0 or pH 7.5 as indicated

*Western blot wash buffer*  
1 x TBS, pH 8.0  
0.05 % (v/v) Triton X-100

*Cross-link buffer (CLB)*

10 mM HEPES  
140 mM NaCl  
1 mM MgCl<sub>2</sub>  
0.1 mM EGTA  
0.02 % (w/v) NaN<sub>3</sub>  
pH 8.0

*FACS buffer*

1 x PBS, pH 7.5  
0.1 % (w/v) BSA  
0.1 % (w/v) NaN<sub>3</sub>

*Lysis buffer 1*

1 x HBS, pH 7.5  
1 % (v/v) Triton X-100  
1 % (v/v) NP-40  
10 mM MgCl<sub>2</sub>  
10 U/mL Dnase  
1 mM PMSF  
10 µg/mL Aprotinin  
100 µg/mL Soybean Trypsin Inhibitor  
10 µg/mL Leupeptin

*Coupling buffer*

0.1 M NaHCO<sub>3</sub>  
0.5 M NaCl  
pH 8.3

*Acetate buffer*

0.1 M acetic acid  
0.5 M NaCl  
pH 4.0

*Lysis buffer 2*

1 x CLB, pH 8.0  
0.5 % (w/v) Sodium deoxycholate  
0.1 % (w/v) SDS  
1 % (v/v) NP-40

*Cy3 labelling buffer*

0.1 M NaHCO<sub>3</sub>  
pH 9.3

*Trypsin buffer*

25 mM Tris  
1 M Urea  
10 mM CaCl<sub>2</sub>  
pH 8.5

### *SDS-PAGE sample buffer*

*(non-reducing)*

62.5 mM Tris

0.025 % (w/v) bromophenolblue

2 % (w/v) SDS

10 % (v/v) Glycerol

adjust pH to 6.8 prior to addition of

bromophenol blue, SDS and glycerol

### *Fluorescein labelling buffer*

0.1 M NaHCO<sub>3</sub>

pH 8.3

### *Coomassie staining solution*

10 % (v/v) Methanol

10 % (v/v) Acetic acid

0.1 % (w/v) Coomassie Brilliant Blue

All buffers were made up with RO (reverse osmosis) water produced in-house.

## **2.4 Chemicals**

The following chemicals were used in the experiments described in this thesis

<b>Chemical</b>	<b>Source</b>	<b>Location</b>
6-(fluorescein-5-carboxamido)hexanoic acid, succinimidyl ester	Molecular Probes	Eugene, OR, USA
Acetic acid	ChemSupply	Gillman, SA, Australia
Acetonitrile	Sigma	St Louis, MO, USA
Ammoniumbicarbonate	Sigma	St Louis, MO, USA
Aprotinin	Sigma	St Louis, MO, USA
Bromophenolblue	Sigma	St Louis, MO, USA
BSA	Sigma	St Louis, MO, USA
Calcium chloride	ICN chemicals	Seven Hills, NSW, Australia
Coomassie brilliant blue	Sigma	St Louis, MO, USA
Cy3 NHS ester	Amersham-Pharmacia	Uppsala, Sweden
D-Biotin	Pierce	Rockford, IL, USA
Dithiothreitol	Sigma	St Louis, MO, USA
DMSO	Amresco	Solon, OH, USA
DSS	Pierce	Rockford, IL, USA
DSP	Pierce	Rockford, IL, USA
EDTA	Sigma	St Louis, MO, USA
EGTA	BDH/Merck	Kilsyth, Vic, Australia
Ethanol	ChemSupply	Gillman, SA, Australia
Ethanolamine	Sigma	St Louis, MO, USA

Formic acid	Sigma	St Louis, MO, USA
Glycerol	Sigma	St Louis, MO, USA
Glycine	Amresco	Solon, OH, USA
HEPES	Amresco	Solon, OH, USA
Hydrochloric acid	BDH/Merck	Kilsyth, Vic, Australia
Isopropyl alcohol	ICN chemicals	Seven Hills, NSW, Australia
Leupeptin	Sigma	St Louis, MO, USA
Magnesium chloride	BDH/Merck	Kilsyth, Vic, Australia
Methanol	ChemSupply	Gillman, SA, Australia
N-(biotinoyl)-N'-(iodoacetyl)-ethylenediamine	Molecular Probes	Eugene, OR, USA
NP-40	Sigma	St Louis, MO, USA
PMA	Sigma	St Louis, MO, USA
PMSF	Boehringer	Mannheim, Germany
Potassium chloride	BDH/Merck	Kilsyth, Vic, Australia
SBTI	Sigma	St Louis, MO, USA
SDS	Sigma	St Louis, MO, USA
Sodium azide	Sigma	St Louis, MO, USA
Sodium bicarbonate	Sigma	St Louis, MO, USA
Sodium chloride	BDH/Merck	Kilsyth, Vic, Australia
Sodium deoxycholate	Sigma	St Louis, MO, USA
Sodium hydroxide	BDH/Merck	Kilsyth, Vic, Australia
TNBP	gift from Dr Jennifer Burgess	University of Wollongong Wollongong, NSW, Australia
TCEP	Pierce	Rockford, IL, USA
Tris	Sigma	St Louis, MO, USA
Triton X-100	Sigma	St Louis, MO, USA
Urea, ultra pure	ICN Chemicals	Seven Hills, NSW, Australia
Water, HPLC grade	Sigma	St Louis, MO, USA

## 2.5 Immunochemicals

The horseradish peroxidase (HRP)-labelled, secondary, anti mouse polyclonal antibody was raised in sheep and obtained from Silenus/Chemicon (Hawthorn, VIC, Australia). The HRP-labelled, secondary, anti rabbit polyclonal antibody was raised in donkey and obtained from Amersham-Pharmacia (Uppsala, Sweden). Fluorescein isothiocyanate (FITC)-labelled, secondary, anti mouse polyclonal antibody was raised in goat and obtained from Beckton-Dickinson (San Jose, CA, USA). The non-specific mouse

immunoglobulin IgG2b was obtained from ICN Biochemicals (Seven Hills, NSW, Australia). Mouse IgG2a and mouse IgG1 were obtained from Sigma (St. Louis, MO, USA). The FITC labelled mouse immunoglobulins IgG1 and IgG2a were from BD-Pharmingen (San Diego, CA, USA). HIV-1 gp120 (Clade E, #3234) was obtained from the NIH AIDS Research and Reference Program.

Table 2.2 lists all the primary antibodies employed in this study

**Table 2.2:** Primary antibodies employed in this study:

Antigen	Antibody name	Isotype	Source
CD4 Domain 1	Q4120	mouse IgG1	purified from hybridoma supernatant
CD4 Domain 3	Q425	mouse IgG1	purified from hybridoma supernatant
CD4 Domain 4	OKT4	mouse IgG2b	purified from hybridoma supernatant
CD71	WM82	mouse IgG2b	purified from hybridoma supernatant
CD45	HI30	mouse IgG1	BD Pharmingen <sup>1</sup> , Cat# 555480
CD98	UM7F8	mouse IgG1	BD Pharmingen, Cat# 556074
DC-SIGN	120507	mouse IgG2b	R & D Systems <sup>2</sup> , Cat# MAB161
CD4	PolyT4-5	rabbit IgG	generous gift from Dr Ray Sweet <sup>3</sup>
CD71	polyclonal	rabbit IgG	Santa Cruz Biotechnology <sup>4</sup> , Cat# sc-9099
DC-SIGN	polyclonal	rabbit IgG	Santa Cruz Biotechnology, Cat# sc-20081
gp120	polyclonal	sheep IgG	Centres for Biologicals Eval. and Res. <sup>5</sup> Lot DV-012

<sup>1</sup>San Diego, CA, USA

<sup>2</sup>Minneapolis, MN, USA

<sup>3</sup>Smith Kline Beecham, King of Prussia, PA, USA

<sup>4</sup>Santa Cruz, CA, USA

<sup>5</sup>Centres for Biologicals Evaluation and Research, FDA, Bethesda, MD, USA

## 2.6 Cell lysate preparation

Cells were grown to the required number and lysed in either lysis buffer 1 or 2. For lysis in buffer 1, the cells were washed twice with ice cold PBS and resuspended in lysis buffer 1 at a concentration of  $10^7$  cells/mL. For lysis in buffer 2, cells washed twice with ice cold CLB and then lysed in lysis buffer 2 at a concentration of  $10^7$  cells/mL. After a 1 h incubation at 4 °C, insoluble material was pelleted at 10 000 x g and the supernatant was passed through a 0.22 µm sterile filter (Millipore, Bedford, MA, USA).

## **2.7 Affinity purification of CD4 complexes**

Affinity purification of CD4 complexes was performed as previously described (Lynch et al. 1999). Briefly this involved preparing lysate from around  $10^9$  cells. The affinity chromatography was carried out using approximately 1 mL Q425 sepharose in an Econo-Column Chromatography Column (BioRad, Regents Park, NSW, Australia). A separate column was used for different preparations employing different lysis buffers or cells treated with a cross-linker. If indicated, a column containing 1 mL IgG1 sepharose preceded the Q425 column. Both columns were connected to a BioLogic™ liquid purification system and followed by a model 2110 fraction collector (both BioRad, Regents Park, NSW, Australia). The columns were equilibrated with 20 mL HBS containing 1 % (v/v) Triton X-100, 1 % (v/v) NP-40 and 10 mM CaCl<sub>2</sub>. The lysate was supplemented with 10 mM CaCl<sub>2</sub> and then applied to the columns at a flow rate of around 1 mL/min. If more than one column were used they were disconnected and each one washed and eluted separately. Each column was washed with 50 mL TBS, pH 8.0, 1 % (v/v) Triton X-100, 1 % (v/v) NP-40 and 10 mM CaCl<sub>2</sub> followed by 20 mL of TBS, pH 8.0, 1 % (v/v) Triton X-100, 1 % (v/v) NP-40. Proteins were then eluted with TBS, pH 8.0, containing 10 mM EDTA and 0.1 % (v/v) Triton X-100 and 0.1 % (v/v) NP-40. Alternatively, they were eluted with 0.2 M Glycine, pH 2.5 – 2.8. Then, acidic fractions were immediately supplemented with 40 µL of a 1 M Tris pH 9.0 solution per 1 mL fraction. Elution fractions were tested for CD4 using poly T4-5 antibody and Donkey anti rabbit antibody coupled to HRP (Amersham-Pharmacia, Uppsala, Sweden).

## **2.8 Biotin labelling of cysteines and tryptic digest:**

Proteins were labelled with N-(biotinoyl)-N'-(iodoacetyl)-ethylenediamine (BIAM, Molecular Probes, Eugene, OR, USA) as previously described (Gygi et al. 1999). This involved first denaturing of the proteins by adding 0.36 g urea per 750 µL of the CD4-containing elution fractions and control fractions. This yields 1 mL of a 6 M urea solution containing the proteins. Proteins were then reduced by adding tributylphosphine (TNBP) to a final concentration of 5 mM and incubating for 1 h at 37 °C. Free sulphydryls were labelled with biotin by addition of 1 % of a 10 mM solution of BIAM in DMSO. The

reaction was incubated for 1 h in the dark at 37 °C. Proteins were then purified by gel filtration into Trypsin buffer using NAP-10 columns (Amersham-Pharmacia, Uppsala, Sweden). Columns were first equilibrated with 3 x 5 mL Trypsin buffer followed by application of 1 mL of the protein solution whilst the flow-through was discarded. Another 1.5 mL Trypsin buffer were added and the flow through containing the proteins was collected. Modified, sequencing grade trypsin (Promega, Madison, WI, USA) was added to a concentration of 1 µg/mL and the protein digestion was performed over night at 37 °C.

### **2.9 Avidin chromatography of biotin-containing peptides**

Approximately 200 µL of a 50 % slurry of immobilised monomeric avidin (Pierce, Rockford, IL, USA) were used per sample. The beads were equilibrated and the strong binding sites were blocked with D-Biotin (Pierce, Rockford, IL, USA) according to the manufacturer's recommendations. Briefly this involved washing the beads 2 x with PBS and then incubating the beads for 30 mins in a solution of 2 mM D-Biotin in PBS. The beads were subsequently washed 3 x with PBS. The tryptic digest was supplemented with 1 mM PMSF to inactivate the trypsin. The beads were then added to the tryptic digest and incubated for 1 h at 4 °C. The suspension was subsequently transferred into a disposable column (Pierce, Rockford, IL, USA) and the beads were washed with 20 mL PBS and 10 mL water. Biotin-labelled peptides were eluted for direct mass spectrometry (MS) analysis in 100 µL fractions with 80 % acetonitrile containing 5 % formic acid.

### **2.10 Sample preparation for SDS-PAGE analysis of CD4 complexes**

The two or three elution fractions containing the majority of CD4 were combined and 100 µL of Strataclean resin (Stratagene, La Jolla, CA, USA) were added. Proteins were allowed to bind to the resin for 2 h at 4 °C. The resin was then pelleted and proteins were eluted with 2 x 30 µL SDS-PAGE sample buffer containing 25 mM TCEP (Pierce, Rockford, IL, USA) at 95 °C. TCEP was omitted when indicated.

### **2.11 Immunoprecipitations of CD4 and CD71**

Cell lysate (0.5 – 1 mL) was precipitated by incubation at 4°C for 1 h with 2.5 µg of the respective antibody and 20 µL Protein G Sepharose (Amersham-Pharmacia, Uppsala, Sweden). The beads were washed exhaustively with HBS, pH 7.5, containing 10 mM CaCl<sub>2</sub> and 0.1 % (v/v) Triton X-100. Bound protein was eluted into sample buffer by incubation for 5 min at 95 °C. TCEP was omitted from the sample buffer when indicated.

### **2.12 SDS-PAGE, Western and Dot blotting and Coomassie Brilliant Blue staining**

Immunoprecipitation- or affinity chromatography-derived samples were separated by polyacrylamide gel electrophoresis on an 8-16 % gradient gel (Gradipore, Frenchs Forest, NSW) using a Mino-PROTEAN II electrophoresis system (BioRad, Regents Park, NSW, Australia). Protein gels for mass spectrometry analysis were stained in Coomassie Brilliant Blue staining solution for 4 h or over night and destained in 40 % (v/v) methanol, 10 % (v/v) acetic acid. Protein gels for western blotting experiments were subjected to electrophoretic transfer of the proteins to nitrocellulose membranes using a Mini Trans-Blot Transfer Cell (BioRad, Regents Park, NSW, Australia). After blocking with 5 % (w/v) Diploma skim milk powder in wash buffer, the membranes were incubated for 1 h with primary antibody and then 1 h with horseradish peroxidase-conjugated secondary antibody. Antibody incubations were carried out in 1 % (w/v) Diploma skim milk powder in wash buffer. Blots were developed, using the western lightening detection kit (Perkin Elmer, Boston, MA, USA). For Dot Blots, 1 µL of the respective sample was spotted onto the dry Nitrocellulose membrane and allowed to dry. The membrane was then incubated in 5 % milk in TBS, pH 8.0, containing 0.1 % (v/v) Triton X-100 and proteins were detected as for western blots.

### **2.13 In-gel digest and peptide extraction**

Gel-slices were excised from the Coomassie-stained gels and dehydrated with methanol for 5 min and rehydrated with 30 % methanol for 5 min. Slices were washed twice with water and then three times for 10 min each with 100 mM ammoniumbicarbonate, 30 % (v/v) acetonitrile. Gel slices were then crushed into fragments, washed with 30 % (v/v) acetonitrile and dehydrated for 5 min with 100 % ethanol. Fragments were dried for 1 h

in vacuum, 50 – 80  $\mu\text{L}$  of trypsin solution (5  $\text{ng}/\mu\text{L}$  sequencing grade modified trypsin in 25 mM ammoniumbicarbonate solution) was added and enzymatic digestion was carried out at 37 °C over night. The supernatant was aspirated and proteins were extracted three times by addition of 50  $\mu\text{L}$  extraction solution (50 % acetonitrile, 5 % formic acid) for 30 min. In between extractions, gel-slices were rehydrated by addition of 30  $\mu\text{L}$  water and 20 min incubation. The aspirated extraction solutions and water supernatants were combined with the aspirated trypsin solution. The samples were then concentrated and acetonitrile was evaporated using a “Speed-Vac”™ vacuum centrifuge with moderate heating. The resulting peptides were purified for MS analysis using “ZipTips” (Millipore, Bedford, MD, USA). The “Zip-Tip” resin was first wetted with 10  $\mu\text{L}$  50 % Acetonitrile and washed twice with 10  $\mu\text{L}$  5 % (v/v) formic acid. The peptide sample was acidified by adding 15  $\mu\text{L}$  5 % (v/v) formic acid and applied to the resin twice. The resin was then washed 5 x with 10  $\mu\text{L}$  5 % (v/v) formic acid and peptides were eluted into 10  $\mu\text{L}$  50 % (v/v) acetonitrile containing 5 % (v/v) formic acid. All solutions used for experiments described in this section had been made using HPLC grade water.

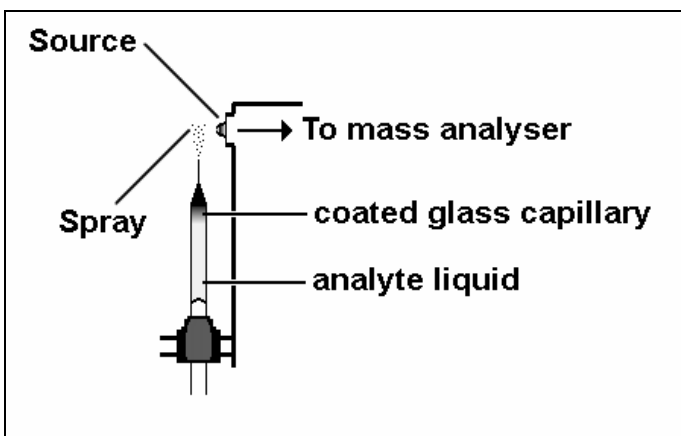
## **2.14 Introduction to mass spectrometry**

Mass spectrometry (MS) involves the measurement of mass an intrinsic property of all molecules. A mass spectrometer is made up of three major parts: an ionisation source, a mass analyser and a detector. The source generally both vaporises and ionises the molecules of the sample by a range of techniques. The generated ions are then accelerated in an electrical field and enter one of a range of different devices where they are analysed or separated according to their mass (or more accurately mass-to-charge ratio). A detector detects the separated ions, and via a digital interface collects data, which is transferred to a computer for analysis.

The major technological advances that lead to the use of mass spectrometry for the analysis of large biomolecules were the introduction of ionisation techniques that enabled transfer of the biomolecules to the gas phase without degradation. The two main techniques used now are matrix assisted laser desorption ionisation (MALDI) and electrospray ionisation (ESI). In MALDI-MS, the analyte is crystallised on an analytical target together with a matrix, which is generally an aromatic organic acid, that absorbs

UV light. To ionise the analyte, a nitrogen laser beam targeted onto the crystallised matrix/analyte spot transfers energy rapidly to the matrix leading to vaporisation of both matrix and analyte molecules. The molecules are ionised via proton transfer reactions in the matrix (Karas et al. 2000; Spengler 2001).

ESI-MS is a fundamentally different ionisation technique, which is based on direct injection of the analyte dissolved in an aqueous/organic solvent under the influence of an electric field. The solution evaporates in source with the assistance of heat or a nebulising gas leading to the formation of small droplets from which solvent and analyte molecules are formed. The precise mechanism by which ions are formed is not fully understood but two models exist describing the formation of ions. The charge residue model suggests that repeated droplet fission occurs until there is only one analyte ion left in the droplet. Evaporation of the remaining solvent then releases the ion into the gas phase. The ion evaporation model suggests that during the process of droplet shrinking owing to evaporation, the Coulombic forces become so strong that individual analyte ions are ejected from the droplet (Smith, R. D. et al. 1990). Nanoelectrospray-MS (nanoESI-MS) is a variant of ESI-MS where the analyte solvent is not pumped into the source but introduced into a metal-coated glass capillary. Capillary forces and the influence of the electric field lead to the formation of a steady spray out of the tip of the capillary from which analyte molecules vaporise again into the source. Charges are acquired by the ions in a mechanism(s) similar to ESI-MS except that droplets are smaller and ion transfer is more efficient. The advantage of nanoESI-MS is the requirement of only a small volume of analyte (at similar or lower concentrations) so in comparison to ESI-MS much less sample is required. Figure 2.1 shows a diagram of a typical nano-ESI source.



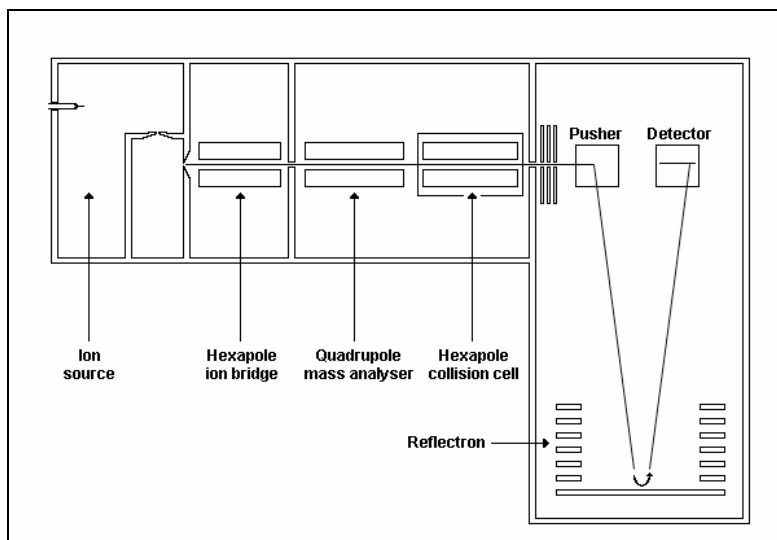
**Figure 2.1**  
Diagram of a nanoESI Source showing analyte in a coated glass capillary spraying towards the source of the MS that directs the ions to the mass analyser

The main two mass analysers, which are employed for biomolecular MS, are the quadrupole mass filter (Q) and the time-of-flight (TOF) mass analyser. In the Q mass filter, the ions are filtered by the application of both direct current (DC) and an alternating current (AC) radio frequency (RF) electric fields. The quadrupole filters are comprised of four parallel rods and selectively transmit ions of a given mass-to-charge ( $m/z$ ) window as the voltage applied to the rods is scanned, thereby generating a spectrum of ion abundance as a function of  $m/z$ . A TOF mass analyser takes advantage of the different velocities that ions have according to their mass-to-charge ratio when subjected to acceleration in an applied electric field. The velocity of the ions depends on their  $m/z$  ratio so ions with a lower  $m/z$  reach the detector faster than molecules with a higher  $m/z$  do when passed through the field free region of the flight tube in a TOF analyser.

A tandem mass spectrometer is essentially a source followed by two mass analysers separated by a collision cell or region and then the detector. The advantage of such an instrument is that in the first mass analyser (usually a quadrupole), ions can be mass-selected. Fragment or product ions are produced in the collision cell via collisions with an inert gas such as argon in a process known as collision-induced dissociation or decomposition (CID). The resulting ions are analysed in the second mass analyser which might be either a quadrupole or TOF analyser\*. Structural data from the selected ion can therefore be obtained from the tandem or MS/MS spectra. See general literature or the reviews from (Corthals et al. ; Yates 2000; Aebersold 2003) for more information. Figure 2.2 shows a diagram of the Quadrupole time-of-flight (Q-tof) instrument used throughout this study. It consists of a nanoESI source followed by a quadrupole and a time-of-flight mass analyser. A hexapole collision cell is located between the two mass analysers to allow for fragmentation of selected ions.

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\* Other forms of tandem mass spectrometry used for biomolecules involve quadrupole ion traps, Fourier transform ion cyclotron resonance and most recently TOF/TOF analysers, but a discussion of these analysers is beyond the scope of this thesis



**Figure 2.2:**  
Diagram of the  
Quadrupole-time-of-  
flight mass  
spectrometer (Q-  
TOF) used in all the  
studies conducted in  
this thesis.

## 2.15 Introduction to peptide analysis via mass spectrometry

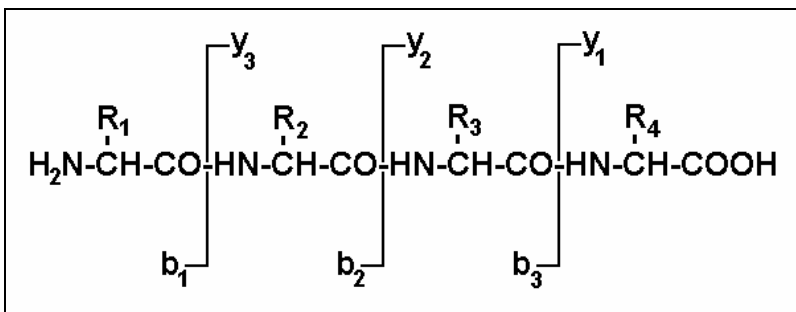
One of the most powerful applications of MS in protein research is the analysis of peptide mixtures. The strength of MS lies not only in its ability to determine the mass of the individual peptides in mixtures, but it is especially able to determine a part or the full amino acid sequence of a peptide. This is often sufficient to determine the protein from which the peptide is derived in combination with information available in protein databases. Thus MS has become an invaluable tool in protein identification.

A peptide is any molecule consisting of two or more amino acids joined by a peptide bond but usually peptides with the length of at least 6 amino acids up to about 30 amino acids can be analysed by tandem MS to obtain sequence information. The molecular masses of much larger peptides and proteins can be determined by ESI-MS or MALDI-MS but sequencing by MS is usually confined to those peptides generated through proteolytic digest of the protein. The most common protease used for generating peptides for MS is trypsin, which cleaves proteins after lysine or arginine except if a proline follows the basic amino acid.

ESI-MS analyses of peptides yield ions of one or more of its mass-to-charge ratio and or charge state. When using low resolution mass analysers such as quadrupole mass filters, the charge must be determined by solving two simultaneous equations and hence requires the presence of two ions for each peptide. In the case of the Q-Tof instrument used in this work (and also quadrupole ion traps and FT-MS instruments) it is possible to

determine the charge on the ion from the spacing between adjacent isotopic peaks. Each peptide (or other molecule species) gives more than one ion in an MS spectrum owing to the occurrence of heavier isotopes of the elements that are naturally incorporated into the peptide. The peak with the lowest mass is usually the “monoisotopic” peak and the molecule from which the peak is derived consists completely of atoms of the most abundant (=lightest) isotope of each element. The molecule to which the second peak can be assigned contains exactly one atom of a heavier isotope like deuterium or  $^{13}\text{C}$ . The rule continues for the other peaks, with the relative intensity of each peak is depending only on relative probability of isotopic combination. The spacing between peaks differing in mass by 1 yields the charge. That is doubly charged ions are separated by 0.5 m/z, triply by 0.33 m/z etc. The total mass of the peptide can be calculated by multiplying the mass to charge ratio with the charge-state and subtracting one mass unit for each charge state since for positive ions the charge arises from addition of a proton.

Tandem MS has the ability to select an individual peptide ion and subject it to collision-induced dissociation (CID). Under normal CID conditions, peptides fragmentate at the peptide bond. Figure 2.3 shows the typical fragmentation of a peptide and the nomenclature of the ions that are produced. Sequence information can be obtained by analysing the mass differences between the ions in each series which are characteristic of the individual amino acids. For tryptic peptides the most abundant precursor ions are usually doubly or triply charged since each tryptic peptide has at least two basic sites for protonation. These ions usually produce abundant y ion series. See general literature of the reviews from (Corthals et al. ; Yates 2000; Aebersold 2003) for a more detailed description of tandem MS.



**Figure 2.3:** Diagram of the typical fragmentation of a peptide in MS/MS. Fragmentation occurs at the peptide bonds.

## 2.16 Mass spectrometry analysis

Electrospray mass spectrometry (ESI-MS) analyses were carried out using a Q-Tof2 (Micromass, Manchester, UK) in nanoelectrospray mode. Typically 5  $\mu$ L sample was loaded into a coated glass capillary (Protana, Odense, Denmark). The ESI parameters were as follows: Capillary voltage, 800 – 1000 V; Cone voltage, 30-40 V; desolvation temperature, 40 °C; desolvation gas flow rate, 50 - 100 L/h. Peptides were detected by acquisition of the  $m/z$  range from 50 – 2000 using a 2.4 s integration time and a 0.1 s delay. Precursor ions were selected for fragmentation within a mass window of 4  $m/z$ . MS/MS spectra were collected using collision cell voltages from 25 V to 45 V with argon as the collision gas. The instrument was calibrated with a solution of 100 fM [Glu<sup>1</sup>]-fibrinopeptide B in 40 % acetonitrile, 2.5 % formic acid. To reduce the intensity of detergent-derived singly charged species, the voltage on the MCP detector was reduced to 2000 V during MS acquisitions whilst kept at 2300 V during MS/MS acquisitions (Loo and Ogorzalek-Loo, 1995). Typically, all multiply charged ions that could be easily distinguished from the background were subjected for fragmentation. Exceptions were made for ions that were known to be peptides derived from certain proteins, which were sometimes omitted when the protein had already been identified. Also a targeted ion approach was sometimes used when a multiply charged ion that could not be clearly distinguished from the background was subjected to fragmentation because the presence of a peptide ion at the  $m/z$  was expected.

## 2.17 Protein identification

Amino acid sequence tags (a series of consecutive amino acids representing a part of the peptide sequence that were identified from the fragmentation spectra via their residue mass) obtained from the MS/MS spectra were used for database searches with the PepSea software (Protana, Odense, Denmark, <http://195.41.108.38/PepSeaIntro.html>) or the ScanProsite software (SWISS-PROT database, Swiss Institute of Bioinformatics (SIB), Geneva, Switzerland, <http://au.expasy.org/tools/scanprosite/>). Proteins containing the sequence tag were subjected to a theoretical digest with trypsin and the resulting peptides searched for the peptide that contained the sequence-tag. To be identified as the sequence of a fragmented ion, the total mass of the peptide including the additional masses of

amino acid modifications (biotin tag: 326.1 Da, Met oxidation: 16 Da, carboxamidomethylcysteine: 71 Da, N-terminal acetylation: 42 Da) needed to be equivalent to the precursor ion mass. Additionally, a series of product ions from the MS/MS spectrum had to match the predicted y and b ions of the sequence. The full peptide sequence was again entered into the ScanProsite search engine and the protein was considered to be identified conclusively in the affinity chromatography-derived sample if it was the only human protein producing this peptide after a tryptic digest.

Alternatively, fragmentation spectra were matched using MASCOT ([http://www.matrixscience.com/cgi/index.pl?page=/search\\_form\\_select.html](http://www.matrixscience.com/cgi/index.pl?page=/search_form_select.html)). Obtained matches were generally confirmed via manual sequencing.

### **2.18 Immunoprecipitations and Co-immunoprecipitations of DC-SIGN complexes**

Unless otherwise indicated, 0.5 mL lysate were precipitated with either 2.5 µg anti DC-SIGN antibody or 10 µL anti gp120 serum bound to 20 µL Protein G beads for 1 h at RT. For co-immunoprecipitations, 2.5 µg gp120 was incubated with the lysate for 1 h at RT prior to addition to the antibody bound to the beads. For precipitations with mannan beads, the beads were washed with HBS, pH 7.5, 10 mM CaCl<sub>2</sub> and 0.1 % (v/v) Triton X-100 and then incubated with the lysate.

After binding, the beads were washed 5 x with HBS, pH 7.5, 10 mM CaCl<sub>2</sub> and 0.1 % (v/v) Triton X-100 and resuspended in 1 x SDS sample buffer. If indicated, DTT was added to a concentration of 50 mM for reduction of the disulfide bonds. Proteins were analysed via western blotting and detected with a polyclonal anti DC-SIGN antibody.

### **2.19 Cross-linking of cell surface molecules and cell lysate preparation**

Cells were washed twice with cross-link buffer (CLB) and then resuspended in CLB at  $5 \times 10^6$  cells/mL. DSP or DSS in dimethylsulfoxide was added to the cell suspension (2 mL of 25 mM per 100 mL suspension unless otherwise indicated). Cross-linking was carried out for 30 min at room temperature. Unreacted cross-linker was quenched by adding TBS, pH 7.5, and resuspending the cells in TBS, followed by a 15 min incubation at room temperature. Cells were then pelleted and lysed in lysis buffer 2 at a concentration

of  $10^7$  cells/mL. After 60 min at 4°C, insoluble debris was pelleted and the supernatant passed through a 0.22 µm syringe-driven filter unit (Millipore, Bedford, MD, USA). The lysate was then supplemented with 10 mM CaCl<sub>2</sub>. For preparation of mock-cross-linked cell lysates, dimethylsulfoxide was added without dissolved cross-linker.

## **2.20 Generation of affinity chromatography medium**

Antibodies were purified from hybridoma supernatant using HiTrap Protein G HP columns (Amersham Pharmacia , Uppsala, Sweden). After application of the antibody, the column was washed exhaustively with PBS and eluted with 0.2 M Glycine, pH 2.5-2.8. Purified antibody was then dialysed into a buffer suitable for the next preparation using dialysis tubing (Life Technologies, Gaithersburg, MD, USA). For generation of affinity beads antibody was dialysed into coupling buffer and the concentration was determined spectrophotometrically by measuring protein absorption at 280 nm. The concentration was calculated according to equation 1 with the denominator 1.3 derived from the extinction coefficient for IgG molecules:

$$\text{concentration}[\mu\text{g/ml}] = A(280) / 1.3 \quad \text{Equation (1)}$$

The antibody was then coupled to Cyanogen-bromide activated sepharose (Sigma, St. Louis, MO, USA). Sepharose (0.4 g will produce about 1 mL gel) was swelled in 1mM HCl and washed with 100 mL 1 mM HCl per 1 g sepharose used. Beads were then washed twice with water and once with coupling buffer followed by addition of the antibody solution. The reaction was performed for 2 h at RT followed by an incubation at 4 °C over night. The beads were then washed with coupling buffer and unreacted sites were blocked by resuspending in coupling buffer containing 1 M ethanolamine for 2 h. The resin was then washed alternately with coupling buffer and acetate buffer for five times and resuspended in HBS containing 1 % (v/v) Triton X-100, 1 % (v/v) NP-40, 10 mM EDTA and 0.1 % (w/v) NaN<sub>3</sub>. Coupling efficiency was determined by Dot Blot and was generally above 90 %. For coupling of commercially obtained antibody, the antibody was buffer exchanged into coupling buffer at a smaller scale using Amicon Ultra Centrifugal filter devices (Millipore, Bedford, MD, USA). The obtained antibody was

added to 1 mL coupling buffer and the solution transferred into a filter device. The antibody solution was concentrated by centrifuging at 7000 x g until 100 µL remained. Another 1 mL aliquot of coupling buffer was added and the concentration/dilution repeated for a total of four times. The concentration of the antibody was again determined spectrophotometrically (as above) and coupling of the antibody was performed similar to antibody derived from hybridoma supernatant but usually at a smaller scale.

### 2.21 Labelling of antibodies with fluorophores

Antibodies were labelled with either 6-(fluorescein-5-carboxamido)hexanoic acid, succinimidyl ester (Molecular Probes, Eugene, OR, USA) or Cy3 (Amersham-Pharmacia, Uppsala, Sweden). Antibodies were buffer exchanged into Cy3 labelling buffer or fluorescein labelling buffer as described in 2.20. For labelling with fluorescein, per 50 µg antibody, 1 µL of a solution of 0.05 mg 6-(fluorescein-5-carboxamido)hexanoic acid, succinimidyl ester in 50 µL DMSO was added. For labelling with Cy3, one aliquot Cy3 NHS ester supplied by the manufacturer was suitable for labelling 1 mg antibody. For smaller quantities the Cy3 NHS ester was dissolved in DMSO and the respective part was added to the protein solution. The reaction was allowed to proceed for 30 min at RT in the dark and labelled antibodies were separated from unconjugated dyes using gel filtration on Sephadex G-25 (Amersham-Pharmacia, Uppsala, Sweden) and PBS. For spectrophotometric determination of the concentration and the degree of labelling, the absorption was determined at 280 nm and 494 nm for fluorescein conjugates and 280 nm and 552 nm for Cy3 conjugates. The concentration was determined using equation 2 and 3 respectively:

$$\text{For fluorescein: } \text{conc } (\mu\text{g/mL}) = (A_{280} - 0.2 * A_{494}) / 1.3 \quad \text{Equation (2)}$$

$$\text{For Cy3: } \text{conc } (\mu\text{g/mL}) = (A_{280} - 0.08 * A_{552}) / 1.3 \quad \text{Equation (3)}$$

The degree of labelling (DOL) was determined using equation 4 and 5 respectively:

$$\text{For fluorescein: } \text{DOL} = A_{494} * 2.2 / \text{conc } (\mu\text{g/mL}) \quad \text{Equation (4)}$$

$$\text{For Cy3: } \text{DOL} = A_{552} * 1.13 / \text{conc } (\mu\text{g/mL}) \quad \text{Equation (5)}$$

## **2.22 Flow cytometry measurements**

For each flow cytometry sample,  $5 \times 10^5$  cells were washed 2 x with FACS buffer. Cells were resuspended in 35  $\mu$ L heat-inactivated fetal calf serum (CSL, Parkville, Vic.) and incubated for 15 min at 4 °C. Cells were then labelled with the antibodies. Usually 0.5  $\mu$ g antibody was used per antibody per sample and the labelling was carried out at 4°C for 30 min. Cells were then washed 2 x with FACS buffer and resuspended in 300  $\mu$ L FACS buffer. Flow cytometry measurements were carried out using a FACScalibur (Becton-Dickenson, San Diego, CA, USA) using a 488 nm laser for excitation. Fluorescein fluorescence was measured at 530 nm. Cy3 fluorescence was measured at 585 nm. Live cells were gated to exclude debris and dead cells and no compensation was used. Data were collected in “List” mode and mean fluorescence intensities were used for evaluation.

## **2.23 FRET**

Fluorescence Resonance Energy Transfer (FRET) technology has become a versatile tool for the study of protein-protein interactions especially because it can be used for membrane protein interactions on the surface of living cells. FRET is a physical phenomenon that involves energy transfer from an excited donor molecule (e.g. fluorescein) to a suitable acceptor molecule (e.g. Cy3 or rhodamine). The transfer efficiency is highly dependent on distance and does not take place if donor and acceptor are separated by more than 5 to 10 nm thus acting as a molecular ruler to investigate proximity between cell surface proteins. FRET is measured as energy transfer efficiency (E), which is the percentage of excited donor molecules that transfer their energy to an acceptor molecule. An E value of more than 5 to 7 % indicates a protein interaction (Matyus et al. 1995; Dornan et al. 2002). Here we measured FRET using flow cytometry and fluorescein and Cy3 labeled monoclonal antibodies using either donor quenching for heteroassociations or acceptor sensitizing for homoassociations (Szollosi et al. 1984; Tron et al. 1984; Guo, C. et al. 1995a; Kenworthy et al. 2000; Szollosi et al. 2002).

## 2.24 FRET measurements on protein homoassociations

Cells were labelled similarly as for flow cytometry analysis. Cells were labelled with either unconjugated antibody, with fluorescein (donor) or with Cy-3 (acceptor) conjugated antibody for determining the control values. For FRET measurements, cells were labelled with a mixture of fluorescein and Cy-3 conjugated antibody employing various Ab-fluorescein/Ab-Cy3 ratios. Flow cytometry measurement was carried out using a FACSVantage SE DiVa from Becton-Dickinson (San Diego, CA, USA). Three fluorescence intensities were collected, two with excitation at 488 nm and detection at 530 nm (I1) and 585 nm (I2). The third fluorescence intensity was collected with excitation at 514 nm and detection at 585 nm (I3). No compensation was used. Cells were gated to exclude debris and dead cells and both mean and median fluorescence intensities were used for analysis. To eliminate the influence of cellular autofluorescence, test values were obtained using equation 6:

$$(\text{test value}) = (\text{mean fluorescence intensity obtained with conjugated antibody}) - (\text{mean fluorescence obtained with unconjugated antibody}). \quad \text{Equation (6)}$$

Values I530 and I585 (see below) were derived from two samples labelled to saturation with either WM82-fluorescein or WM82-Cy3. I530 was detected at 530 nm with the WM82-fluorescein sample and excitation at 488 nm whereas I585 was detected at 585 nm with the WM82-Cy3 sample and excitation at 488 nm. Energy transfer efficiencies were calculated from the test values using equations 7-9 (Szollosi et al. 1984; Tron et al. 1984):

$$a = \frac{I585 * \text{DOL (WM82-Fluorescein)}}{I515 * \text{DOL (WM82-Cy3)}} \times 2.15 \quad \text{Equation (7)}$$

$$A = \frac{\frac{I2 - S2 \times I3}{1 - \frac{S3 \times S2}{S1}}}{S1} - S1 \quad \text{Equation (8)}$$

$$E = A / (a + A) \quad \text{Equation (9)}$$

The values for S1 to S3 were obtained as follows:  $S1 = I2/I1$  for cells labelled only with the donor,  $S2 = I2/I3$  for cells labelled only with the acceptor and  $S3 = I3/I1$  for cells labelled only with the donor. E values were excluded when the value obtained from the median fluorescence intensities differed from the value obtained from the mean fluorescence intensities by more than 50 %. The FACSVantage DiVa was operated by Tara McDonald (Centennial Institute, Camperdown, NSW, Australia) at the Centennial Institute.

### **2.25 FRET measurements on protein heteroassociations**

For FRET measurements on associations between different proteins, cells were labelled similarly as for flow cytometry analysis. The less abundant molecule was hereby used as donor and the higher abundant molecule as acceptor. Four samples were prepared for each measurement. Cells were stained with unconjugated donor and acceptor antibody (blank sample), with unconjugated acceptor antibody and fluorescein conjugated donor antibody (donor sample), with Cy3 conjugated acceptor antibody and unconjugated donor antibody (acceptor sample) and Cy3 conjugated acceptor antibody and fluorescein conjugated donor antibody (FRET sample). Flow cytometry analysis was carried out using a FACScalibur (Becton Dickinson, San Diego, CA, USA) with a 488 nm laser. Cells were gated to exclude debris and dead cells and mean fluorescence intensities were used for analysis. Two fluorescence intensities were collected, FL1 at 530 nm and FL2 at 585 nm. All samples were stored on ice until analyzed. Cells were subsequently incubated at 37 °C for a given time and then analyzed again. For study of the influence of PMA on the association, 100 ng/mL PMA was added to each sample before the incubation period. Nothing was added to mock treated samples. Energy transfer efficiencies were determined as donor fluorescence quenching and calculated using equation 10:

$$E = \left[ \frac{\{FL1 (\text{Donor}) - FL1 (\text{FRET})\}}{\{FL1 (\text{Donor}) - FL1 (\text{blank})\}} \right] \times 100 \%$$

Equation (10)

### **2.26 Purification of DC-SIGN complexes**

The monoclonal anti DC-SIGN antibody was coupled to cyanogen bromide-activated sepharose as described in 2.20. Lysate from about  $9 \times 10^7$  MDDCs that had been treated with DSS as described in 2.19 was first passed over  $\sim 100 \mu\text{L}$  of an irrelevant antibody coupled to sepharose to absorb non-specifically binding proteins and subsequently passed over  $\sim 100 \mu\text{L}$  anti DC-SIGN beads (containing approximately  $100 - 150 \mu\text{g}$  immobilized antibody). The beads were then washed with TBS, pH 8.0, containing 0.1 % (v/v) Triton X-100. Complexes were eluted by boiling the beads for 5 min in 1 x SDS sample buffer.

**CHAPTER THREE:**

**SEARCH FOR PROTEINS CO-PURIFYING  
WITH THE CD4 RECEPTOR COMPLEX USING  
MASS SPECTROMETRY**

**EVALUATION OF AFFINITY-TAG MEDIATED PURIFICATION  
OF TRYPTIC PEPTIDES TO IDENTIFY PROTEINS PURIFIED  
WITH Q425 AFFINITY CHROMATOGRAPHY**

### **3.1 Aims**

The work described in this chapter aimed to:

- (i) Develop a method to identify the proteins present in an affinity chromatography-derived purification of the CD4 complex using affinity tag-mediated purification of tryptic peptides;
- (ii) Evaluate the strategy with respect to both the capacity to identify protein-protein interactions and the extent to which the possible proteins in each sample are detected;
- (iii) Confirm (or otherwise) the possible interactions between CD4 and the detected proteins using control experiments.

### 3.2 Introduction

The CD4 protein as described in chapter 1 is an integral membrane protein of 55 kDa consisting of four extracellular domains, a transmembrane domain and a cytoplasmic tail (Maddon et al. 1987). CD4 functions as a coreceptor in the activation of CD4<sup>+</sup> T-cells (Gay et al. 1987), as a chemotactic receptor for IL-16 in various cells (Cruikshank, W. W. et al. 1994) and together with CCR-5 and CXCR-4 as the receptor for the entry of HIV-1 into its target cells (Lapham et al. 1996; Wu, L. et al. 1996). CD4 cannot transmit signals into the interior of the cell by itself nor on its own allow entry of HIV-1 (reviewed in chapter one). The correct function of the CD4 molecule is mediated by its associating molecules. Thus, to function correctly as a co-receptor in MHC class II recognition, CD4 associates with the tyrosine kinase lck via its cytoplasmic tail and with the tyrosine phosphatase CD45 via a lateral interaction in the membrane. For entry of HIV-1 into a T cell, as reviewed in chapter 1, the virus first interacts with CD4 which is not sufficient for entry. Only recruitment of a co-receptor, CCR5 or CXCR4 and subsequent membrane fusion allows for infection of the cell. Pre-assembled complexes of CD4 with those co-receptors have been suggested but little data is available on such an interaction (see section 1.2.3.2). Furthermore studies at our centre have suggested host cell-derived effects on HIV replication that could be derived from CD4-associated proteins (Naif et al. 1999). The mechanism by which IL-16 mediates chemotaxis is poorly understood and little is known about the CD4-associating molecules in non-lymphoid cells that area also susceptible to IL-16, though some evidence for the presence of associating proteins has been reported (Graziani-Bowering et al. 2002). Several molecules have been described associating with CD4 in T-lymphocytes that could be involved in CD4 function namely, ACP33 (Zeitlmann et al. 2001), LAT-1 (Bosselut et al. 1999), raf-related polypeptide (Prasad and Rudd, 1992) and the T-cell receptor (Vignali and Vignali, 1999). However, those reports have yet to be confirmed so apart from its extracellular ligands IL-16, MHC class II and HIV-gp120, only the interactions with p56lck and CD45 are generally accepted. Most of the proteins listed above, such as p56lck or ACP33 that were reported to associate with CD4 could be detected using co-immunoprecipitation techniques followed by western detection. Thus affinity purification of CD4 from a detergent solubilized lysate, which is technically identical to co-immunoprecipitation at a larger

scale, should purify any associating proteins along CD4. CD4 affinity purification had been established in our laboratory (Lynch et al. 1999) so this study aimed to identify the proteins present in the affinity purified sample to detect any associating molecules that could play a role in CD4 function. For this purpose a method is required that detects all proteins present via determining the amino acid sequence of the molecules present in a mixture of proteins.

Recent years have seen the introduction of powerful techniques to obtain rapid information on protein identity, relative protein expression or protein interactions owing to the demand of rapid information of protein function. Aebersold and co-workers demonstrated quantitative analysis of changes in protein expression after changes in growth conditions using isotope-coded affinity tags (ICAT) (Gygi et al. 1999). This method has also been used successfully to determine alterations in membrane protein expression (Han et al. 2001) and a similar approach was employed to identify proteins released from permeabilized mitochondria (Spahr et al. 2000). Membrane proteins are generally very difficult to study and often the low abundance of important receptor molecules requires additional strategies for their detection. Simpson and co-workers have used a "targeted" ion approach to identify "predicted" peptides from a low-abundance protein in a digested membrane fraction. They demonstrated that even when low abundance proteins are known to be present, the resulting peptide ions are normally not detected in the presence of ions from more abundant proteins (Simpson et al. 2000). To overcome the problem of low abundance, trace enrichment procedures are commonly used to purify low copy proteins together with associating molecules. MS analysis of the purified complex, usually done using 1-D PAGE followed by in-gel digest and MS, yields very valuable data on protein interactions as demonstrated in the publication of the yeast interaction map (Ho, Y. et al. 2002).

In this study, a method is developed for the identification of proteins present in an affinity-purified CD4 sample to investigate CD4-associating molecules. To identify the proteins present in the purification of the CD4 receptor complex, a tryptic digest of the whole protein mixture was employed to circumvent the 1-D PAGE step generally used in similar studies. The resulting complex peptide mixture, however, requires separation of peptides to facilitate detection. Common approaches to achieve this are multidimensional

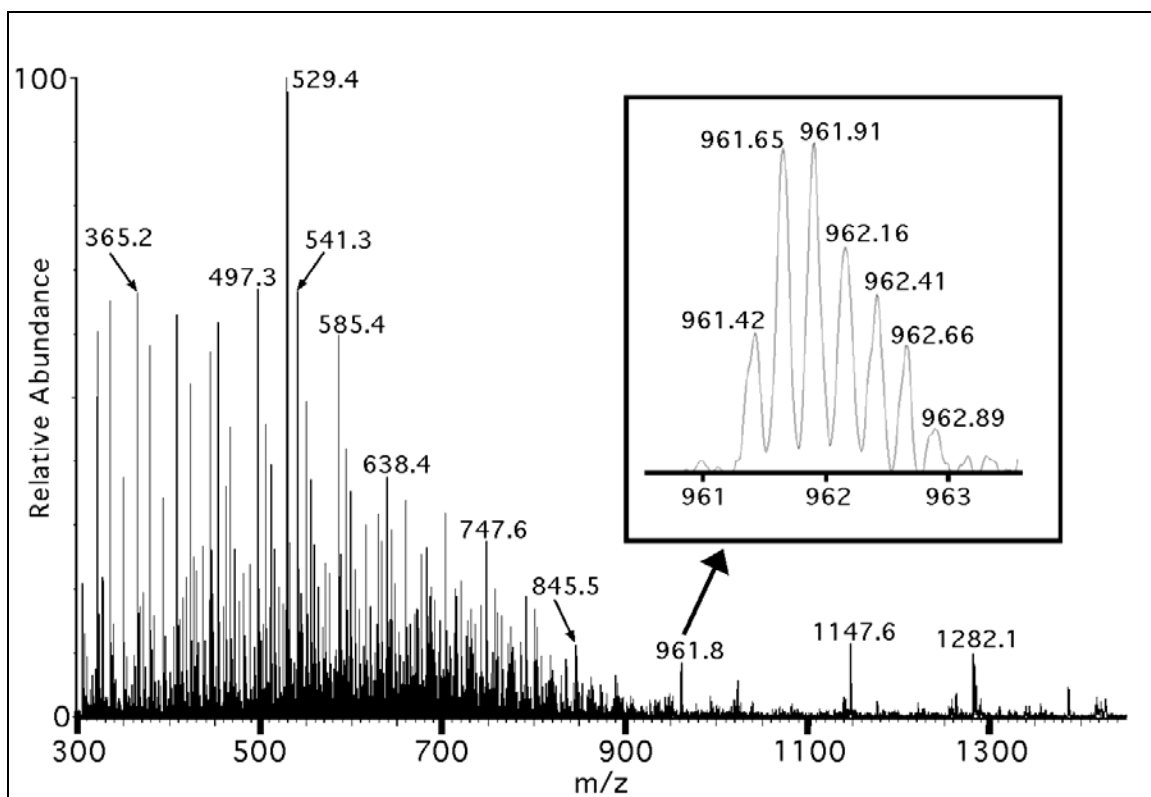
chromatography, usually ion-exchange chromatography followed by reverse phase (RP)-chromatography (Spahr et al. 2000). To avoid the use of a multidimensional chromatography a variation of the cysteinyl affinity capture method of Spahr et al. (2000) was used to generate a representative subset of cysteine-containing peptides for subsequent MS analysis without prior RP-separation of peptides.

This method is shown as a rapid alternative to 1D-PAGE-MS for the study of protein interactions involving membrane proteins. The proteins present in the CD4-affinity purification were identified and information on whether they specifically interact with CD4 was obtained. The reduction of sample complexity using this strategy has been evaluated and also some potential limitations of this approach are delineated.

### **3.3 Results and Discussion**

#### **3.3.1 Mass spectrometry analysis of affinity purified tryptic peptides.**

To identify the proteins associated with the purified CD4 receptor complex,  $10^9$  CemT4 cells were lysed (see 2.6) and purified (see 2.7) using a CD4-specific affinity column (see 2.20 for column generation). The eluted proteins were labelled with BIAM at cysteine residues and digested with trypsin (see 2.8). The cysteine-containing peptides carrying the biotin tag were purified by affinity chromatography using monomeric avidin gel (see 2.9) and the eluted fractions subsequently analysed by ESI MS (see 2.16). Figure 3.1 shows the MS screen of the affinity-purified peptide mixture with one enlarged region showing isotopic resolution of peptide ions.



**Figure 3.1:** Mass spectrometry identification of peptides derived from the purification of CD4 associating proteins. ESI-mass spectrum of a purified mixture of cysteine-containing peptides derived from the CD4 receptor complex is shown together with the ion at  $m/z$  961 enlarged.

The spectrum shows strong peaks from contaminating singly charged species in the  $m/z$  range up to 700. The distance of 44 Da between the peaks strongly points to a polyethyleneglycol (PEG)-based contamination. As both Triton X-100 and NP-40 are PEG-based polymers and were used for efficient solubilization of CD4, it is obvious that those detergents were not completely eliminated during the purification process. The carry over of the detergents through the purification is undesirable because it interferes with MS analysis owing to strong singly charged ions derived from the polymeric molecules. For washing away the detergents, similar studies used additional separation steps like cation exchange chromatography (Han et al. 2001), which were not desired in this study. Alternatively, SDS has been employed for protein solubilization (Han et al. 2001), however, SDS could not be used here as it might have destroyed the protein interactions we aimed to investigate in high concentrations.

Despite the singly-charged background, numerous ions attributed to peptides can be distinguished in the spectrum. The inset shows the region around  $m/z$  961 where the

individual isotopic peaks are clearly resolved and the difference in  $m/z$  of 0.25 between the peaks shows the ions are quadruply charged yielding a parent ion mass of 3841 Da. A comparison of the detected parent ions with the masses of trypsin autolysis fragments (2211Da, 1045 Da and 842 Da) showed that none of those commonly-seen contaminating trypsin peptides were present, demonstrating the ability of the method to eliminate those species. Many other ions that could be assigned to peptides were detected, enabling a total of 18 to be identified and the protein of origin determined as described below (see also 2.17 for a description of methods used for protein identification).

### **3.3.2 Identification of the proteins present in the affinity-purification of CD4.**

Table 3.1 shows all peptides that were identified in the affinity-chromatography purified sample and Figure 3.2 shows a representative MS/MS spectrum used for identification of a tryptic peptide from CD4. With the exception of avidin-derived peptides, all identified peptides contained a cysteine carrying the biotin tag (C\*) and no peptide resulted from a tryptic miscleavage. In the spectrum shown in figure 3.2 the precursor ion at  $m/z$  1176.3 was shown to be triply charged yielding a mass of 3526 Da. The spectrum showed a series of singly charged, C-terminal sequence ions from  $y_1^+$  through to  $y_8^+$ . This identifies the C-terminal portion of the sequence as V[LI][LI]ESN[LI]K (notation according to the ScanProsite software tool from the SwissProt database). Note that a mass difference of 113 can be equally derived from a leucine or an isoleucine residue so the software used for database searching requires that both residues are entered at the position representing a mass difference of 113. Here this is done by putting into square brackets both residues permitted at the respective position. It is also not possible to distinguish between lysine (K) and glutamine (Q) on the basis of mass; however, in this case lysine can be placed at the C-terminus because it is a tryptic peptide. Entering the above pattern into the ScanProsite software searching all human entries in the SwissProt database identifies CD4 as the only match out of 14,353 entries. Searching the CD4 sequence for the peptide containing the tag identifies the peptide at position 357-385 to contain the sequence at the C-terminus. Its mass of 3200.6 Da together with the mass of the biotin tag of 326.1 yields 3526.7 Da matching the detected mass of the precursor ion. Inspection of the predicted fragmentation of the peptide shows that many other ions detected in the MS/MS spectrum

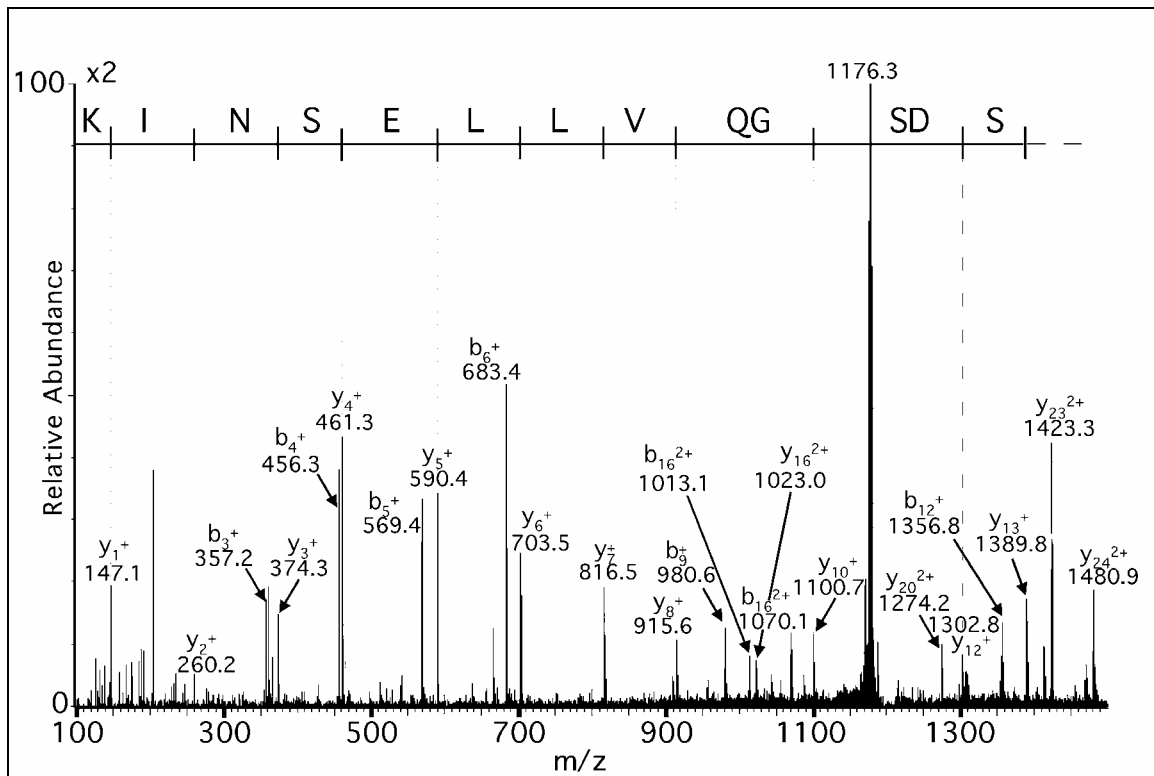
can be assigned to predicted product ions of the peptide. Figure 3.2 also shows evidence of a b-ion series representing fragments containing the N-terminal part of the peptide as well as doubly charged ions from fragments with a higher mass that can also be assigned to the peptide leading to its unambiguous identification.

Analogous MS/MS spectra were obtained from the peptides listed in Table 3.1 allowing their unambiguous identification by the same type of strategy. Fifteen cysteine-containing tryptic peptides were identified by MS/MS analysis in three subsequent experiments (Table 3.1). Of the 15 peptides identified, two were from CD4 and one from the binding partner p56lck. This demonstrates that the strategy under investigation in this study is a suitable method for rapid identification of proteins present in a mixture derived from affinity chromatography using a single chromatographic step. The detection of both CD4 and p56lck highlights the ability of the method to detect protein complexes. Since CD4 and p56lck are known to interact in T-lymphocytes (Veillette et al. 1988) and the identification of both proteins serves as a proof-of-principle for the versatility of this approach. The detection of avidin-derived peptides has a different origin. Avidin is present in the peptide sample owing to tryptic hydrolysis of the avidin beads during incubation of the tryptic peptide mixture with the beads. PMSF, a trypsin inhibitor was added to the digest in order to inactivate trypsin, however, traces of the avidin-derived peptides were always present. For the remaining peptides identified additional experiments were conducted to assess the specificity of binding to CD4 as outlined below.

**Table 3.1.** Peptides and proteins identified by ESI-MS/MS

mass	Peptide sequence	Protein matched	Acc. Number SWISS-PROT
3189.5	Ac-ASVSELAC*IYSALILHDDEVTVTEDK <sup>a)</sup>	60 S Acidic ribosomal protein P1	P05386
4078.0	ALANVNIGSLIC*NVGAGGPAPAAGAAPAGG- PAPSTAAAPAEK	60 S Acidic ribosomal protein P1	P05386
2928.3	YGWSADESGQIFIC*SQEESIKPK	HSPC029	Q9Y6D1
1899.9	ALTNLPHDTFTLC*K	HSPC029	Q9Y6D1
2619.1	NAFAC*FDEEATGTIQEDYLR	Myosin regulatory light chain	P19105
3398.5	MTEEEVEMLVAGHEDSNGC*INYEAFVR	Myosin essential light chain	P16475
2977.5	LTTPTYGDLNHLVSATMSGVTTC*LR	Tubulin $\beta$ -5 chain	P04350
3256.6	ITSAVWGPLGEC*IIAGHESGELNQYSAK	eIF-3 $\beta$	Q13347
3259.6	LSRPC*QTQKPQKPWWEDEWEVPR	p56lck	P06239
3526.7	AVWVLNPEAGMWQC*LLSDSGQVLLESNIK	CD4	P01730
3005.5	TLSVSQLQLQDSGTWTC*TVLQNQK	CD4	P01730
1330.7	LGEWVGLC*K	40 S ribosomal protein S12	P25398
2545.2	HLYTLDDGGDIINALC*FSPNR	Guanine nucleotide binding protein	P25388
2251.0	YTVQDESHSEWVSC*VR	Guanine nucleotide binding protein	P25388
3012.4	TNHIGHTGYLNTVTVSPDGSLC*ASGGK	Guanine nucleotide binding protein	P25388
919.5	VGINIFTR	Chicken avidin	P02701
3264.7	TMWLLRSSVNDIGDDWKATRVGINIFTR	Chicken avidin <sup>b</sup>	P02701
3219.7	SSVNDIGDDWKATRVGINIFTRLRTQKE	Chicken avidin <sup>b, c</sup>	P02701

- a) C\* represents cysteine carrying the biotin tag  
b) two avidin-derived peptides contain miscleavages  
c) comprises of the C-terminal part of avidin

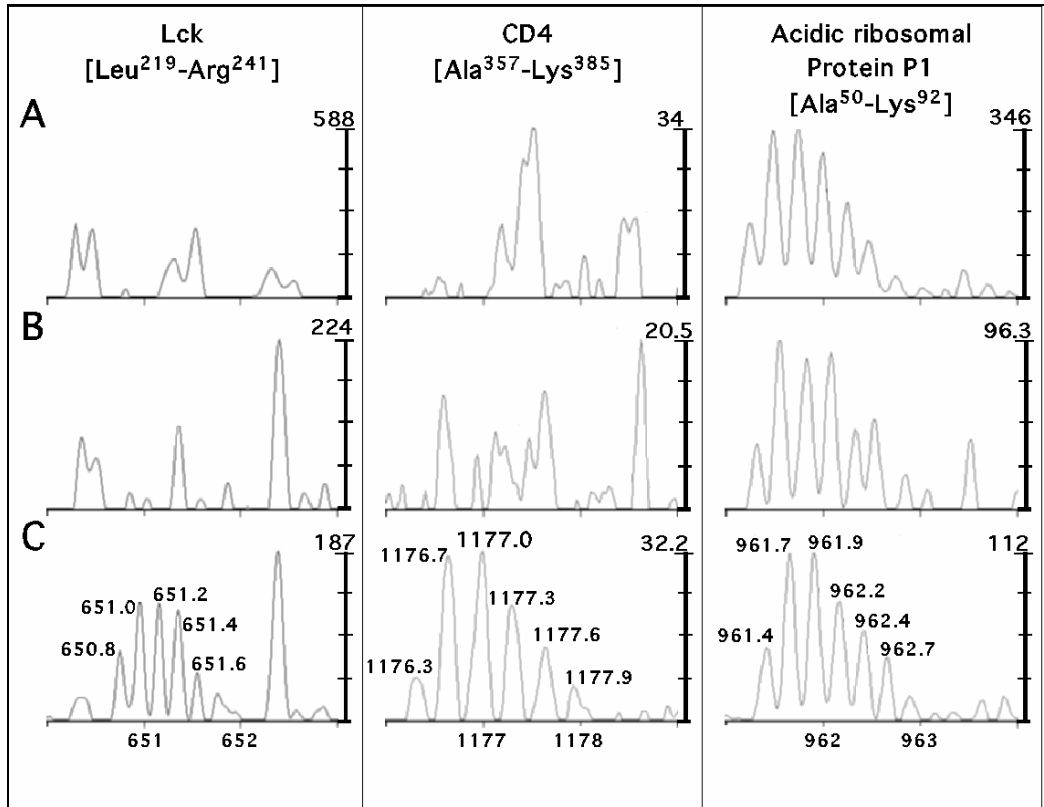


**Figure 3.2:** Identification of CD4 via tandem mass spectrometry. The MS/MS spectrum of the ion at  $m/z$  1176 ( $3+$ , precursor mass 3526 Da) is shown. The full peptide sequence is AVWVLNPEAGMQCLLSDSGQVLLESNIK. Singly and doubly charged product ions are labelled.

### 3.3.3 Specificity of the interaction with CD4

To determine whether the other proteins identified in the affinity purification of the CD4 receptor complex were molecules that specifically interact with CD4, two different control experiments were performed. These included the analysis of an affinity purification of a CD4 negative cell line (A2.01) and an affinity purification using immobilised IgG1 to identify the proteins that bind non-specifically to the column. Figure 3.3 shows representative portions of the ESI mass spectra for 3 peptides. These show the presence or absence of ions in the purified sample derived from the CD4 affinity chromatography and the control sample. Table 3.2 summarises the data for all proteins and indicates whether peptides derived from their tryptic digest were detected in the control experiments. It can be seen that only CD4 and p56lck are uniquely present in the purification of the CD4 receptor complex demonstrating the versatility of this approach to distinguish specific components of a receptor complex from non-specific interactors. Though present in the control sample, cytoskeletal proteins are often found to be

associated with membrane receptors through intermediate adaptor proteins and an association between CD4/p56lck and the cytoskeleton has been reported (Kinch et al. 1994; Thuillier et al. 1994). Thus we cannot exclude the possibility that in addition to the non-specific binding to the resin, cytoskeletal components are also associated with CD4 and p56lck via intermediate linker proteins. The results indicate that the affinity purification procedure probably results in a loss of specific binding partners as none of the other described interacting molecules such as ACP33 or LAT (section 1.1.4.2) are observed. Further, the method also promotes non-specific protein interactions, therefore more refinement was needed to promote the preservation of specific interactions without promoting nonspecific binding.



**Figure 3.3:** Comparison of regions of ESI mass spectra of representative peptides showing ions at  $m/z$  651 (p56lck),  $m/z$  1176 (CD4) and  $m/z$  961 (60 S acidic ribosomal protein P1). Shown are the same regions from (A) CD4 negative control; (B) the IgG1 affinity chromatography control; and (C) the sample of the CD4 receptor complex. Scales with the ion abundances are indicated at the right of each section.

**Table 3.2.** Proteins detected by ESI-MS and ESI-MS/MS analysis of the CD4 preparation and control experiments

Protein	CD4 Purification	IgG1 control	CD4-negative control
60 S Acidic ribosomal protein P1	MS/MS <sup>a)</sup>	MS/MS	MS <sup>b)</sup>
40 S Ribosomal protein S12	MS	MS/MS	
Chicken avidin	MS/MS	MS/MS	MS
Myosin regulatory light chain	MS/MS	MS/MS	
Myosin essential light chain	MS/MS	MS	MS/MS
Tubulin $\beta$ -5 chain	MS/MS	MS/MS	
eIF-3 $\beta$	MS/MS	MS	
Guanine nucleotide binding protein	MS/MS	MS/MS	
HSPC029	MS/MS	MS	
p56lck	MS/MS		
CD4	MS/MS		

a) indicates that identity was confirmed by MS/MS analysis

b) indicates that the peptide was detected by MS analysis

### 3.3.4 Evaluation of the reduction of sample complexity

Using tandem mass spectrometry, the determination of the amino acid sequence of a single tryptic peptide is usually sufficient to identify unambiguously the protein from which this peptide originated (Mangasarian and Trono, 1997). When studying the digest of an unseparated protein mixture, it is therefore desirable to have only a few peptides per protein (ideally one unique peptide allowing the identification of the protein) in order to reduce the complexity of the peptide sample used for protein identification. Therefore a modification of the cysteinyl affinity capture method of Spahr and co-workers (Spahr et al. 2000) was employed here to purify only cysteine-containing peptides. Cysteine is a relatively rare amino acid and a protein usually produces only a few tryptic peptides, which contain a cysteine residue, but 96.7 % of the human protein entries in the SwissProt database do contain at least one cysteine residue. Hence, apart from the information on the remaining 3.3 % of proteins, which would not be accessible with this method, it was investigated how far the reduction of sample complexity reaches and whether it is likely that information on additional proteins is not obtained with this

method. To do this the number of peptides that were detected in the MS analysis were compared with the number of cysteine-containing peptides and the total number of tryptic peptides derived from the proteins that were identified. As a minimum of about 7 amino acids is required for a peptide to obtain sufficient sequence information based on a single protein for identification and to have a high enough mass to be seen in the MS (relatively free from singly charged background ions), only those peptides were considered. To ensure that no peptides from the detected proteins were missed, all spectra from the CD4 affinity purification were screened for the expected range of multiply charged ions from tryptic peptides that contain one or more cysteine assuming charge states between +2 and +5 depending on peptide size. This search identified only one ion that could have been an additional peptide from the guanine nucleotide binding protein. The original MS/MS analysis of this ion did not yield fragment ions in high enough abundance to be sufficient for peptide identification. This shows that almost all peptides that could be detected from those proteins were indeed identified. Table 3.3 summarises the data from this approach and shows that from a total of 148 tryptic peptides, 15 had been identified in the sample leading to a reduction of sample complexity of about 90 %. This is a very desirable value as a mixture of 15 peptides is much easier to analyse than a mixture of 150 peptides. However, the 15 identified peptides represent only ~ 30 % of a total of 47 peptides, which contain a cysteine residue. A similar result was reported by Gygi et al. (1999), where 15 out of 44 cysteine-containing tryptic peptides derived from a model protein mixture were identified (Gygi et al. 1999). It also correlates with the results from Spahr et al. (2000) who report identification of 22 out of 68 cysteine-containing peptides from a model protein mixture consisting of 17 proteins (Spahr et al. 2000). In that study, the authors used a different method for biotin labelling and avidin affinity chromatography. They employed a thiol exchange reaction for the labelling of reduced cysteine-containing peptides and used DTT to elute bound peptides from the immobilised avidin by cleaving the disulfide bond in the linker region of the affinity tag. Despite the difference in the methodology, the loss of a large portion of cysteine-containing peptides tends to be a characteristic of this procedure. Additionally, it can be seen that, in this study, predominantly relatively large peptides were identified (about half of the detected

peptides have a mass of > 3 kDa) and no peptides having two cysteine residues (of 6 possible ones) were detected.

**Table 3.3.** Summary of proportion of peptides detected for each protein.

Protein	number of cysteines <sup>a)</sup>	number of tryptic peptides <sup>b)</sup>	tryptic peptides with cysteine <sup>c)</sup>	number of detected peptides
60 S Acidic ribosomal protein P1	2	4	2	2
40 S Ribosomal protein S12	6	7	3	1
Myosin regulatory light chain	1	9	1	1
Myosin essential light chain	3	10	3	1
Tubulin $\beta$ -5 chain	8	22	6	1
eIF-3 $\beta$	7	6	6	1
HSPC029	5	13	5	2
Guanine nucleotide binding protein	8	20	8	3
p56lck	9	25	5	1
CD4	11	18	8	2
Total	60	148	47	15

a) number of cysteine residues in the whole protein

b) number of all tryptic peptides that are at least 7 amino acids long

c) number of tryptic peptides of 7 amino acids or longer which contain one or more cysteine residues

The reasons for the loss of so many peptides remain elusive. First, the possibility was considered that it arises from the unsuccessful reduction of some disulfide bridges. The two peptides originating from CD4 contain one cysteine involved in the domain D4 disulfide-bridge and one cysteine involved in the domain D2 disulfide-bridge (Maddon et al. 1987), but the peptides containing the other cysteines involved in those disulfide bridges were not detected despite one of them having a convenient size of about 1760 Da. It is thus more likely that the absence of a large portion of cysteine-containing peptides is a consequence of the affinity tag-mediated purification. It is possible that smaller peptides, and peptides containing multiple cysteine residues, bind more tightly to the avidin resin and cannot be eluted as easily as the larger peptides that contain only one affinity tag. Further it would be unrealistic to expect that all peptides carrying a cysteine could be detected in the MS analysis, some peptides are just too small or too large to be

detected or to yield useful MS/MS data. Additionally, for example, the peptide spanning the transmembrane domain of CD4 also contains a cysteine and such a hydrophobic peptide is unlikely to be readily purified and/or detected in the MS analysis. However, coverage of only one third of cysteine-containing peptides suggests that more protein information is lost than originally expected. It is possible that other proteins are present in the sample but were not identified. According to Spahr and co-workers, they identified only 9 out of 13 cysteine containing proteins (out of 17 total proteins) by analysing the peptides that bound to the avidin resin (Spahr et al. 2000). To detect proteins that are present in the affinity chromatography-derived sample but missed with this strategy, the flow-through fraction of the avidin affinity column could be subjected to MS analysis. However this was not performed owing to the presence of detergents in the flow-through fraction which would have required additional purification steps. To put this in context, however, no method of protein identification using MS will identify all proteins. It is known that 2D-PAGE-MS analysis lacks coverage of small, acidic and hydrophobic proteins (Moseley 2001). As with so many analytical techniques, it is best to have several complementary techniques available to achieve the best possible coverage. According to the results presented in this study, this method adds to the repertoire of protein identification techniques and demonstrates its usefulness for studying interactions of membrane proteins. The advantage of this method lies in the simplicity of the analysis. It does not require PAGE separation of proteins, staining of the gel and the dissection of the gel into slices. It also does not employ multidimensional chromatography. Instead of analysing several gel slices or several chromatography fractions independently, this method allows the identification of a proportion of the proteins of interest in one single mass spectrometry experiment.

### **3.4 Conclusion**

The results described in this chapter demonstrate the versatility of a modified ICAT strategy to determine the components of a protein mixture and investigates its limitations. An affinity chromatography purification of the CD4 receptor complex was studied and the proteins present in the sample were identified. A relatively low coverage of cysteine-containing peptides was observed making it possible that some proteins are not identified.

This shows the need of additional strategies to achieve a higher coverage of the components of the CD4 receptor complex. The strategy proved to be successful for the study of CD4 and its associating molecules as both CD4 and p56lck, which is known to be associated with CD4, were identified. However, the majority of the proteins found in the affinity chromatography derived sample were probably present because of non-specific binding to the matrix during the affinity chromatography step. The results described in subsequent chapters will therefore investigate approaches to reduce non-specific binding as well as preserving labile and/or transient protein interactions. Besides, a different technological approach (1D-PAGE) has been evaluated for its ability to detect proteins that may have missed detection using the approach outlined in this chapter.

### **3.5 Summary**

The study of protein interactions using mass spectrometry (MS) for identification of the components of purified protein complexes is leading to the description of increasingly valuable data on protein function. Commonly proteins in a given complex are identified via MS analysis of in-gel digests of gel-electrophoretically separated proteins. In this study, the use of an approach employing the digest of the whole protein complex to identify directly the proteins present in a purification of the CD4 receptor complex is evaluated. A cysteinyl affinity capture method was used to reduce the complexity of the peptide mixture that was obtained from the tryptic digest of the whole protein complex to the rather limited mixture of only cysteine-containing peptides.

Here the use of this approach with mass spectrometry is described for identification of the CD4 receptor complex components CD4 and p56lck, along with several other proteins present in the detergent-solubilized fractions from the purification. These proteins were identified using peptide sequence data obtained from cysteine-containing peptides. With appropriate control experiments, the specific nature of the CD4-p56lck interaction was demonstrated. In contrast, the other proteins identified are shown to arise from non-specific interactions during the affinity chromatography purification suggesting a possible loss of specific interactions during the chromatography procedure. The complexity of the mixture was reduced such that only 10% of the peptides derived from tryptic digest of the identified proteins were detected. This represents only one third of the cysteine-containing peptides, however, suggesting that this approach does not enable detection of all individual proteins.

## **CHAPTER FOUR:**

### **SEARCH FOR PROTEINS CO-PURIFYING WITH THE CD4 RECEPTOR COMPLEX USING MASS SPECTROMETRY**

### **EVALUATION OF ONE-DIMENSIONAL POLYACRYLAMIDE GEL ELECTROPHORESIS FOLLOWED BY IN-GEL DIGEST TO IDENTIFY PROTEINS PURIFIED WITH Q425 CHROMATOGRAPHY.**

### **EVALUATION OF DIFFERENT SOLUBILISATION METHODS.**

#### **4.1 Aims**

The work outlined in this chapter aimed to achieve the following:

- (i) To evaluate the use of one-dimensional polyacrylamide gel electrophoresis (1D-PAGE) followed by in-gel digest and mass spectrometry analysis for identifying the proteins present in an affinity chromatography-derived purification of the CD4 receptor complex;
- (ii) To discuss that strategy with respect to its ability to identify the proteins present and compare the strategy to the method described in chapter 3;
- (iii) To investigate the use of a different detergent system containing SDS for the solubilisation of the CD4 receptor complex and determine its effect on (non-)specific binding; and
- (iv) To evaluate all detected proteins in terms of an interaction with CD4; finally a summary of the data from results in chapters 3 and 4 is provided and the potential of the approach of the methods in chapters 3 and 4 for identifying novel CD4 binding proteins is discussed.

## 4.2 Introduction:

Most proteins, including CD4, carry out their function in association with other proteins with which they interact either transiently or permanently as reviewed in chapter 1. Often the proteins that are involved in a specific task such as ligand binding (followed by transmission of signals) preassemble in large complexes. Identification of the individual subunits of such a complex often provides valuable data on the function of individual components. The increasing demand for rapid information on protein function has therefore seen an increase in studies aiming to define the components of protein complexes. Complexes are normally affinity-purified and the mixture of proteins that is obtained is then analysed using one of several different approaches that are now available. When purifying protein complexes for MS analysis, several factors need to be considered. The purification strategy needs to preserve the protein interactions that hold the complex together without introducing non-specific binding of abundant cellular proteins. This can be a problem when the use of detergents is required for solubilizing membrane proteins as it has been suggested that different detergents can influence the protein interactions ultimately detected (Beyers et al. 1992). Furthermore, cross-linking of protein interactions may be helpful for preserving and identifying specific protein interactions (Wine et al. 2002).

After purification of the complex, the protein mixture needs to be digested with trypsin and the tryptic peptides analysed by MS. As noted earlier, because tryptic cleavage of only a few proteins already produces a large number of peptides, the mixture of which is often difficult to analyse, usually at least one more separation step is required prior to MS analysis. This separation can be achieved via SDS polyacrylamide gel electrophoresis (SDS-PAGE) of the protein mixture followed by analysis of the individual slices (Wine et al. 2002) or by liquid chromatography-MS (LC-MS) analysis of the tryptic peptides derived from all proteins (Link et al. 1999). A 2D-PAGE separation of the purified complex, albeit possible, is not normally employed. This is because a purified complex is a mixture of a rather limited number of proteins that can be sufficiently separated by 1D-PAGE whereas 2D-PAGE is able to resolve large numbers of proteins. It was also not undertaken here because hydrophobic proteins such as membrane proteins generally separate poorly during the isoelectric focussing step. In

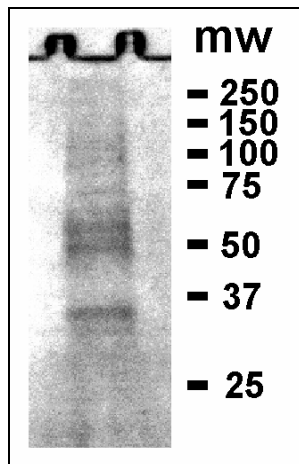
chapter 3 a strategy was evaluated using affinity tags introduced at cysteine residues and purification of peptides containing the affinity tag to create a representative subset of only few tryptic peptides per original protein thereby increasing the numbers of proteins that can be detected in one MS experiment (Bernhard et al. 2003). These results showed the identification of the components of the CD4 receptor complex CD4 and lck, a tyrosine kinase that has been reported to associate with CD4 (Veillette et al. 1988) functioning in T cell activation. In addition, to those two proteins several unrelated proteins were identified, which were shown using different control experiments to result from non-specific binding. It was assumed that non-specific binding was promoted by the use of the non-ionic detergents Triton X-100 and NP-40. Specific protein-protein interactions may have been obscured because of the background from the detergents or ions derived from non-specifically binding proteins. Proteins that did not contain a cysteine residue or where the peptides containing cysteine residues were not detectable with mass spectrometry would also not be identified with that method (Bernhard et al. 2003).

In this chapter, the use of one-dimensional gel electrophoresis followed by mass spectrometry analysis of in-gel digested protein bands was investigated as an alternative strategy to identify proteins present in the detergent solubilized sample. It describes the identification of many different copurifying proteins and provides a comparison between the SDS-PAGE-based method and the method described in chapter 3 based on the experience obtained in this study. In order to reduce non-specific binding, the use of a detergent combination of NP-40, sodium deoxycholate (DOC) and SDS was evaluated for cell lysis. This combination was chosen because it has been shown previously that interactions persist in this detergent combination (Sheng et al. 1994) when different subunits of the *N*-methyl-D-aspartate (NMDA) complex could be co-immunoprecipitated. The presence of SDS, which is a potent detergent for disrupting non-covalent protein interactions suggests that weak protein interactions such as non-specific binding will be reduced. It was therefore chosen to compare the extent of non-specific binding when using this lysis buffer with the non-specific binding detected when Triton/NP-40 was used for cell lysis.

## 4.3 Results

### 4.3.1 Proteins detected visually in the coomassie stained gels

To identify proteins purifying together with CD4, the affinity-purified (section 2.6 & 2.7) complexes from  $\sim 1 \times 10^9$  cells were concentrated (section 2.10) and separated on an 8-16 % gradient polyacrylamide gel and the gel was stained with Coomassie Brilliant Blue (section 2.12). Figure 4.1 shows a representative example for all experiments of a Coomassie stained gel containing the proteins of a CD4 affinity purification using the SDS containing lysis buffer. The visible pattern is very weak and apart from major bands at 60, 50 and 37 kDa, several minor bands of different strengths (but all very weak) can be seen.



**Figure 4.1:** Visualisation of proteins present in the affinity-purification of the CD4 receptor complex. CD4 and associating proteins from approximately  $10^9$  cells were separated on an SDS-Polyacrylamide gel and stained with Coomassie Brilliant Blue.

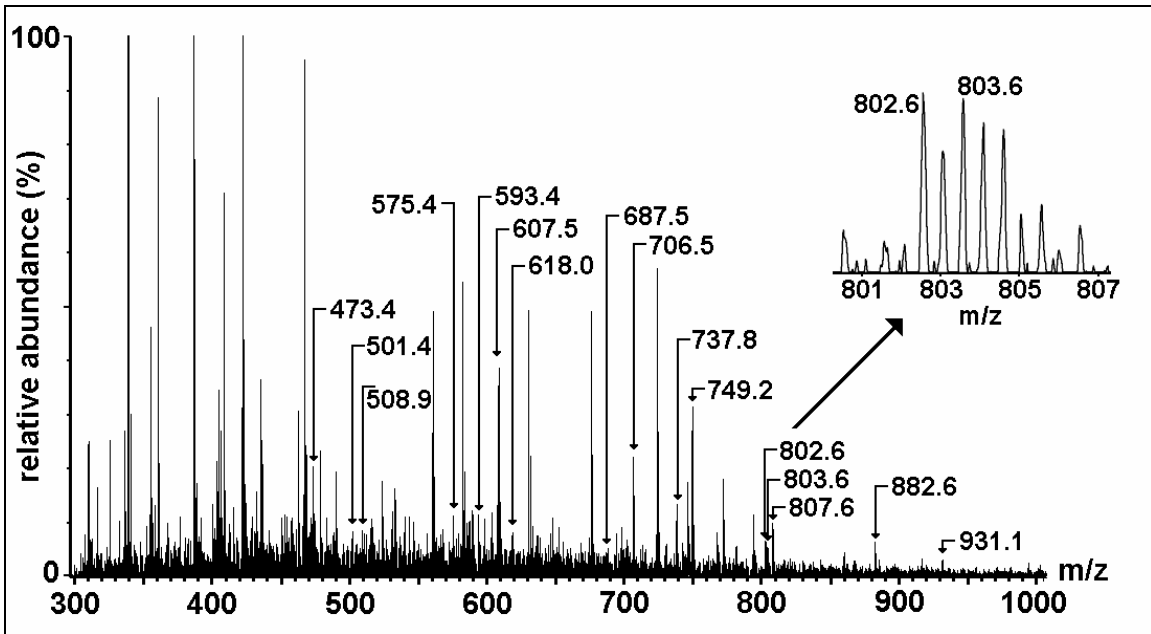
### 4.3.2 Proteins identified in the sample prepared with Triton X-100/NP-40 lysis buffer

Two experiments were carried out to determine proteins present in an affinity purification of CD4 employing the Triton X-100/NP-40 based lysis buffer. Whereas in the first experiment, only visible protein bands were excised and analysed (sections 2.13, 2.16 & 2.17), in the second series of experiments, the entire lane of the gel was dissected into slices from the very top of the gel down to the area representing the molecular weight of 15 kDa. A total of 23 proteins were identified in the two different experiments using the 1D-PAGE-MS strategy. Table 4.1 lists all identified proteins. In all, 8 proteins were identified in the first experiment, 6 of them were identified again in the second

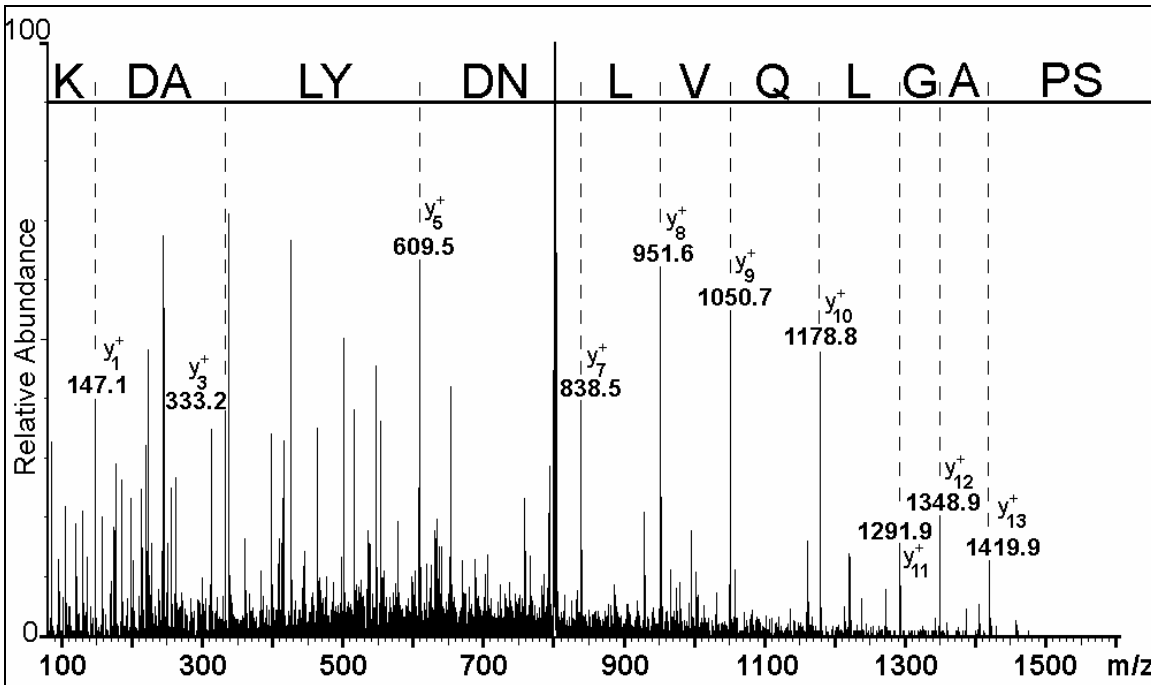
experiment along with 15 other proteins. Proteins were mainly derived from the transcription/translation machinery, besides, some cytoskeletal proteins were identified along with chaperones such as heat shock proteins.

#### **4.3.3 Proteins identified in the sample prepared with NP-40/DOC/SDS lysis buffer**

To investigate whether the use of a more stringent detergent combination has a positive effect on reducing non-specific binding, three experiments were carried out employing a different detergent combination including SDS in the lysis buffer. CD4 complexes were purified (section 2.6 & 2.7) as described above and it was determined that CD4 binding to the antibody is not abrogated in the presence of SDS (shown in a different context in 5.4.1). Figure 4.2 shows a representative mass spectrometry analysis of the purified peptide mixture obtained from a tryptic digest of an excised band containing only a barely visible amount of protein. The inset shows the expansion of a part of the spectrum allowing the identification of doubly charged ions at  $m/z$  802.6 and 803.6. Figure 4.3 shows a MS/MS spectrum of the ion at  $m/z$  802.6 leading to the unambiguous identification of elongation factor 1-beta. Sixteen peptide ions were identified in this gel slice as indicated with the labels resulting in the identification of 9 different proteins (excluding trypsin). Table 4.2 lists all peptides and proteins that were identified in this gel slice. A total of 54 proteins were identified in three independent experiments using 1D-PAGE MS analysis of purified CD4 derived from cells lysed with NP-40/DOC/SDS buffer. Table 4.1 lists all proteins that were identified in these experiments. It can be seen that again, most proteins are part of the transcription/translation machinery along with cytoskeletal proteins and chaperones. However, in this study other proteins were identified such as importins, ion channel subunits or the leucine-rich protein which have different functions than the usually seen non-specifically binding proteins.



**Figure 4.2:** Mass spectrometry identification of peptides derived from the tryptic digest of an excised gel slice. The ESI-mass spectrum of the tryptic peptides derived from the proteins in a gel slice of about 30 kDa is shown together with the ions at  $m/z$  802.6 and 803.6 enlarged.



**Figure 4.3:** Identification of proteins via tandem mass spectrometry. The MS/MS spectrum of the ion at  $m/z$  802.6 ( $2+$ , precursor mass 1604.2 Da) is shown. Singly charged product ions are labelled. The peptide sequence SPAGLQVLNDYLADK identified it as derived from the Elongation factor 1-beta.

**Table 4.1: Proteins identified by ESI-MS/MS in all experiments**

Protein Name	Spot# <sup>a</sup>	MW kDa	number of Cys	Peptides identified in experiment					
				[Biotin] <sup>b</sup>	1A <sup>c</sup>	1B	2A	2B	2C
Leucine-rich protein	P42704	145	21	.	.	.	8	.	.
Eukaryotic translation initiation factor 3 subunit 8	Q99613	105	12	.	.	2	.	.	.
Importin beta-2 subunit	Q92973	101	26	.	.	.	1	.	.
Importin beta-1 subunit	Q14974	97	23	.	.	.	3	.	.
Eukaryotic translation initiation factor 3 subunit 9	P55884	92	7	.	.	1	.	.	.
Heat-shock protein HSP 90-alpha	P07900	85	7	.	.	.	3	.	.
Nephrilysin	P08473	85	12	.	.	.	.	1	.
Heat-shock protein HSP 90-beta	P08238	83	6	.	.	.	8	.	.
Structure-specific recognition protein 1	Q08945	81	6	.	2	4	.	.	4
Annexin A6	P08133	76	6	.	.	.	.	9	11
Nucleolin	P19338	76	1	.	.	5	.	.	5
DEAD-box protein 3	O00571	73	7	.	.	1	.	.	.
Heat shock cognate 71 kDa protein	P11142	71	4	.	4	2	3	.	.
Probable RNA-dependent helicase p68	P17844	69	9	.	.	2	.	.	.
Phenylalanyl-tRNA synthetase beta chain	Q9NSD9	66	11	.	.	1	.	.	.
Eukaryotic translation initiation factor 3 subunit 7	O15371	64	11	.	.	4	.	.	.
60 kDa Heat shock protein	P10809	58	3	.	.	.	4	.	.
P56lck	P06239	58	9	1	2	2	9	16	2 <sup>d</sup>
ATP synthase alpha chain, mitochondrial	P25705	55	2	.	.	.	3	.	.
CD4	P01730	55	11	2	3	1	3	7	5 <sup>d</sup>
Vimentin	P08670	54	1	.	.	.	1	.	.
Eukaryotic translation initiation factor 3 subunit 6	Q64252	52	7	.	2	6	.	.	.
Tubulin alpha-1 chain	P05209	50	12	.	.	1	.	.	.
Tubulin beta-1 chain	P07437	50	9	.	2 <sup>e</sup>	1	.	.	.
Tubulin beta-5 chain	P04350	50	8	1	.	1	.	.	.
Heterogeneous nuclear ribonucleoprotein H	P31943	49	5	.	.	1	.	.	.
Alpha enolase	P06733	47	6	.	.	.	8	.	.
Eukaryotic initiation factor 4A-II	Q14240	46	4	.	.	.	1	.	.
GA17 protein	O60735	43	8	.	1	.	.	.	.
Actin, cytoplasmic I	P02570	42	6	.	.	.	2	.	.
Guanine nucleotide-binding protein G(i), alpha-2 subunit	P04899	40	10	.	.	.	.	.	1
Annexin A1	P04083	39	4	.	.	.	.	2	2
Heterogeneous nuclear ribonucleoprotein A1	P09651	39	2	.	.	1	1	.	.
Annexin A2	P07355	38	4	.	.	.	.	8	7
Heterogeneous nuclear Ribonucleoprotein D0	Q14103	38	3	.	.	1	.	.	.
Voltage-dependent anion-selective channel protein 2	P45880	38	10	.	.	.	1	.	.
Heterogeneous nuclear ribonucleoproteins A2/B1	P22626	37	1	.	.	1	.	.	.
Eukaryotic translation initiation factor 3	Q13347	37	7	1	1	.	.	.	.

subunit 2									
Glyceraldehyde 3-phosphate dehydrogenase	P04406	36	3	.	.	.	6	2	.
Nuclease sensitive element binding protein 1	P16991	36	0	.	.	.	.	.	2
Guanine nucleotide binding protein	P25388	35	8	3	.	.	.	.	.
60 S acidic ribosomal protein P0	P05388	34	3	.	.	2	.	.	1
40 S ribosomal protein SA	P08865	33	2	.	.	4	.	.	.
Nucleophosmin	P06748	33	3	.	.	1	.	.	.
Voltage-dependent anion-selective channel protein 1	P21796	31	2	.	.	.	1	.	.
40 S ribosomal protein S3A	P49241	30	4	.	.	1	1	.	.
60 S ribosomal protein L7A	P11518	30	3	.	.	.	.	.	1
Voltage-dependent anion-selective channel protein 3	Q9Y277	30	6	.	.	.	1	.	.
Prohibitin	P35232	30	1	.	.	.	2	.	.
40 S ribosomal protein S4	P12750	29	4	.	.	.	.	.	1
60 S ribosomal protein L7	P18124	29	1	.	.	.	.	.	1
Dehydrogenase/reductase SDR family member 2	Q13268	27	6	.	.	.	2	.	.
Elongation factor 1-beta	P24534	25	3	.	.	.	2	.	.
HSPC029	Q9Y6D1	25	5	2	.	.	.	.	.
40 S ribosomal protein S8	P09058	24	5	.	.	.	.	.	1
GTP-binding nuclear protein RAN	P17080	24	3	.	.	.	1	1	.
40 S ribosomal protein S7	P23821	22	0	.	.	.	.	.	1
40 S ribosomal protein S9	P46781	22	1	.	.	.	.	.	1
60 S ribosomal protein L18	Q07020	22	2	.	.	.	.	.	1
60 S ribosomal protein L11	P39026	20	4	.	.	.	.	.	1
Myosin regulatory light chain	P19105	20	1	1	.	.	.	.	.
40 S ribosomal protein S10	P46783	19	0	.	.	.	.	.	1
40 S ribosomal protein S18	P25232	18	0	.	.	.	.	.	4
40 S ribosomal protein S13	Q02546	17	0	.	.	.	.	.	1
Myosin essential light chain	P16475	17	3	1	.	.	.	.	.
40 S ribosomal protein S19	P39019	16	0	.	.	.	.	.	1
40 S ribosomal protein S24	P16632	15	0	.	.	.	.	.	1
60 S ribosomal protein L22	P35268	15	1	.	.	.	.	.	1
40 S ribosomal protein S12	P25398	14	6	1	.	.	.	.	.
Histone 2B	P02278 <sup>f</sup>	14	0	.	.	.	.	.	2
60 S Acidic Ribosomal Protein P1	P05386	12	2	2	.	.	.	.	.
Ubiquitin	P02248	8.5	0	.	.	.	.	.	1
Total: 66 proteins (not including [Biotin])					8	23	26	8	26

<sup>a</sup> Swiss-Prot accession number

<sup>b</sup> detected in the study described in chapter 3 (Bernhard et al. 2003)

<sup>c</sup> + indicates in which of five experiments the protein was detected. Experiment labels 1/2 refer to the respective lysis buffer Triton X-100/NP-40 or NP-40/DOC/SDS and A/B/C refer to one of two or three independent experiments.

<sup>d</sup> Several ions typical of CD4- or lck-derived peptides were detected but not subjected to fragmentation

<sup>e</sup> the detected peptide matched two or more tubulin beta chains

<sup>f</sup> the detected peptide matched various alleles of the same protein, one allele is listed representative for all matches.

**Table 4.2:** Peptides and proteins identified in an excised gel-slice of ~ 30 kDa

Precursor m/z <sup>a</sup>	Peptide mass <sup>b</sup>	Sequence <sup>c</sup>	MASCOT Score	Protein Name	Swiss Prot#	MW kDa
473.4	944.6	LVPVGYGIK	12	Elongation factor 1-beta	P24534	24.6
501.4	1001.5	LITEDVQGK	25	40 S ribosomal protein S3A	P49241	29.8
508.9	1015.6	LTLSALVDGK	34	Voltage-dependent anion-selective channel protein 2	P45880	38.1
575.4	1029.6	LTLSALIDGK	33	Voltage-dependent anion-selective channel protein 3	Q9Y277	30.7
593.4	1184.7	DLQNVNITLR	26	Prohibitin	P35232	29.8
607.5	1212.7	VLPSITTEILK	34	Prohibitin	P35232	29.8
618.0	1233.7	IEVIEIM*TDR	29	Heterogeneous nuclear ribonucleoprotein A1	P09651	38.7
687.5	1373.7	WTEYGLTFTEK	7	Voltage-dependent anion-selective channel protein 1	P21796	30.6
706.5	1410.8	GALQNIIPASTGAAK	32	Glyceraldehyde 3-phosphate dehydrogenase	P04406	35.9
737.8	2210.1	LGEHNIDVLEGNEQFIN AAK	47	Trypsin	P00761	23.5
749.2	2244.1	VIIISAPSADAPM*FVM* GVNHEK	13	Glyceraldehyde 3-phosphate dehydrogenase	P04406	35.9
802.6	1602.8	SPAGLQVLNDYLADK	57	Elongation factor 1-beta	P24534	24.6
803.6	1604.9	VAVVTGSGTGIGFAIAR	17	Dehydrogenase/reductase SDR family member 2	Q13268	27.3
807.6	1612.9	LIVINGNPITIFQER	46	Glyceraldehyde 3-phosphate dehydrogenase	P04406	35.9
882.6	1762.8	LISWYDNEFGYSNR	42	Glyceraldehyde 3-phosphate dehydrogenase	P04406	35.9
931.1	1860.0	SPALLSQQLLPYM*ENR	45	Dehydrogenase/reductase SDR family member 2	Q13268	27.3

<sup>a</sup> given is the experimentally determined m/z value

<sup>b</sup> given is the theoretical uncharged monoisotopic peptide mass

<sup>c</sup> M\* indicates oxidized methionine

## 4.4 Discussion

### 4.4.1 Identification of peptides and proteins

Proteins usually carry out their function in complexes and the knowledge of the interacting partners is crucial to determine the function of a given protein. CD4 has multiple functions in T cell activation (Gay et al. 1987), as an interleukin-16 receptor (Cruikshank, W. W. et al. 1994) and in HIV-1 entry (Lapham et al. 1996; Wu, L. et al. 1996). In chapter 3 proteins which copurify with CD4 were sought in a search for novel binding partners that could contribute to the function of this receptor (Bernhard et al. 2003). In that study proteins were identified using affinity tag-mediated purification of

tryptic peptides which carried a biotin tag at cysteine residues. However, only the CD4/lck complex was identified along with several non-specifically binding proteins. It was therefore suggested that proteins present in the affinity chromatography-derived sample could have escaped detection because they did not contain a cysteine residue required for biotin labelling or the labelled peptide was not detectable via ESI-MS analysis (Bernhard et al. 2003). To investigate the possibility that potential protein partners were missed, in this study the use of SDS-PAGE followed by in-gel digest and mass spectrometry analysis was evaluated as an alternative method to the affinity tag-mediated purification of biotin labelled peptides reported in chapter 3 (Bernhard et al. 2003). SDS-PAGE has the advantage that the protein pattern can be visualised with staining and that individual bands can be excised and the approximate molecular weight assists in their later identification. As figure 4.1 shows, in all the experiments, only a very faint pattern of proteins was visible after the gel was stained showing that affinity purification of CD4 from lymphoid cells produces only a small amount of protein, roughly estimated as less than 1  $\mu\text{g}$  from the intensity of the band. Still, the employed strategy led to the identification of many CD4-derived peptides for which clean and easy-to-interpret MS/MS spectra were obtained. The same applies to the tyrosine kinase lck. Most of the CD4 is associated with lck in lymphoid cells (Veillette et al. 1988), so the purification strategy led to the isolation of a similar amount of lck so that this protein was identified as easily as CD4.

Besides a band at  $\sim 55$  kDa, that could represent either or both CD4 (55 kDa) and lck (58 kDa), several other (barely visible) bands were present in the gel. Analysis of gel slices containing those proteins was still able to unambiguously identify the proteins that were contained in the slices. Even regions of the gel that did not contain any visible protein band yielded protein data upon mass spectrometry analysis demonstrating the ability of the strategy to detect proteins in sub Coomassie-stainable amounts (i.e.  $< \text{pmols}$ ). In one instance, up to nine different proteins along with trypsin could be identified in one mass spectrometry analysis of a single gel slice that contained only a barely detectable amount of protein (Figures 4.2 and 4.3, Table 4.2).

#### **4.4.2 Comparison of this method with the method described in chapter 3**

A total of 23 different proteins were identified in two different experiments in the affinity purification of CD4 employing similar purification methods as in described in chapter 3 (Bernhard et al. 2003). However, in that study, only a total of 10 proteins were detected in three different experiments indicating that the SDS-PAGE based method is a more powerful approach for identifying most of the proteins present. CD4 and Ick were detected in all experiments, but only two other proteins were identified by both technological approaches. Significant run-to-run differences in the intensity of non-specific binding to CD4 probably account for much of these differences but the differential abilities of the two methods to identify proteins is also a factor. Whereas the SDS-PAGE based method can identify a protein based on any of its tryptic peptides, the affinity tag-based method specifically detects only peptides containing a cysteine. As not all proteins contain a cysteine residue and not all peptides containing a cysteine residue can be observed in a mass spectrometry experiment (Bernhard et al. 2003), the SDS-PAGE based method has the potential to identify more of the proteins present in the sample. Of the 66 proteins we identified in this study, 9 do not contain a cysteine residue and therefore would have been missed using the affinity tag-mediated purification. In contrast, non-PAGE based methods have the advantage of identifying proteins which migrate poorly in gels (Moseley 2001) and the detection of the small acidic myosin and ribosomal proteins in the affinity tag-based method could be because this method does not involve such a separation. Therefore the observed difference in the identified proteins is likely to be a not only a result of the differential protein binding in different experiments. Also the different sensitivities of the methods but also the lower overall sensitivity of the affinity tag-based method because of the interference by the detergents contribute to the differences between the two studies.

When choosing the appropriate method for an investigation, several other factors must be taken into account concerning the speed with which the experiments and the protein identification can be carried out. Protein identification fundamentally relies on how efficiently tandem mass spectra can be matched to tryptic peptides of proteins. The affinity tag-based method led to detection of mainly peptides of high molecular weight (Bernhard et al. 2003), which produced complicated fragmentation spectra containing

multiply charged peptides that complicated manual analysis. In contrary the gel-based method mainly detected peptides in the mass range of 1000 to 2000 Da the spectra of which mostly showed a predominant y ion series comprising of most of the peptide sequence facilitating fast manual identification of the peptide. Also the samples were less contaminated with singly charged species as the SDS-PAGE separation eliminated the non-ionic detergents that caused a significant background in the affinity tag-based method (Bernhard et al. 2003). The time for sample preparation is another factor. The affinity tag-based method has the advantage that representative peptides obtained from all proteins are present in a single fraction, which is relatively easy to investigate. Moreover the SDS-PAGE based method leads to a high degree of redundant protein identification. For example more than 15 peptides have been observed for lck in one experiment (Table 4.1). Also each gel slice needs to be analysed separately leading to a much more time-consuming analysis when using this method. Nevertheless the investigation of slices, provides the analyst with the approximate molecular weight of the protein (as noted above), which can be an advantage when doing a database search for protein identification. Clearly, this study demonstrates that no one method is capable of identifying all proteins present in such a complex mixture. Each method has its particular advantages. However for our aims the SDS-PAGE based method seemed more efficient because more proteins were detected and the spectra obtained were generally cleaner. When choosing the appropriate method for an investigation, those factors need to be taken into account. To achieve detection of as many proteins as possible, the use of both strategies may be necessary.

#### **4.4.3 Evaluation of a different lysis buffer**

Because of the high degree of non-specific binding to CD4 detected in chapter 3, a different purification strategy was evaluated that reduces the non-specific binding to CD4. Also, some CD4 has been reported to participate in detergent-insoluble microdomains (Percherancier et al. 2003). This compartment together with any possible associating molecules, is likely to escape detection in a method employing cell lysis in cold Triton X-100. To reduce contamination with abundant proteins from the transcription/translation machinery and to generally increase solubility of membrane

proteins a detergent combination was used for cell lysis that had been reported to efficiently solubilize proteins from the NMDA receptor complex (Tingley et al. 1993). Co-immunoprecipitation studies on the NMDA receptor complex have demonstrated that interactions between different subunits of the complex persist even in the presence of up to 0.2 % SDS (Tingley et al. 1993; Sheng et al. 1994). However, SDS is a potent detergent for inhibiting noncovalent protein-protein interactions and its incorporation should reduce non-specific protein interactions. Table 4.1 lists all proteins identified in three independent experiments, in which a total of 49 proteins were identified together with CD4 and Ick. Only these two were identified in all experiments. Otherwise the pattern of copurifying proteins is different between the individual experiments and also different from the experiments employing the Triton X-100/NP-40 lysis buffer. However, there was no obvious reduction in non-specific binding and any conclusions on the nature of binding proteins in relation to the employed lysis buffer cannot be drawn because of the variability between the experiments. Based on these data, it cannot be stated whether employing the NP-40/DOC/SDS lysis buffer has any beneficial influence on non-specific protein binding. It could be that it does decrease non-specific binding but also induces partial unfolding of CD4 and/or Ick-domains that are then accessible to non-specific binding, counteracting such benefits. Thus the choice of lysis buffer in this experiment apparently has no significant influence on purification of both the CD4 receptor complex and on non-specific binding.

Whereas this strategy aimed to reduce non-specific binding by using SDS as a detergent for cell lysis, tandem affinity purification (TAP) would be an alternative way of reducing the presence of non-specifically binding proteins when purifying complexes for mass spectrometry analysis. It is normally performed by expressing the recombinant protein of interest with two different affinity tags and then perform two different affinity purifications. The sequential purification leads to a high purity of the complex, increasing the chance that detected proteins are specific interactors. It has been successfully used for several proteomic studies, including the human Smad proteins for example (Knuesel et al. 2003). Those strategies are especially efficient when complexes that contain large numbers of subunits are purified where non-specific binding might be expected. In this study it could have been performed using two different monoclonal antibodies for

affinity-purification of CD4, even using two columns with the same antibody would have been an option. Alternatively, CD4 could have been expressed as a recombinant tagged protein such as (His)<sub>6</sub>-tagged in a CD4-negative cell line like A2.01 and TAP carried out via Ni-NTA and Q425 affinity chromatography. However, some preliminary studies have shown difficulties when eluting CD4 from columns made of other immobilised CD4 antibodies such as OKT4 (data not shown). Recombinantly expressing tagged CD4 is also rather laborious so tandem-affinity purification was only considered to be a method of choice if the results obtained in this study had shown evidence that a large complex were present. As no evidence of a large CD4-containing complex could be found, this option was not further evaluated.

#### **4.4.4 Evaluation of the proteins regarding an association with CD4**

Eight proteins were found to copurify with CD4 in chapter 3 (Bernhard et al. 2003) but none of them was functionally related to CD4 and using control experiments, it was shown that all of them are non-specifically binding proteins. In this study, a further 62 proteins were detected. As none of the detected proteins showed consistent signals in all experiments as observed for p56lck, the detected proteins were unlikely to be functionally related to CD4, similarly to the proteins detected in chapter 3 (Bernhard et al. 2003). Therefore no experiments were conducted to test the specificity of the interaction. This could have been done using independent technologies or by conducting control experiments with non-specifically absorbing beads it was done in chapter 3 (Bernhard et al. 2003) and by others (Ranish et al. 2003). Along with the vast majority of the detected proteins, which are derived from the abundant protein synthesis, transcription or RNA processing machinery, several proteins from the cytoskeleton (actin, tubulin, and vimentin) were detected. As it was previously stated, a connection between CD4 and the cytoskeleton has been reported and the detection of components of the cytoskeleton could be a result thereof (Kinch et al. 1994; Thuillier et al. 1994) (and reviewed in chapter 1.1.4.1). However, more investigation is required to further clarify the mechanism of an association between CD4 and the cytoskeleton. Also because of the possibility that weak protein interactions might not be detected in every experiment, it could be possible that one of the detected proteins does specifically interact with CD4 via

a weak protein-protein interaction. However, this was not followed, because there appeared to be no direct functional connection between CD4 and the proteins identified here. Given the large number of non-specifically binding proteins and the different protein families they belong to, however this data also shows the importance of reproducibility and control experiments to validate protein interactions detected in such settings.

#### **4.4.5 General discussion of the method to determine novel CD4 binding proteins**

Taken together with chapter 3 (Bernhard et al. 2003), a total of about 70 proteins were identified co-purifying with the CD4 receptor complex the majority of which are likely to be non-specifically binding proteins. Different methods of protein identification were used and different lysis buffer systems were evaluated. Altogether this suggests that no specific CD4 binding proteins will be detected using this approach. This is probably due to such interactions being weak and/or transient in nature so that they will be lost during cell lysis or affinity purification. The next chapter therefore evaluates the use of chemical cross-linking prior to cell lysis to preserve such interactions. Besides, interactions can be cell type- and state- dependent and it may well be that in the resting lymphoid cells that were used in this study there are no strong protein interactions involving CD4 except for lck. Protein interactions could be induced by ligand binding and/or cell activation. Thus, investigation of CD4 interactions in activated cells or different cells is also a potential future study.

#### **4.5 Summary**

Mass spectrometry-based identification of the components of affinity purified protein complexes after polyacrylamide gel electrophoresis (PAGE) and in-gel digest has become very popular for the detection of novel protein interactions. As an alternative, the entire protein complex can be subjected to proteolytic cleavage followed by chromatographic separation of the peptides. Based on a method using affinity tag-mediated purification of cysteine-containing peptides to analyse proteins present in an affinity purification of the CD4/lck receptor complex described in chapter 3, here the use of one-dimensional polyacrylamide gel electrophoresis for analysis of the same receptor complex purification

is evaluated. Using electrospray and tandem mass spectrometry analyses of tryptic peptides from in-gel digested proteins the components of the CD4 receptor complex were identified along with 23 other proteins that were all likely to be non-specifically binding proteins and mainly different from the proteins detected in our previous study. This strategy is compared with the affinity tag-based method that was described in chapter 3. It is shown that the PAGE-based method enables more proteins to be identified. Also the use of a more stringent lysis buffer for the CD4 purification is evaluated to minimise non-specific binding and 52 proteins along with CD4 were identified in three independent experiments suggesting that the choice of lysis buffer had no significant effect on the extent of non-specific binding. Non-specific binding was inconsistent and involved various types of proteins underlining the importance of reproducibility and control experiments in proteomic studies especially given the discrepancies in the identity of non-specifically binding proteins detected in identical experiments.

## **CHAPTER FIVE:**

### **LATERAL MEMBRANE PROTEIN ASSOCIATIONS OF CD4 IN LYMPHOID CELLS DETECTED BY CROSS-LINKING AND MASS SPECTROMETRY**

## **5.1 Aims**

The work outlined in this chapter was conducted with the following aims:

- (i) To develop a method based on cross-linking of cell surface molecules followed by affinity purification and mass spectrometry to define lateral protein associations of CD4; and
- (ii) To use Fluorescence Resonance Energy Transfer (FRET) measurements to independently confirm new protein interactions.

## 5.2 Introduction:

Studies involving the large scale identification of protein interactions via identifying the components of purified protein complexes using mass spectrometry, as evaluated for CD4 in chapters 3 and 4 have become very popular in the biosciences community (Mann et al. 2001). Complexes can be either purified directly with immuno affinity chromatography such as performed in chapters 3 and 4 or after introduction of a tagged bait protein into the cells followed by affinity-tag mediated purification (Walsh et al. 2002). Alternatively, the bait protein is expressed recombinantly and binding partners are pulled down from cell lysates (Carrascal et al. 2002). Different methods are available for identifying the proteins in the purified complexes with mass spectrometry as thoroughly discussed in chapters 3 and 4. Successful studies employing such strategies have included the yeast interaction map (Ho, Y. et al. 2002) and characterisation of the brain *N*-methyl-D-aspartate receptor complex (Husi and Grant, 2000). However, to be detected in such a strategy, a protein interaction needs to be sufficiently strong to withstand dissociation during the cell lysis and/or affinity purification steps. Strong protein interactions that occur, for example, between antibodies and their antigens, are characterized by dissociation constants in the low nM to pM range. For CD4 and the monoclonal antibody IOT4a  $K_D$  values between 38 nM (Velge-Roussel et al. 1995) and 0.5 nM (Idziorek et al. 1992) have been measured. Weaker (but not less important) protein interactions have  $K_D$  values much less than this. For example the interaction of CD4 and the adaptor protein AP-2, which mediates its endocytosis has a  $K_D$  value of only 90  $\mu$ M (Pitcher et al. 1999). Such weak protein complexes are likely to dissociate during the cell lysis and/or affinity-purification steps and may not be detected. Detection of similar weak interactions between membrane proteins could provide important information on the function of those proteins (Szollosi et al. 2002).

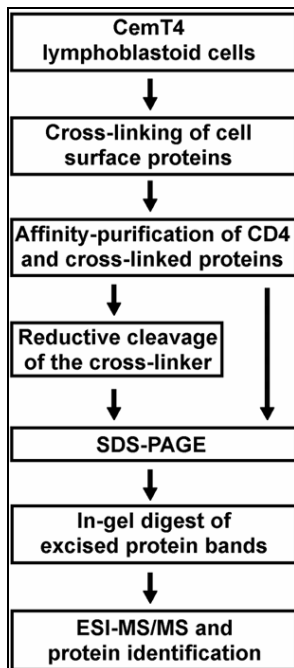
Known protein interactions of CD4 are with the kinase lck (Veillette et al. 1988) and the membrane phosphatase CD45 (Dornan et al. 2002). In chapters 3 and 4 different approaches aiming at the identification of CD4-associated proteins in T-lymphoblastoid cells were described using affinity-purification of CD4 complexes (Bernhard et al. 2003) (Bernhard et al. 2004a). The versatility of the methods was shown by identifying CD4 along with its associated kinase lck. However, a large number of functionally unrelated

proteins were also detected in the studies, which were attributed to the promotion of nonspecific binding and the dissociation of weak protein associations in the presence of detergents, which probably accounted for the inability to detect the transmembrane phosphatase CD45.

Chemical cross-linking has become a useful tool for the study of protein complexes including, for example, determination of the oligomerization state of proteins (Young et al. 2000). More sophisticated approaches combined with mass spectrometry are aimed at the determination of the individual cross-linked peptides to draw conclusions about spatial arrangements in a protein complex (Wallon et al. 2000). To determine whether a peptide connected to a cross-linker has arisen from cross-linking of the protein with itself or with another protein, an approach was delineated to distinguish peptides derived from inter and intramolecular cross-linking of proteins using differentially isotope labeled proteins (Taverner et al. 2002). Cross-linking in conjunction with mass spectrometry has also been used to detect novel protein interactions of a given protein. It has been used to cross-link the components of complexes bound to affinity-beads. After cleavage of the cross-linker, proteins that were cross-linked to the protein of interest can be identified by PAGE and MS analysis (Wine et al. 2002). Another strategy used cross-linking to connect associated proteins in purified endosomes. The protein under investigation, apolipoprotein B, was then purified along with the proteins that were cross-linked to it. Using SDS-PAGE and liquid-chromatography-mass spectrometry (LC/MS), Rashid and co-workers identified an impressive number of proteins that are possibly linked to the function of apolipoprotein B (Rashid et al. 2002).

The work outlined in this chapter involves the use of cross-linking followed by affinity-purification and mass spectrometry analysis for the identification of proteins that associate with the CD4 receptor in the cell membrane. Figure 5.1 shows schematic the strategy for identifying associating proteins. After purifying cross-linked complexes, the three transmembrane proteins CD45, CD71 and CD98 were identified along with CD4 indicating that those proteins are close to CD4 on the cell surface. Using co-immunoprecipitation and fluorescence resonance energy transfer (FRET), evidence that at least some CD4 associates with (clusters of) CD71 was found and that this association

increases upon treatment of the cells with phorbol 12-myristate 13-acetate (PMA). An explanation for this based on the endocytic properties of the molecules is provided.



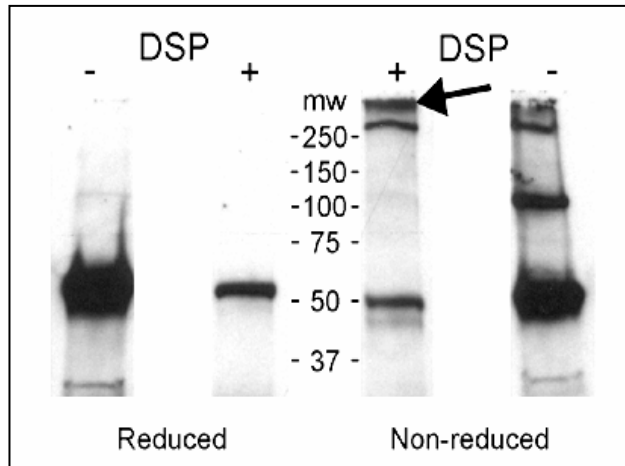
**Figure 5.1:** A strategy to identify proteins that come in close contact with CD4 on the cell surface. See experimental procedures sections 2.19, 2.7, 2.10, 2.12, 2.13, 2.16 and 2.17 for details

## 5.3 Results

### 5.3.1 Cross-linking of cell surface molecules and purification of cross-linked CD4 complexes.

Dithobis[succinimidylpropionate] (DSP), a cleavable, amine-reactive homobifunctional and membrane-permeable cross-linker with a 12Å linker was used to cross-link cell-surface molecules that are closely associated (Section 2.19). To investigate whether cross-linked complexes that include CD4 could be affinity purified with the Q425 antibody, small-scale immunoprecipitations were conducted and presence of CD4 was detected using Poly T4-5 antiserum (described in sections 2.6, 2.11 & 2.12). Figure 5.2 shows a western-blot of affinity-purified CD4 from cells that had or had not been cross-linked with DSP. The purified CD4 complexes were either left non-reduced or reduced so that cross-linked proteins were released as monomers. The data indicate that a significant part of the CD4 that purifies with Q425 is incorporated in complexes of high molecular weight if cell surface molecules had been cross-linked. Without cross-linking, the large majority of CD4 is present in the monomeric form. The complexes are observed to

disappear upon reduction. CD4 dimers and higher oligomers as detected in the non-reduced DSP- lane have been described previously (Lynch et al. 1999) and are disulfide bridged dimers and oligomers with undetermined physiological function.

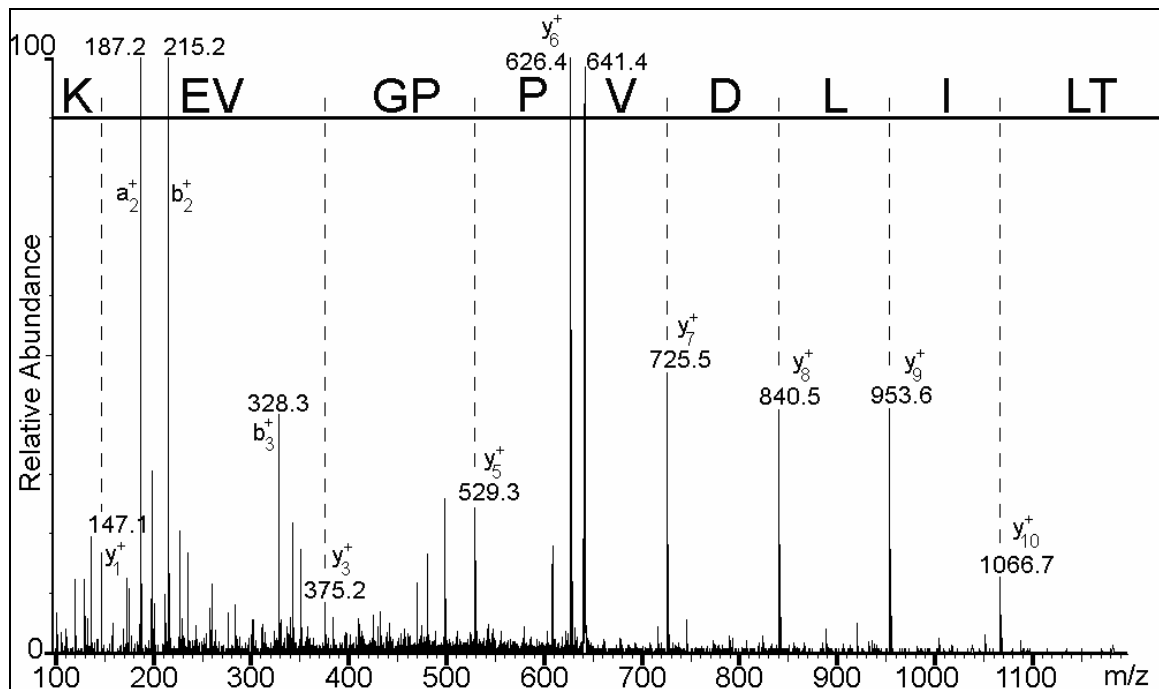


**Figure 5.2.** Western Blot showing CD4 purification with the Q425 antibody with or without preceding cross-linking of cell-surface proteins. CD4 was purified from CemT4 lysates with the Q425 antibody after the cells were cross-linked with DSP (+) or not (-). CD4 was detected with Poly T4-5 antibody and high MW complexes are indicated with an arrow. Reduction of the complexes before SDS-PAGE, where indicated, produces monomeric CD4 owing to the cleavage of the cross-linker. The difference in the intensity of the CD4 bands detected with or without cross-linking is arising from inactivation of antibody binding epitopes on CD4 by the cross-linker.

### 5.3.2 Mass spectrometry identification of the proteins cross-linked to CD4.

The purification results suggested that part of CD4 is indeed cross-linked into complexes with a sufficiently high molecular weight so that the complexes only just migrate into the very top section of the gel. To investigate other molecules that were present in those complexes, they were affinity purified from  $10^9$  cells and separated by SDS-PAGE followed by Coomassie Brilliant Blue staining (2.7, 2.10 & 2.12). A very faint band at the very top section of the gel was observed (data not shown). Because the employed MS strategy can detect proteins even in sub-Coomassie stainable amounts (Chapter 4) (Bernhard et al. 2004a) the region above 150 kDa was dissected into slices and subjected the protein identification strategy (2.13, 2.16 & 2.17). Three transmembrane proteins CD45, CD71 (transferrin receptor) and CD98 (4F2 lymphocyte antigen) were identified along with the CD4 receptor complex CD4 and Ick. To test for reproducibility, a total of three independent experiments were carried out. Whereas in the first experiments, the complexes were left in their high MW form, experiments 2 and 3 used TCEP to cleave the cross-linked complexes into their monomeric constituents so that individual proteins migrated according to their molecular weight. In the latter two experiments, the gel was

dissected into individual slices from the very top to about 15 kDa. To determine any effect of the temperature on the composition of the cross-linked complexes, experiment 3 involved cross-linking carried out at 4 °C. Table 5.1 lists the proteins that were identified in the cross-linked complexes in the three experiments. See also section 8.3 for more details. It also shows how many peptides were identified by MS/MS in each individual experiment. Peptides from CD71 and CD45 were identified in each experiment, whereas the peptide derived from CD98 was observed only weakly in experiments 2 and 3. The second experiment had less abundant peptide ions. Figure 5.3 shows a MS/MS spectrum of a tryptic peptide from CD45 representative for MS/MS spectra leading to the identification of the constituents of the CD4 receptor complex, CD4 and lck along with the three transmembrane proteins CD45, CD71 and CD98. The strategy for manual interpretation of MS/MS spectra and protein identification was the same as outlined in section 2.17 and 3.4.2. Two peptides derived from annexin II were identified in the third experiment. None of the recorded MS/MS spectra showed evidence that it was derived from a DSP-linked peptide providing information on the epitope of the protein interaction.



**Figure 5.3:** Identification of proteins that were cross-linked to CD4 via tandem MS spectrometry: A representative tandem MS spectrum from peptide with a precursor  $m/z$  of 641.0 (2+, peptide mass 1280.9) is shown. The full sequence is TLIDVPPGVEK and identified it as derived from CD45. Similar spectra were obtained from the other proteins (see 5.6.1).

**Table 5.1:** Peptides and proteins identified via MS/MS in the cross-linked sample.

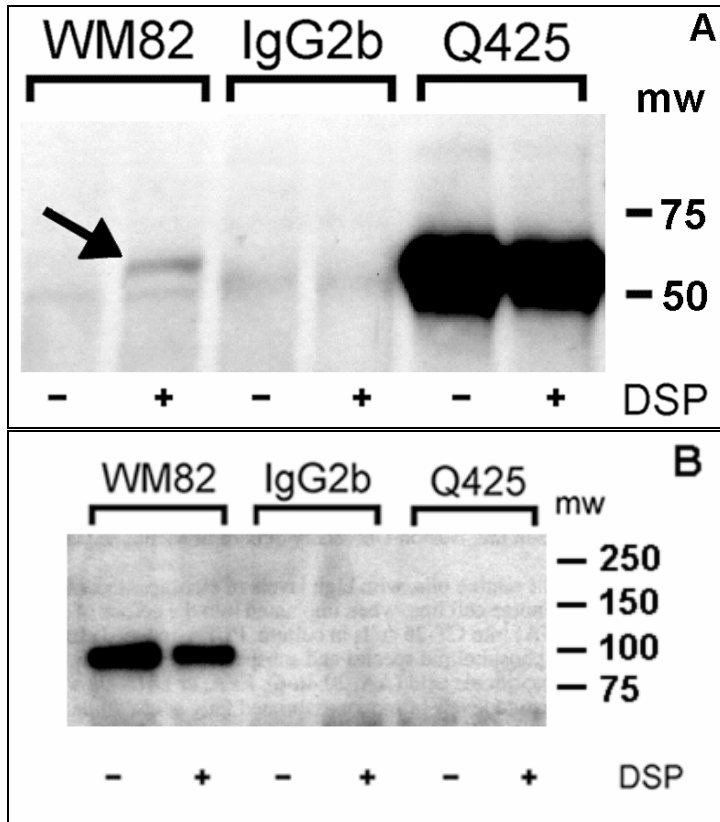
Protein Name	MW (kDa)	SwissProt#	Number of Peptides detected via MS/MS in Experiment number			
			1	2	3	total
CD45	144	P08575	3	1	2	3
CD71 (TFR)	84	P02786	14	2	15	17
CD98 (4F2 antigen)	60	P08195	1	(1) <sup>a</sup>	(1)	1
p56lck	58	P06239	(1)	0	8	8
CD4 receptor	55	P01730	9	1	4 <sup>b</sup>	10
Annexin II	38	P07355	0	0	2	2

<sup>a</sup>peptides in brackets were only detected in low abundance using a targeted ion approach

<sup>b</sup>several ions corresponding to typical CD4-derived peptides were present but not subjected to MS/MS

### 5.3.3 A fraction of CD4 copurifies with CD71 only after cell surface molecules were cross-linked.

Small-scale immunoprecipitations were conducted to investigate the specificity of the detection of CD71 along with CD4. Figure 5.4 shows detection of CD4 by Western blot after immunoprecipitation with WM82 antibody specific for CD71, Q425 antibody specific for CD4 or IgG2b isotype control. CD4 was detected co-purifying with CD71 only after the cell-surface molecules had been cross-linked. The CD4-specific band was persistently observed in three independent experiments. No CD4 could be detected when the IgG2b isotype control experiment was carried out. No CD4 could be detected in similar experiments using anti CD98 and anti CD45 antibodies (data not shown). The reverse blot shows detection of CD71 after precipitation with the anti CD71 antibody WM82. No CD71 could be detected in the precipitation with Q425 presumably owing to the sensitivity of the polyclonal anti CD71 antibody which does not recognise its antigen as well as the highly sensitive Poly T4-5 antibody.

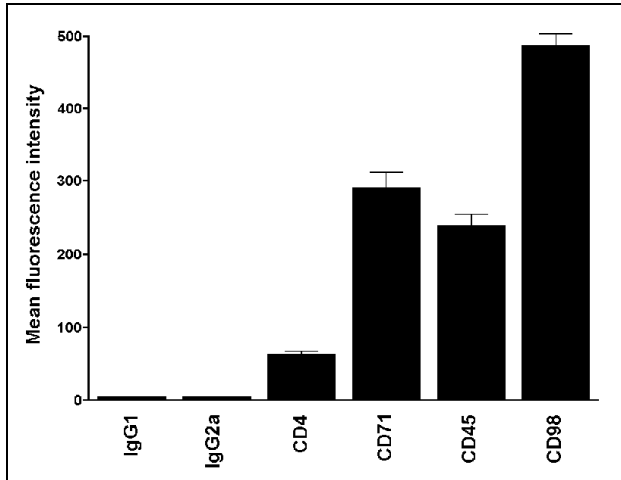


**Figure 5.4:** Western Blot detection of CD4 and CD71 purified with antibodies for CD71 (WM82), for CD4 (Q425) or with IgG2b (isotype control) with or without cross-linking. CemT4 cells were either subjected to cell-surface cross-linking with DSP (+) or not (-). Complexes were precipitated with either WM82 antibody against CD71, Q425 against CD4 or with an isotype control (IgG2b) for the WM82 antibody. The cross-linker was reductively cleaved and proteins were separated by SDS-PAGE. A, CD4 was detected with PolyT4-5 antibody. The arrow indicates a small amount of CD4 that co-purifies with CD71 only after cell-surface molecules had been cross-linked. B, reverse Blot against CD71 with a rabbit polyclonal antibody.

### 5.3.4 Cell surface expression of CD4, CD71, CD45 and CD98 does not correlate with the cross-linking efficiency.

To evaluate a possible correlation between the degree of cell surface expression of the CD45, CD71 and CD98 molecules with the efficiency of cross-linking, the cell surface expression of those molecules was determined using standard flow cytometry procedures (2.22). Figure 5.5 shows that of the three molecules cross-linked to CD4, CD98 is the most abundant on the surface of CemT4 cells followed by CD71 and CD45. CD4 is the least abundant. A protein that is cross-linked more effectively to CD4 will be present in the gel to a higher amount. Even though protein abundance in the gel and peptide abundance as detected with mass spectrometry cannot be correlated quantitatively, it is obvious that CD98, which was only represented by one low abundant peptide throughout the study is less abundant in the gel than CD71, of which 17 peptides had been detected in sometimes very high abundance. Consequently, the efficiency with which those proteins were cross-linked to CD4 is not correlated with the cell surface expression of

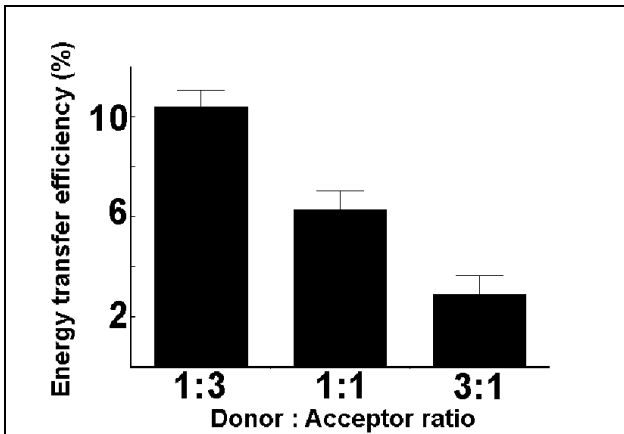
those proteins: CD98 was the most abundant protein on the cell surface but only one CD98-derived peptide was detected per experiment.



**Figure 5.5:** Cell surface expression of the identified proteins on CemT4 cells. Shown are the mean fluorescence intensities of 3-4 flow cytometry experiments using the fluorescein labeled antibodies Q4120 (CD4), WM82 (CD71), anti-CD45 and anti-CD98. FITC labeled IgG1 and IgG2a were used as isotype controls. The degree of labelling for the fluorescein conjugated antibodies was similar allowing for direct comparison using the mean fluorescence intensities.

### 5.3.5 FRET experiments detect clusters of CD71 molecules on CemT4 cells.

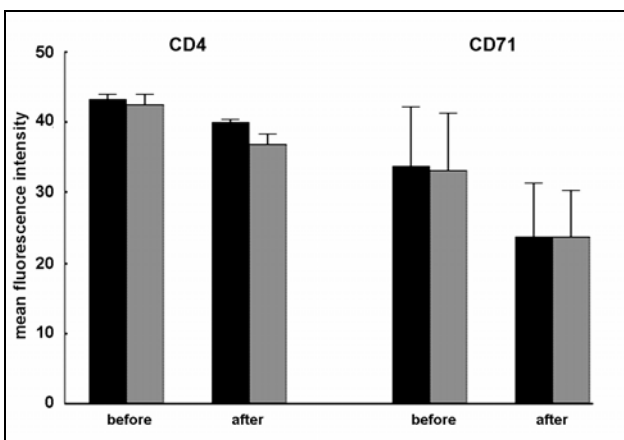
To investigate if CD71 forms clusters on CemT4 cells, as has been reported on other cell types (Matyus et al. 1995), FRET measurements were conducted using the CD71 specific WM82 antibody labeled with either fluorescein or Cy3 (described in 2.24 & 2.25). Figure 5.6 shows energy transfer efficiencies measured on CemT4 cells that were labeled with mixtures of fluorescein and Cy3 labeled WM82 antibodies employing different donor/acceptor ratios. Significant E values could be detected that vary with the donor/acceptor ratio. Experiments using WM82 as acceptor in combination with anti CD4 antibodies as donor molecules yielded FRET efficiencies of below 5 % (see below) showing that the energy transfer efficiencies observed between CD71 molecules are not a result of the high acceptor surface concentration. This behaviour is characteristic for FRET determination of receptor clustering (Kenworthy et al. 2000). However it indicates no strong association between CD4 and CD71 suggesting only a small fraction of CD4 associates with CD71.



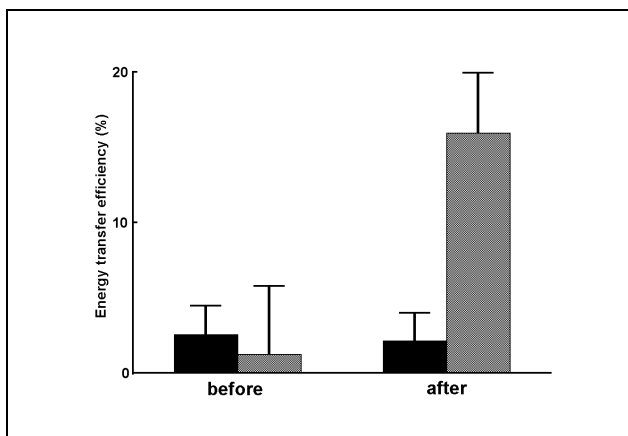
**Figure 5.6:** FRET detection of clustering of the CD71 molecule. Energy transfer efficiencies were measured on CemT4 cells that were labeled with different ratios of fluorescein and Cy3 labeled WM82 antibodies. Energy transfer efficiencies are shown for different ratios of donor:acceptor labeled antibodies.

### 5.3.6 PMA treatment of cells induces an association between CD4 and CD71.

The cell activator PMA is known to induce CD4 endocytosis in lymphoid cells (Pelchen-Matthews et al. 1993). To investigate further whether the association between CD4 and CD71 is mediated through their endocytic properties, cell surface expressions of CD4 and CD71 were investigated as well as energy transfer efficiencies between CD4 and CD71 before and after treatment with PMA or a mock treatment (section 2.25). Figure 5.7 shows expression of CD4 and CD71, before and after activation, showing a reduction of CD4 detection in both samples with the reduction being slightly stronger when cells were treated with PMA. The expression of CD71 is similarly reduced after PMA and mock treatment. Figure 5.8 shows the energy transfer efficiency between CD4 and CD71 before and after activation and it can clearly be seen that only after PMA treatment, can significant association between CD4 and CD71 was detected.



**Figure 5.7:** Cell surface expression of CD4 and CD71 before and after 8 min treatment with PMA (shaded bars) or mock treatment (filled bars) was measured using the fluorescein conjugated Q4120 antibody and detection at 530 nm and the Cy3 conjugated WM82 antibody and detection at 585 nm. Shown are the mean fluorescence intensities of three independent experiments.



**Figure 5.8:** Energy transfer between CD4 and CD71 before and after 8 min treatment with PMA (shaded bars) or mock treatment (filled bars) was measured using fluorescein conjugated Q4120 and Cy3 conjugated WM82 antibody. Shown are the mean energy transfer efficiencies of three independent experiments.

## 5.4 Discussion

Membrane proteins have very important functions, for example in cell signalling, growth inhibition and as virus receptors. However, membrane proteins are intrinsically difficult to study because, for example, detergents are required for solubilisation of the proteins which, in turn can interfere with their interactions. Further, their function is more subtly controlled by the membrane environment in which they reside. Participation in, or exclusion from, lipid rafts has an influence on protein function (Simons and Toomre, 2000) as does the conformation and other proteins that permanently or transiently associate with the membrane (Szollosi et al. 2002). Detecting proteins that reside in close proximity to a protein of interest can provide crucial information on how the function of a protein is influenced by those proteins. Standard methods such as immunoprecipitation are not always suited for detecting such associating proteins as they rely on membrane solubilisation, which is likely to interfere with weak lateral protein associations. Chemical cross-linking is a tool that can maintain protein associations because the proteins become covalently linked. In conjunction with immunoprecipitation, cross-linking can be used to detect proteins that are associated in the cell membrane. Here a strategy was developed based on this method that allows *de-novo* identification of proteins that can be cross-linked to the cell-surface receptor CD4. Lateral associations of CD4 are especially important because of its function in the immune system and as a receptor for HIV-1. There is still controversy about its function as a receptor for IL-16 as researchers have postulated that the real IL-16 receptor is a different molecule in close

proximity to CD4 (Mathy et al. 2000). Differences in susceptibility to different HIV-1 strains that cannot be explained by differences in tropism (Naif et al. 1999) could also be connected to different CD4 associating proteins in the membrane.

To cross-link adjoining proteins on the cell membrane, a protocol was used employing DSP that has previously been used to determine the oligomerization state of CD45 (Takeda et al. 1992). DSP is a cross-linker with a short range of only 12Å, which minimizes non-specific cross-linking. To maximize solubilisation of cross-linked complexes, a detergent combination containing SDS was employed because it had been shown to efficiently solubilise membrane proteins (Husi et al. 2001). Then it was investigated whether CD4 is still bound by the Q425 antibody after cross-linking, as this is a prerequisite for a purification of CD4-containing complexes generated by cross-linking. Using small-scale immunoprecipitations with Q425 followed by detection of CD4 by polyT4-5 a significant reduction in the amount of CD4 that was precipitated with Q425 was found. Such a reduction is likely to originate from the modification of lysine residues by the cross-linking reaction that leads to a decreased recognition of CD4 both by the Q425 antibody as well as by the polyT4-5 antibody. Still it can be seen that a large fraction of CD4 becomes part of high molecular weight complexes that are present when surface molecules had been cross-linked. Reduction cleaves the cross-linker and yields monomeric CD4. Without cross-linking, the majority of CD4 remains in its monomeric form. The above result suggested that part of CD4 is indeed cross-linked into complexes with a sufficiently high molecular weight such, that the complexes only just migrate into the very top section of the gel. Following purification of CD4-containing complexes out of a large number of cells, three transmembrane proteins CD45, CD71 (transferrin receptor) CD98 (4F2 lymphocyte antigen) were identified along CD4, Ick and in one of three experiments annexin II.

The detection of CD71 and CD45 together with CD4 is very unlikely to arise from non-specific cross-linking with CD4 on the cell surface. If this were the case, the level of cell-surface expression of those molecules should have correlated with the efficiency with which those proteins were cross-linked to CD4. Obviously a highly abundant protein is more likely to be cross-linked to CD4 after non-specific lateral diffusion than less abundant molecules. However, this is not the case here as CD98 was the most abundant

molecule of those three on the surface of CemT4 cells in comparison with CD71 and CD45 but only one, low abundant peptide from CD98 was detected by MS/MS. This shows that the method is not artificially favoring the abundant molecules. CD71, however, is much less prominent on the cell surface but is cross-linked extremely efficiently to CD4 as shown by the detection of 17, CD71-derived peptides in the three experiments. Furthermore, given the large number of cell surface molecules, if non-specific cross-linking were significant, one would then expect a broad band of many molecules in low amounts co-purifying with CD4. Such a very high background level of tryptic peptides derived from many non-specifically cross-linked proteins would make any MS/MS analysis extremely difficult. This was not the case, however, as peptides from CD45 and CD71 were easily distinguishable from the background and MS/MS spectra obtained were of a very good quality. Furthermore, cross-linking at 4°C should result in a reduction in lateral mobility of cell-surface molecules and therefore should produce a decrease in cross-linking of proteins that have no affinity for each other. Because cross-linking at 4 °C may subject the membrane to a phase change, detected interactions would less likely resemble the true state on the native cell and data obtained from such a setting needs to be interpreted more carefully. However, little reduction was observed for CD71 and CD45, supporting the notion that specific cross-linking was observed. CD71 or CD45 did not non-specifically bind to the resin. Chapter 3 and 4 reported extensive studies searching for molecules that co-purify with CD4 without cross-linking under similar conditions but CD45, CD71 or CD98 were never identified in any of those experiments (Bernhard et al. 2003) (Bernhard et al. 2004a). Annexin-II, however, was identified without cross-linking (Chapter 4) (Bernhard et al. 2004a), and, given its described ability to bind calcium ions, it is most likely to bind non-specifically to the Q425 antibody via a calcium bridge. CD98, which is the most abundant molecule on the cell surface of the three, was only once detected in high abundance and then only one peptide was detected so that non-specific cross-linking for this protein cannot be excluded.

These results suggest that there is an association of CD4 with both CD45 and CD71 on the cell surface. The interaction or association between CD4 and CD45 has been reported previously using techniques such as co-capping (Leitenberg et al. 1996),

co-immunoprecipitation (Bonnard et al. 1997) and FRET measurements (Dornan et al. 2002). CD45 is thought to play a role in controlling the kinase activity of lck via its function as a tyrosine phosphatase. Whether the CD4-CD45 interaction involves a direct or indirect binding has been controversial and it has been suggested that the T cell receptor and an associated protein mediates the interaction between CD4 and CD45 (Veillette et al. 1999). Were this the case, one would have expected that the proteins would have been cross-linked to CD4 and identified in this study. These results therefore suggest that the CD4-CD45 interaction is either direct or that the bridging molecule is the transferrin receptor CD71 forming a cluster with CD4 and CD45.

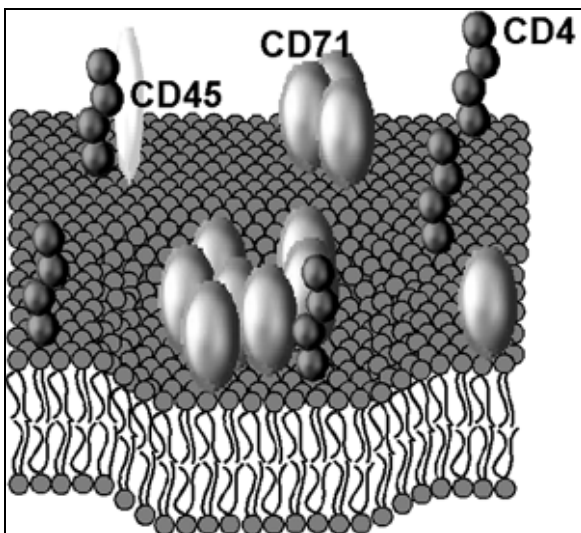
The human transferrin receptor CD71 is a ubiquitous type II transmembrane glycoprotein. The number of CD71 molecules on the cell surface depends on cell type and activation state (Cerny et al. 1996). The receptor plays a role in iron metabolism and regulates the cellular uptake of iron, which is thought to control the proliferation of T cells (Cano et al. 1990). Whereas our studies suggest a direct CD4-CD71 interaction, previous studies have suggested an indirect interaction in large non-covalent complexes of CD71 with CD4, CD3, CD45 and a range of other molecules on lymphocytes (Cerny et al. 1996). The transferrin receptor has been shown to form clusters on a number of cell types that incorporate various other molecules such as HLA-A, -B and -C (Matyus et al. 1995). The large number of peptides that were identified from CD71 and the good quality of the MS/MS spectra suggest that a significant amount of CD71 was cross-linked to CD4. It was also shown that a part of CD4 can be cross-linked to CD71 and that CD71 forms clusters on the investigated cells.

Because of the differences in function between CD4 and CD71, the reason for their association remains elusive, like the described association between CD71 and HLA molecules (Matyus et al. 1995). However, Matyus and co-workers, who reported the association between CD71 and the HLA molecules, suggested an explanation based on the endocytic properties of the molecules. Both HLA and CD71 molecules are internalized via coated pits and coated vesicles on the cells where an association between them had been shown indicating that the interaction results from clustering of the molecules during the endocytic process (Matyus et al. 1995). On T lymphocytes and T cell lines, CD4 is actively endocytosed owing to a dileucine motif on its cytoplasmic tail.

However, only a very small fraction of CD4 is endocytosed at any time owing to the interaction with p56lck, which prevents targeting of CD4 to coated pits (Pitcher et al. 1999). CD71 carries a different endocytic signal on its cytoplasmic tail but both signals target proteins to similar endocytic compartments (Marks et al. 1996). To investigate whether the observed association of a fraction of CD4 with clusters of CD71 is because of their endocytic properties, the association between CD4 and CD71 was investigated after treatment with PMA. PMA is a cell activator that strongly enhances endocytosis of CD4 in lymphoid cells (Pelchen-Matthews et al. 1993). Thus, if the association of CD4 and CD71 was because of their combined endocytosis, an increased association should be detectable after PMA stimulation and hence an increased participation of CD4 in the endocytosis process. Using FRET measurements, a significant increase in energy transfer between CD4 and CD71 after PMA treatment was observed demonstrating that an association of those molecules can be induced via stimulating CD4 endocytosis. The observed energy transfer was not a result of a PMA induced reduction in CD4 expression because the energy transfer was dependent on the difference in fluorescence signal between the donor and the FRET sample, both of which were treated with PMA. CD4 levels decreased only slightly in the PMA and in the mock treated sample with the decrease being slightly more intense in the PMA treated sample where an increased CD4 endocytosis was expected. The similarity between the mock and PMA treated samples relates to the labeling of CD4 prior to PMA induced endocytosis and/or CD4 still present in open endocytic pits. CD71 expression was monitored to check that the energy transfer efficiency was not a result of a change in CD71 levels. However, despite a significantly decreased detection of CD71 after the (mock) activation, no difference was observed between the PMA the mock treated samples. These results suggest that in unstimulated cells only CD71 is subject to bulk endocytosis and that a small fraction of CD4 is being endocytosed at the time resulting in the detected cross-linking with CD71 clusters. These clusters are also targeted for endocytosis in the same coated pits. Treatment of cells with PMA then leads to a large portion of CD4 being targeted for endocytosis and a significant association can be detected with FRET. Figure 5.9 shows a schematic diagram of the cell-surface arrangement of the three molecules CD4, CD45 and CD71 without activation according the interpretation proposed from these data. Whereas the majority of CD4 is

not associated with other cell surface molecules, a fraction of it is associated with the tyrosine phosphatase CD45. CD71 forms clusters on the cell surface and probably becomes associated with CD4 during endocytosis via coated pits.

Several other cell surface molecules have been described associated with CD4, most importantly the T cell receptor (Veillette et al. 1999; Vignali and Vignali, 1999) and the HIV coreceptor CCR5 (Xiao et al. 1999). However, no peptides derived from these proteins could be detected probably because either such associations are with cytoplasmic domains or cross-linking of their external domains with CD4 is difficult. The association with AP-2 that mediates CD4 endocytosis (Pitcher et al. 1999) was not detected because it is characterised by a very weak affinity and the cross-linking occurred mainly extracellularly. The expression and associations of cell surface molecules may be altered on malignant cell lines (Elghetany 1998) compared to those on resting native T cells. Further studies will therefore concentrate on primary or activated cells to address these questions.



**Figure 5.9:** Hypothetical arrangement of the cell-surface molecules CD4, CD45 and CD71 according to our interpretation of the reported data: Whereas the majority of CD4 is not associated with other cell-surface molecules, a fraction of it associates with the phosphatase CD45. When undergoing endocytosis, CD4 is targeted to coated pits (indicated by a developing pit at the front) where it becomes associated with clusters of CD71 molecules that are also targeted for endocytosis into the same pit. The sizes of the molecules are not to scale.

In summary, a novel method was used to investigate association of CD4 with external domains of membrane proteins and the previously reported association with CD45 was confirmed and identified a new association, with CD71 within clathrin coated pits on forming endocytic vacuoles was identified. These results contribute to the task of defining the complex and changing quaternary associations of cell surface molecules and demonstrate the general utility of combining cross-linking with mass spectrometry.

Further studies will investigate CD4 associations in different cell lines or primary cells or will address lateral protein interactions of other HIV receptors.

### **5.5 Summary:**

Interactions of membrane proteins are important in various aspects of cell function. However, weak membrane protein-protein interactions are difficult to study using techniques like co-immunoprecipitations. CD4 is a cell surface protein involved in T cell activation and the binding of the human immunodeficiency virus to HIV target cells. Here the use of cross-linking followed by affinity-purification of CD4 in combination with mass spectrometry is reported for identification of proteins that are in close proximity to CD4. Besides the components of the CD4 receptor complex, CD4 and Ick, by tandem mass spectrometry seventeen tryptic peptides from the transferrin receptor CD71 were identified as well as three peptides from the protein phosphatase CD45 and one peptide from the 4F2 lymphocyte activation antigen CD98. The efficiency of the cross-linking did not correlate with the cell surface expression of the detected molecules, excluding a possible bias of the cross-linking towards the most abundant cell-surface molecules. Whereas the association of CD4 with CD45 has been reported, the associations with CD71 and CD98 have not been previously described. Small-scale immunoprecipitation after cross-linking in combination with fluorescence resonance energy transfer (FRET) measurements were used to investigate the association between CD4 and CD71. The data show that CD71 self associates on the cell-surface, that a small fraction of CD4 can be detected co-purifying with CD71 after cross-linking and that the association between CD4 and CD71 significantly increases after phorbol 12-myristate 13-acetate-induced endocytosis of CD4. This suggests that a small fraction of CD4 associates with clusters of CD71. As both molecules undergo endocytic recycling, the association and cross-linking, results from their clustering in the same pit/vesicle. The association CD4-CD98 is probably resulting from non-specific cross-linking.

## **CHAPTER 6**

**DC-SIGN FORMS TETRAMERS ON MONOCYTE-DERIVED  
DENDRITIC CELLS WHICH INCREASES ITS AFFINITY FOR  
MANNAN AND HIV GP120**

## **6.1 Aims**

The work outlined in this chapter had the following aims:

- (i) To apply the proteomic method of cross-linking followed by mass spectrometry described in chapter 5 to the DC-SIGN molecule to determine whether it is engaged in lateral protein interactions on the surface of human monocyte-derived dendritic cells;
- (ii) To determine the protein composition and stoichiometry of the detected complexes; and
- (iii) To investigate whether detected protein interactions play a role in binding of the natural ligands of DC-SIGN, mannan and HIV gp120.

## 6.2 Introduction

Dendritic cells (DCs) are potent antigen presenting cells (APCs) functioning at the interface between adaptive and innate immune system (Banchereau and Steinman, 1998). A variety of different subsets exist in different types of peripheral tissue where they act as sentinels searching for pathogens (Turville et al. 2002). Once a pathogen or foreign antigen is encountered it is internalised and processed for MHC (class I or II)- mediated presentation to T lymphocytes. During this process, dendritic cells mature, migrate through underlying tissues and to lymph nodes where they encounter T cells. Increased expression of co-stimulatory molecules during maturation enhances T cell activation with pathogen-derived peptides presented on MHC class I or II molecules (Banchereau and Steinman, 1998); reviewed in sections 1.1.5.1 and 1.2.3.2.

To internalise pathogens, DCs express a series of pathogen recognition receptors such as toll-like receptors that recognise lipoproteins, lipopolysaccharide or bacterial DNA commonly found on various bacteria. Also expressed are C-type lectin receptors (CLRs) that bind to conserved oligosaccharides that are commonly found on the surface glycoproteins of viruses. CLRs expressed by DCs include the mannose receptor (CD206), DEC-205 (CD205), Langerin (CD207) and DC specific intercellular adhesion molecule 3-grabbing non-integrin (DC-SIGN; CD209) (Figdor et al. 2002). Those receptors differ not only in their expression on various subsets of DCs and other tissues but they also recognise different oligosaccharides thus discriminating between different ligands (Figdor et al. 2002; Frison et al. 2003).

DC-SIGN is a 44 kDa type II transmembrane protein that consists of a carbohydrate recognition domain, a neck domain involved in oligomerisation, a transmembrane domain and a cytoplasmic tail mediating interactions with the endocytosis machinery important for ligand internalisation (Geijtenbeek et al. 2000; Mitchell et al. 2001; Figdor et al. 2002; Frison et al. 2003). DC-SIGN binds and internalises several viruses such as HIV, Ebola virus, CMV, Dengue virus and hepatitis C virus (Alvarez et al. 2002; Halary et al. 2002; Navarro-Sanchez et al. 2003; Pohlmann et al. 2003) though other receptors are also involved (Turville et al. 2002). Other non-viral pathogens can also interact with DC-SIGN (van Kooyk and Geijtenbeek, 2003). Many of these viruses, however, have evolved a mechanism leading to their escape from

lysosomal degradation and allowing them to either infect DCs or to hide inside the cells followed by transfer to and infection of T cells when these cells are being activated by the mature DC. In mature monocyte-derived DCs (MDDCs) it has been shown that HIV binding to DC-SIGN and subsequent internalisation into the DC does not lead to the complete degradation and part of it is protected and subsequently infects T cells (Moris et al. 2003; Turville et al. 2003). The mechanism of this protection is poorly understood (Kwon et al. 2002; van Kooyk and Geijtenbeek, 2003) but it was suggested that it is an HIV-induced change in endocytic routing (Turville et al. 2003; van Kooyk and Geijtenbeek, 2003). A detailed knowledge of the DC-SIGN interaction with HIV glycoproteins is thus crucial for the understanding of DC-SIGN-induced routing of internalised ligands and involves both the selectivity for individual sugar structures as well as the stoichiometry of the DC-SIGN-ligand complex.

In contrary to CD4, which binds gp120 via a protein-protein interaction, DC-SIGN recognises the sugar moieties via a carbohydrate recognition domain. Crystal structural data have revealed that DC-SIGN binds to internal tri-mannose glycosylation structures present in N-linked high-mannose oligosaccharides. The receptor makes contact with the three adjacent mannose residues at an internal branched structure but fails to bind to the core trimannose motif in complex oligosaccharides owing to steric interference resulting from different anomeric linkages (Feinberg et al. 2001). This explained the preference of DC-SIGN for high-mannose carbohydrate structures and is in contrast to binding characteristics of other CLRs such as the mannose receptor, which was suggested to bind to single terminal mannose residues (van Kooyk and Geijtenbeek, 2003). Based on the oligomerisation on its extracellular domain, it was suggested that DC-SIGN forms tetramers and that this oligomerisation enhances the affinity for neoglycoproteins (Mitchell et al. 2001) It is therefore proposed that the interaction between DC-SIGN and glycoproteins involves several molecules of DC-SIGN binding to differential sugar moieties on the glycoprotein spaced at appropriate distances determined by the DC-SIGN oligomerisation (Mitchell et al. 2001). However, there is no direct evidence that DC-SIGN forms oligomers in the membrane environment present on immature DCs. Furthermore, it is still unclear whether any DC-SIGN associated proteins are involved in the carbohydrate recognition and how oligomerisation influences binding

affinity to the natural ligands of DC-SIGN such as HIV-1 gp120. The latter is important as the mechanisms of infection of DCs by HIV suggests a possible association between CD4 and DC-SIGN in the plasma membrane which has been shown by colocalization (Lee, B. et al. 2001).

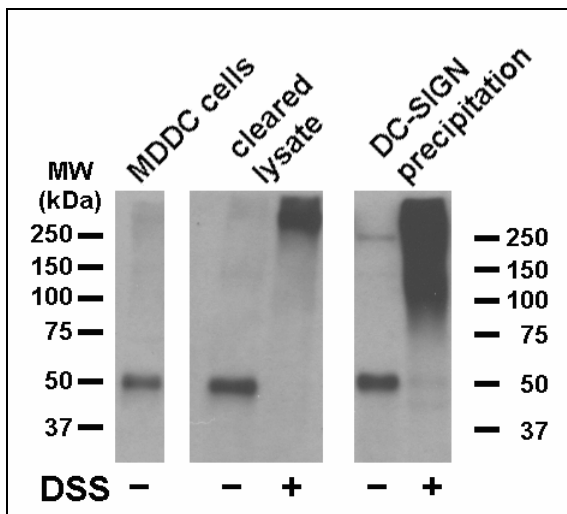
Here the techniques described in chapter 5 based on mass spectrometry (MS) are used to investigate lateral protein associations of native DC-SIGN on MDDCs. Chapter 5 described the versatility of those tools in determining lateral membrane protein associations on lymphoid cells (Bernhard et al. 2004b). In this chapter, cross-linking, immunopurification and western blotting are used to show that DC-SIGN assembles into oligomers of very high apparent molecular weight on the cell-surface of MDDCs. Mass spectrometry analysis of the purified complexes identified them as homo-oligomers of DC-SIGN and cross-linking at different concentrations of the cross-linker suggests that they are tetramers. Fluorescence resonance energy transfer (FRET) measurements did not detect any association between DC-SIGN and CD4. The complexes were also shown to bind with high affinity to immobilized mannan (a yeast-derived polysaccharide) as well as gp120 whereas DC-SIGN monomers are not bound. This confirms the formation of DC-SIGN tetramers on immature MDDCs, shows that they do not associate with other proteins and that the oligomerisation mediated by the lateral organisation in the membrane is required for high-affinity ligand binding.

## **6.3 Results:**

### **6.3.1 Complexes of high molecular weight containing DC-SIGN can be detected after cross-linking**

Because DC-SIGN was reported to form tetramers based on the properties of the recombinantly expressed extracellular domain, it is investigated here, whether DC-SIGN is engaged in lateral protein interactions on the surface of immature MDDCs. Cells were treated with DSS or subjected to a mock treatment (see section 2.19) and DC-SIGN was visualised after PAGE separation and western blotting using a DC-SIGN specific polyclonal antibody (see sections 2.12 & 2.18). Figure 6.1 shows the detection of DC-SIGN in lysates and immunoprecipitates from mock or DSS-treated cells. In a whole cell

lysate from mock treated MDDCs, DC-SIGN shows an apparent molecular weight of around 50 kDa, which is a good approximation to the theoretical molecular weight of 44 kDa from PAGE results alone. DC-SIGN present in a clarified lysate from mock treated cells shows a similar molecular weight to that in untreated cells whereas DC-SIGN present in the lysate from cells treated with DSS has an apparent molecular weight of more than 300 kDa indicating that DC-SIGN was cross-linked into large complexes. Immunoprecipitation of DC-SIGN with a murine monoclonal antibody confirms that the detected species are monomers and large complexes of DC-SIGN. The band at ~250 kDa in the DSS-treated sample from precipitation with anti-DC-SIGN antibody (and similar bands in later figures) is the murine anti DC-SIGN antibody that is weakly recognized by the anti rabbit secondary antibody.

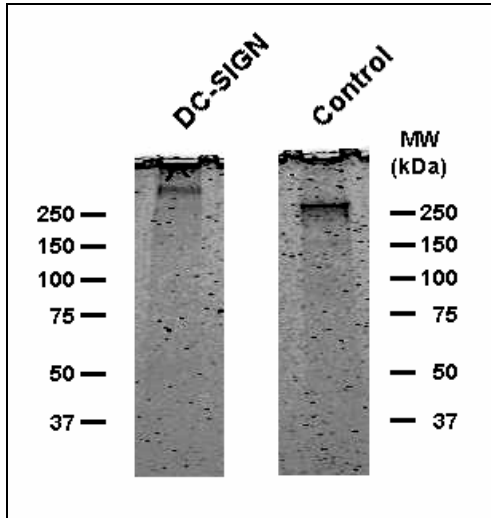


**Figure 6.1:** Detection of high MW oligomers of DC-SIGN after cross-linking of cell surface molecules. MDDCs were subjected to cross-linking with DSS where indicated. Cells were either directly lysed in SDS sample buffer (whole cells) or a cleared lysate was prepared and supplemented with SDS sample buffer (cleared lysate). DC-SIGN was also precipitated out of cleared lysate using a murine antibody (DC-SIGN precipitation). DC-SIGN was detected with a rabbit polyclonal antibody. The band at ~250 kDa in the DSS- sample from precipitation with anti-DC-SIGN antibody (and similar bands in later figures) is the murine anti DC-SIGN antibody that is weakly recognized by the anti rabbit secondary antibody.

### 6.3.2 The complexes are homo-oligomers of DC-SIGN

To investigate whether the identified large complexes of DC-SIGN are homo-oligomers or associations with other molecules, complexes were affinity-purified from approximately  $9 \times 10^7$  MDDCs after DSS treatment (see section 2.26), separated on a polyacrylamide gel and visualized with Coomassie Brilliant Blue (see section 2.12). Purification with an irrelevant antibody prior to the DC-SIGN purification was included as a control for non-specific binding. Figure 6.2 shows the visualised proteins from the DC-SIGN and the control purification. A band of high molecular weight can be seen in

the lane containing the purified DC-SIGN complexes whereas a band of around 250 kDa can be seen in the control lane.



**Figure 6.2:** Purification of the DC-SIGN complexes from MDDCs. MDDCs were treated with DSS and lysate was applied to a control column followed by an anti DC-SIGN column. The eluted proteins were separated on a SDS-PAGE and stained with Coomassie Brilliant Blue. A band of high molecular weight can be seen in the DC-SIGN purification and a band with an apparent molecular weight of 250 kDa is visible in the control purification.

Mass spectrometry analysis was carried out on the excised bands to determine their protein composition (see sections 2.13 - 2.17). Peptide identification was carried out as described in section 2.17 and chapter 3. Table 6.2 shows the peptides detected in the sample derived from the in-gel digest from the visible DC-SIGN complex. Using the sensitive nano-ESI-MS and nano-ESI-MS/MS-techniques, ten tryptic peptides from DC-SIGN were detected whereas no other protein was identified showing that the complexes are most likely homo-oligomers of DC-SIGN (see section 8.4 for MS/MS data). Further, no ions that could represent cross-linked peptides were detected. The band in the control purification was identified as murine IgG1 (data not shown) suggesting that part of the antibody eluted from the beads during boiling of the sample.

**Table 6.1:** DC-SIGN-derived peptides identified in the purified complexes:

Precursor m/z <sup>a</sup>	Peptide mass <sup>b</sup>	Sequence <sup>c</sup>	Position	MASCOT Score
818.9	1635.8	SAEEQNFLQLQSSR	296-309	87
784.9	1567.9	LQQLGLLEEEQLR	9-21	58
760.9	1519.8	QQEIYQELTQLK	235-246	32
753.4	1504.8	LQEIYQELTQLK	97-108	62
663.8	1325.6	M*QEIYQELTR	166-175	70
655.8	1309.6	MQEIYQELTR	166-175	18
654.3	1306.7	QQEIYQELTR	189-198 <sup>d</sup>	37
646.3	1291.7	LQEIYQELTR	120-129	52
609.3	1216.6	VPSSISQEQSR	63-73	53
528.3	1054.6	EVGAQLVVIK	286-295	47
457.3	912.5	AAVGELPEK	109-117 <sup>d</sup>	60

<sup>a</sup> given is the experimentally determined m/z value

<sup>b</sup> given is the theoretical uncharged monoisotopic peptide mass

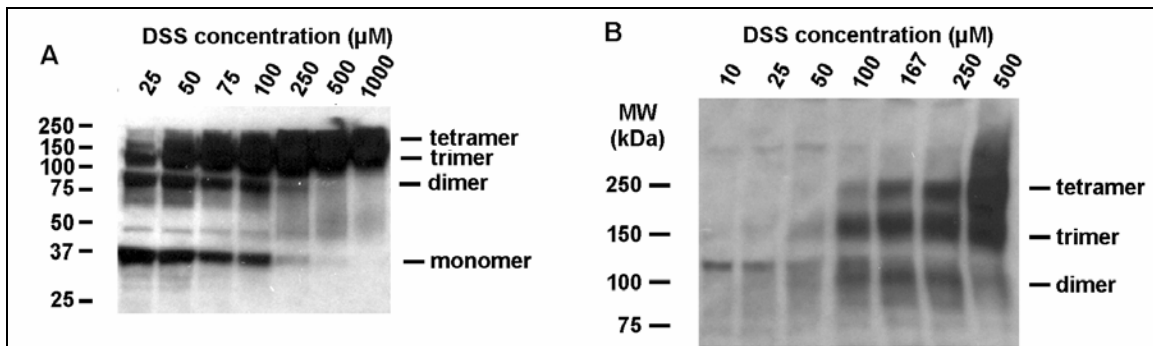
<sup>c</sup> M\* indicates oxidized methionine

<sup>d</sup> the protein produces multiple peptides of the same sequence, the first position only is shown

### 6.3.3 The high molecular weight complexes are tetramers

In order to deduce the oligomerisation state of the complexes, MDDCs were subjected to cross-linking with different concentrations of DSS. Cells were then lysed and the cleared lysate separated on 8-16 % gradient gels as well as on 5 % gels in order to increase the resolution in the high molecular weight region of the gel. Figure 6.3 shows representative DC-SIGN oligomers detected in the cleared lysate after cells had been treated with increasing concentrations of DSS. It can be seen in Figure 6.3A that the amount of monomeric DC-SIGN gradually decreases and that bands of high apparent molecular weight appear with increasing concentration of the cross-linker. Figure 6.3B shows resolution of the high molecular weight bands into three individual bands indicating that they consist of DC-SIGN dimers, trimers and tetramers. Dimers are detected at an

apparent molecular weight of around 100 kDa slightly below a non-specifically recognised band. Trimer bands migrate at approximately 150 kDa whereas the tetramer bands are detected slightly below 250 kDa. Their migration is slightly above the predicted molecular weight, which is probably a consequence of some globularity being retained through the cross-linking that interferes with the normal migration of the proteins through the gel. A similar effect can be seen in Figure 6.2, where the antibody of a molecular weight of 150 kDa migrates at an apparent molecular weight of 250 kDa. This is probably due to the globularity of the antibody that is retained through the intact interchain disulfide bridges.

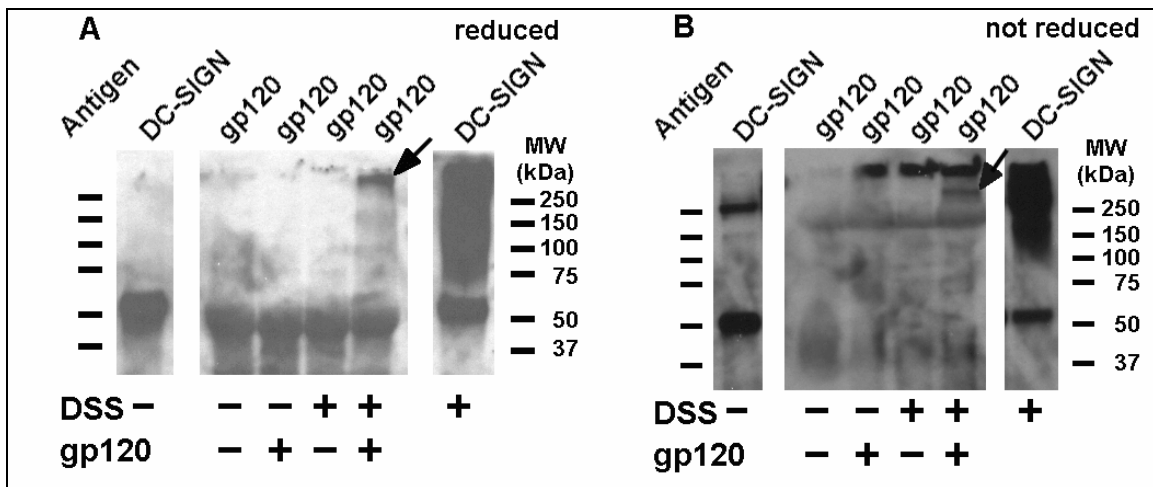


**Figure 6.3:** Detection of DC-SIGN oligomers after cross-linking was performed with different concentrations of DSS. MDDCs were subjected to cross-linking DSS at different concentrations as indicated. Cleared lysates were prepared and directly separated on an 8-16 % gradient gel (6.3A) or a 5 % gel (6.3B). DC-SIGN complexes were detected after western transfer with a polyclonal antibody. Figures 6.3A and 6.3B are derived from two independent experiments and the different onset of detection of multimers suggests some variability between experiments, however, the overall observed pattern is representative for more than three different experiments.

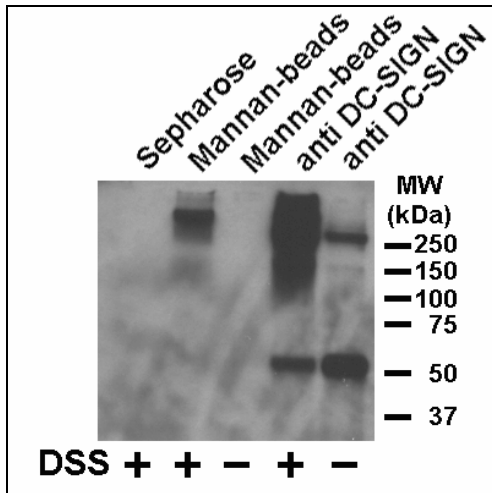
#### 6.3.4 The tetramers bind to mannan and HIV-1 gp120

It has been proposed that DC-SIGN tetramerisation is required for high-affinity ligand binding. An increased affinity of the extracellular domain for mannosylated BSA was detected when the neck region required for oligomerisation was present (Mitchell et al. 2001). Albeit an affinity constant of 1.4 nM has been determined between DC-SIGN and monomeric gp120 (Curtis et al. 1992), no data is available to determine the relative affinity of monomeric, dimeric or tetrameric DC-SIGN to gp120. To investigate directly whether HIV gp120 as well as yeast-derived mannan binds with higher affinity to DC-SIGN tetramers than to monomers, we conducted co-immunoprecipitation experiments between the molecules (see section 2.18). Figure 6.4 shows the co-immunoprecipitation

of DC-SIGN tetramers (arrow) with HIV gp120 whereas DC-SIGN monomers, detected by precipitation with a murine anti-DC-SIGN antibody, were not detected. This shows that the interaction between the complexes and HIV gp120 is strong enough to allow for co-immunoprecipitation and is stronger than the interaction between gp120 and DC-SIGN monomers. Bands derived from non-specific recognition of the anti gp120 antibody can be seen at ~50 kDa in Figure 6.4A and at 150-250 kDa in Figure 6.4B owing to the large amount of antibody present in the sample. Figure 6.5 similarly shows precipitation of DC-SIGN complexes with mannan beads whereas no DC-SIGN monomers are precipitated. Complexes were not detected involving binding by Sepharose itself indicating that the binding occurs predominantly between mannan and DC-SIGN.



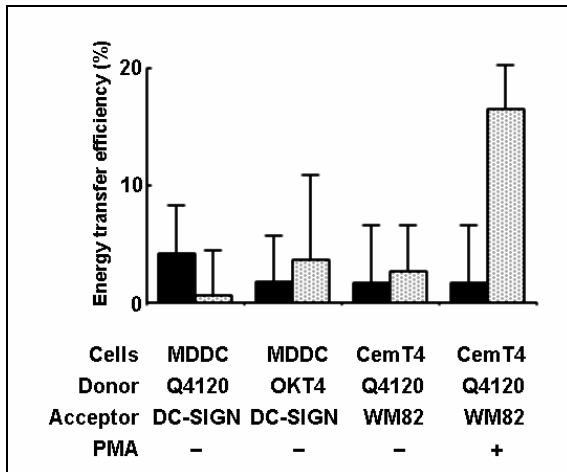
**Figure 6.4:** Co-immunoprecipitation of DC-SIGN complexes and HIV gp120. MDDCs were subjected to cross-linking with DSS or to mock cross-linking and DC-SIGN complexes were precipitated with an anti DC-SIGN or anti gp120 antibody. If indicated, DC-SIGN complexes were allowed to bind gp120 for 1h before precipitation. Samples were either subjected to reduction with DTT (A) or not (B) and DC-SIGN was detected with a rabbit polyclonal antibody. The two blots represent results obtained in two independent experiment and are not reduced/non reduced variants of the same experiment.



**Figure 6.5:** Precipitation of DC-SIGN complexes with mannan beads. MDDCs were subjected to cross-linking with DSS or mock cross-linking as indicated. DC-SIGN was precipitated out of cleared lysate using either sepharose, mannan beads or anti DC-SIGN antibody-Protein G beads. DC-SIGN complexes were detected by western blot using a rabbit polyclonal antibody.

### 6.3.5 FRET measurements do not detect an association between CD4 and DC-SIGN

Because it was suggested that there is an association between CD4 and DC-SIGN, but no peptides from other proteins were detected in the cross-linked complexes, FRET measurements between CD4 and DC-SIGN were conducted similarly as in chapter 5 between CD4 and the transferrin receptor on CemT4 cells which was included as a positive control. Figure 6.6 shows energy transfer efficiency measured between CD4 and DC-SIGN using two different anti-CD4 antibodies as donor molecules. Energy transfer efficiencies between CD4 and the transferrin receptor with or without stimulation with PMA are also included as a positive control for a protein association. It shows that no significant energy transfer (above 5-7 % is considered significant (Bernhard et al. 2004b)) could be detected between CD4 and DC-SIGN. This suggests that there is no interaction between the two molecules that involves a large proportion of both molecules as it is the case for CD4 and the transferrin receptor on CemT4 cells after PMA stimulation. However, it is well possible that only a fraction of either molecule is involved in an association as we have described for CD4 and the transferrin receptor on unstimulated CemT4 cells (Bernhard et al. 2004b).



**Figure 6.6:** FRET measurements between CD4 and DC-SIGN on MDDCs and between CD4 and CD71 on CemT4 cells. Energy transfer efficiencies were measured between different fluorescein conjugated donor and cy3 conjugated acceptor antibodies before (dark bars) and after 15 minutes incubation with or without a stimulus (shaded bars). Shown are the mean energy transfer efficiencies of ten independent experiments (OKT4/DC-SIGN) or four independent experiments (all others).

## 6.4 Discussion

Interactions between DCs and pathogens such as HIV are a key and probably the first step in the immune recognition of the pathogens and initiation of the subsequent cell-mediated response to control them. Alternatively, HIV may use DCs to obtain access to its major cellular targets, CD4<sup>+</sup> T lymphocytes. The complex formation between CLRs on dermal (or epidermal) DCs such as DC-SIGN and HIV-1 gp120 may be the first contact between the virus and its new host. A detailed understanding of this interaction is crucial for the development of any antiviral agents addressing this early stage of HIV infection. DC-SIGN is a CLR that recognises high-mannose oligosaccharides present on pathogen surfaces (Geijtenbeek et al. 2000; Kwon et al. 2002). DC-SIGN has been shown to function as the receptor for numerous viruses including HIV (van Kooyk and Geijtenbeek, 2003) and also to recognise self-proteins, a process that is thought to be involved in cell adhesion processes (Geijtenbeek et al. 2000). Apart from DC-SIGN, other receptors such as the mannose receptor or langerin may also contribute to HIV attachment (Turville et al. 2002). Normally binding is followed by endocytosis and degradation of the pathogen and presentation of pathogen-derived peptides to T cells via MHC molecules (Banchereau and Steinman, 1998). However, some viruses, especially HIV have developed the ability to escape the degradation normally following uptake and endocytosis, allowing infectious viruses to survive in DCs and subsequently infect T cells (Geijtenbeek et al. 2000; Moris et al. 2003; Turville et al. 2003). Alternatively, a minor proportion of HIV is transferred to the CD4/CCR5 infection pathway. To understand how

virus binding results in a differential entry into these pathways it is crucial to obtain a detailed knowledge on all the interactions involved in the binding of the viral glycoprotein including the oligomerisation of the receptor and possible other protein interactions with the CLR.

DC-SIGN has been reported to form tetramers based on the properties of recombinantly expressed extracellular parts of the receptor. However, the properties of extracellular fragments outside the natural membrane environment do not always correlate with the true cell surface arrangement of proteins. For example, CD4 was shown to crystallize as dimers interfacing in domain D4 (Wu, H. et al. 1997) whereas other studies suggested disulfide bridged dimers mediated by domain D2 (Matthias et al. 2002). Chapter 5 described the use of cross-linking/mass spectrometry to efficiently determine lateral protein interactions of this transmembrane protein including interactions in which only a fraction of CD4 was involved (Bernhard et al. 2004b). Here it is shown that DC-SIGN assembles into complexes on the surface of immature MDDCs and that no other proteins were detected in those interactions, especially CD4. Furthermore, because DC-SIGN has been suggested to associate with CD4 to facilitate infection of DCs (Lee, B. et al. 2001), fluorescence resonance energy transfer (FRET) measurements were also conducted between both molecules to investigate whether an association between the molecules could be detected. Although chapter 5 has shown an association between CD4 and the transferrin receptor CD71 using FRET on lymphoid cells after stimulation (Bernhard et al. 2004b), here no significant interaction between CD4 and DC-SIGN could be detected, suggesting that such an association might involve only a small fraction of the receptors and/or depend on gp120 binding to DC-SIGN (Figure 6.6).

Using cross-linking at various concentrations of DSS, evidence was obtained that the complexes are indeed tetramers as has been suggested previously from studies of recombinant fragments of the DC-SIGN extracellular domain (Mitchell et al. 2001). This provides confirmation of the mechanism of ligand recognition of DC-SIGN on cells as first described by Mitchell and co-workers (Mitchell et al. 2001) supporting their hypothesis that DC-SIGN assembles as tetramers on the surface of DCs mediated by the neck region of the molecule. The so defined arrangement of four carbohydrate recognition domains at a predetermined distance provides a platform for recognition of

ligands carrying appropriately spaced carbohydrate structures that are then recognised with high affinity. Unfortunately, studies on DC-SIGN-oligosaccharide interactions have mainly focused on defining ligands for DC-SIGN using synthetic oligosaccharides or purified glycopeptides (Feinberg et al. 2001; Mitchell et al. 2001; Frison et al. 2003). Little information is available on the influence of receptor oligomerisation on ligand binding, especially on binding of native ligands such as HIV gp120. Studies on full length extracellular DC-SIGN and extracellular DC-SIGN lacking the neck region that mediates oligomerisation have suggested that the constructs containing the neck domain bind ligands with higher affinity (Mitchell et al. 2001). This provided evidence that oligomerisation increases the affinity of DC-SIGN for its ligands. However, those studies were conducted using neoglycoproteins such as BSA containing up to 30 individual mannose residues (Mitchell et al. 2001). The sugar structure on those proteins is significantly different from the structure of high mannose oligosaccharides that are efficiently bound by DC-SIGN (Feinberg et al. 2001). Whereas mannosylated BSA contains individual mannose residues that are linked to the protein, DC-SIGN preferentially binds to three anomericly linked mannose residues present on the carbohydrate moiety of high mannose oligosaccharides (Feinberg et al. 2001).

Here, using the more natural ligands of DC-SIGN, yeast-derived mannan and HIV-1 gp120, DC-SIGN tetramers were successfully co-immunoprecipitated with these ligands. The binding is conducted in a buffer system containing 0.1 % SDS, a potent detergent disrupting non-covalent protein interactions. It can thus be assumed that DC-SIGN is present as a monomer after the cells have been solubilised, as the weak associations mediating tetramer formation are destroyed. Cross-linking, however, conserves the spatial arrangement of the tetrameric complexes which are now preserved in the presence of SDS. The cross-linked complexes retain their ability to bind mannan and gp120 with high affinity whereas the solubilised monomers fail to bind the glycoproteins with sufficient affinity to be co-immunoprecipitated in an amount detectable via western blot. This demonstrates that the assembly of DC-SIGN into tetramers in the membrane environment and, in these experiments conserved by cross-linking, is required for high-affinity binding of pathogens by DC-SIGN.

This study thus provides evidence for a binding mechanism involving a DC-SIGN tetramer binding to glycoproteins carrying probably four individual high-mannose carbohydrate residues spaced at a defined distance. This mechanism is distinct from the mechanism by which other lectins such as the mannose-binding protein (MBP) bind their ligand as discussed by Mitchell et al (Mitchell et al. 2001). Each MBP binds to individual mannose residues on the surface of the glycoprotein whereas the assembly of MBP into trimers allows for recognition of broad mannose-containing structures with high affinity. In contrast, DC-SIGN requires a specific arrangement of carbohydrates on its ligands which explains its selectivity.

Further studies will also attempt co-precipitation of HIV-1 gp120 with immobilized DC-SIGN tetramers and examine gp120 trimer and direct viral particle binding to DC-SIGN tetramers to assess whether X4 or R5; T-tropic or M-tropic viral strains are bound with different affinities by DC-SIGN. Mass spectrometry studies will be conducted to search for cross-linked peptides from interchain cross-links to provide spatial information on the residues that are involved in cross-linking.

Thus this is the first demonstration of DC-SIGN tetramer formation on dendritic cells (or any cell type). As the formation of tetramers significantly influences the binding affinity for ligands like HIV, attempts to design molecules to block these interactions must take into account the oligomeric nature of this protein. These quaternary interactions may also provide a paradigm for high affinity gp120 binding to other C-type lectin receptors.

### **6.5 Summary:**

DC-SIGN (dendritic cell specific intracellular adhesion molecule 3 grabbing non-integrin) or CD209 is a type II transmembrane protein expressed by dendritic cell (DC) subsets which binds to high mannose glycoproteins promoting their endocytosis and potential degradation. It also mediates attachment of HIV to DCs along with other receptors. HIV binding to this receptor can subsequently lead to endocytosis or enhancement of CD4/CCR5 dependent infection. Endocytosis of HIV virions does not necessarily lead to their complete degradation. A proportion of the virions remain infective and can be later presented to T cells mediating their infection in *trans*.

Previously, the extracellular domain of recombinant DC-SIGN has been shown to assemble as tetramers and in this study a short-range covalent cross-linker is used to show that DC-SIGN exists as tetramers on the surface of human monocyte-derived dendritic cells (MDDCs). There was no evidence of direct binding between DC-SIGN and CD4 by either cross-linking and fluorescence resonance energy transfer measurements as an explanation for *cis* enhancement of infection. Importantly it is also shown that the tetrameric complexes, in contrast to DC-SIGN monomers, bind with high affinity to high-mannose glycoproteins such as mannan or HIV gp120 suggesting that such an assembly is required for high-affinity binding of glycoproteins to DC-SIGN. Thus these results provide the first direct evidence that DC-SIGN forms tetramers on immature MDDCs and is essential for high affinity interactions with pathogens like HIV.

## **CHAPTER SEVEN:**

### **SUMMARY AND FUTURE DIRECTIONS**

## Summary

Interactions of HIV gp120 with surface receptors of HIV target cells are a key step for virus dissemination throughout the body, for virus entry and an important target for antiretroviral drugs. The understanding of the interactions of HIV with its receptors has increased dramatically during the last few years elucidating the mechanisms of how it infects cells and spreads throughout the body. The first cells to come into contact with HIV during infection are thought to be dendritic cells which express a series of C-type lectin receptors for recognition and internalisation of viruses that is normally followed by degradation and presentation of viral peptides via MHC molecules. HIV is bound via the interaction of C-type lectins such as DC-SIGN, which binds to a trimannose motif present in high-mannose carbohydrates on the HIV envelope glycoprotein gp120. HIV-induced alterations of endocytic routing is thought to allow the virus to remain infective in DCs for transfer of the virus to T cells which are subsequently infected. DCs can also be infected with HIV. HIV infects cells by binding to the cell surface glycoprotein CD4 followed by structural changes in the envelope protein gp120. Newly exposed domains bind to a chemokine receptor, mostly CCR5 or CXCR4 which is followed by the membrane fusion process. CD4 plays a key role in HIV replication as it is the primary receptor for HIV and its structure and protein associations can influence virus binding and subsequent fusion with the host membrane. Furthermore IL-16, the natural ligand of CD4 has been shown to inhibit replication of HIV via a mechanism that is little understood.

This work aimed to develop “proteomics-based” analytic techniques based on protein identification with mass spectrometry to study proteins that associate with CD4 and later to study interactions of DC-SIGN. Based on an affinity-purification of CD4 that had been developed previously in our laboratories, a method was established to identify the proteins present in the affinity chromatography-derived sample in order to search for novel CD4 binding proteins. The method employed a strategy to selectively purify cysteine-containing peptides to reduce the complexity of the sample analysed via mass spectrometry. The method was shown to be successful as both CD4 and Ick, the CD4-associated kinase, were identified. A series of other proteins were also detected, however, using control experiments, they were shown to be non-specifically binding during the

affinity-chromatography step. An evaluation of the reduction of sample complexity via the cysteine-capture method revealed that only a third of the cysteine-containing peptides theoretically present were actually detected. This suggested the possibility that proteins present could have escaped detection because either they did not contain a cysteine or because the cysteine-containing peptide was not detected during the mass spectrometry analysis.

To investigate whether proteins could have been missed using the method employing affinity purification of cysteine containing peptides, an alternative strategy was evaluated, employing 1D-PAGE separation of the proteins in the affinity purification of CD4. Proteins present in the sample were then identified using mass spectrometry analysis of peptides derived from in-gel digests of individual gel slices. A large number of proteins were identified that had no apparent functional connection and were considered to be non-specifically binding. With the aim to reduce non-specifically binding to the resin, a detergent combination was evaluated for cell lysis that did include the anionic detergent SDS. This detergent cocktail had been used by other groups for successful co-immunoprecipitations and here, in several experiments on the CD4 purification, the components of the CD4 receptor complex, CD4 and lck were detected. However, again a large number of proteins were identified which were considered non-specifically binding because they were not functionally related to CD4 and they were not consistently detected in the different experiments. A total of more than 60 non-specifically binding proteins were identified, all with inconsistent binding characteristics suggesting a careful investigation of detected proteins is needed when interacting partners are sought in such studies. The 1D-PAGE-based method was also compared with the strategy employing affinity-tag mediated peptide purification and advantages and limitations of both methods were discussed. Whereas the affinity-tag based method was characterised by a more rapid sample preparation and an easier MS analysis as all peptides were present in one sample, the 1D-PAGE based method allowed for identification of more proteins and delivered cleaner spectra. Thus this method was chosen for the following studies. The absence of any proteins specifically interacting with CD4 in all experiments was interpreted as that the CD4 receptor complex in the

investigated cell type is a rigid unit that is only engaged in weak and/or transient protein interactions that cannot be detected in these types of experiments.

To identify proteins that weakly and/or transiently associate with CD4 in the membrane of CemT4 cells, a novel technology was developed employing covalent cross-linking of cell surface molecules on the intact cells. Subsequently, CD4, together with proteins that were cross-linked to it, was affinity purified and proteins present in the purified sample were identified with mass spectrometry. The method successfully identified the three transmembrane proteins CD98, CD45 and CD71 along with the CD4 receptor complex and annexin II. Whereas annexin II is likely to be non-specifically bound by the resin and CD98 is likely to be non-specifically cross-linked to CD4, the CD45-CD4 and CD71-CD4 interactions were shown to be specific. The CD4-CD45 interaction is well described whereas the CD4-CD71 interaction is novel and was thought to be a result of the combined endocytosis of a fraction of CD4 with complexes of CD71. FRET measurements were developed and demonstrated both an aggregation of CD71 as it has been described for other cell types as well as a strong association of CD4 with CD71 after PMA-induced endocytosis of CD4. This demonstrated that both molecules are subject to a combined endocytosis, that in non-stimulating cells only a fraction of CD4 is endocytosed together with clusters of CD71 and that the mass spectrometry based method identifies this interaction in which only a fraction of CD4 is involved.

The techniques described for CD4 were then applied to DC-SIGN, a C-type lectin receptor on the surface of some types of dendritic cells that, in conjunction with other lectin receptors, mediates initial attachment of HIV. Because studies of the recombinant extracellular domain of DC-SIGN have suggested that it forms tetramers and colocalization studies suggested an interaction with CD4, it was aimed to elucidate protein interactions in which DC-SIGN is involved either with itself or with other molecules. Cross-linking studies showed that DC-SIGN forms large complexes on the surface of MDDCs. Mass spectrometry analysis of the complexes revealed them as homo-oligomers and studies with different concentrations of cross-linker suggested that they were indeed tetramers. This showed for the first time the quaternary structure of DC-SIGN on native MDDCs (or any cells) supporting a novel mechanism for carbohydrate recognition in which the assembly of four receptors contributes to high-

affinity ligand recognition as previously described. Further supporting this hypothesis, co-immunoprecipitation studies revealed that the cross-linked complexes bound with high affinity to mannan and HIV gp120 whereas monomeric DC-SIGN was not bound. This showed for the first time that indeed the tetramerisation of DC-SIGN mediates high affinity binding of its natural ligands and has implications for the developments of antiretroviral agents blocking this step. Any such agents must address the oligomerisation state of DC-SIGN to ensure that the desired blocking is achieved by a high binding affinity.

### **Future directions**

The work described in this thesis shows the successful development of novel “proteomic” tools to investigate interactions of membrane proteins and it also describes their application to CD4 and DC-SIGN revealing novel biological aspects of both molecules. The work described here can therefore be followed both technologically and biologically.

Technologically, the described methods can be applied to any other cell-surface molecule that fits the requirements for the study. Appropriate targets within HIV virology would be other HIV receptors, such as the mannose receptor, or studying the same receptor on different cells or activated cells. Certainly other cell surface markers important in various research fields such as cancer research could be investigated and their protein interactions revealed. FRET proved to be an excellent supportive tool to follow up protein interactions detected in such studies. It has the advantage of enabling real-time monitoring of the changes in protein associations, as demonstrated for the CD4-CD71 interaction after PMA induced endocytosis. Therefore, it is highly suited to refine the mechanism of protein interactions detected by cross-linking/mass spectrometry. Lastly, the methods described can be developed further technically. A very useful way to improve the versatility of the method would be to establish LC-MS analysis of the purified cross-linked complexes. Not only could proteins be identified that are less abundant in the gel and were not detected using nanoESI-MS, but also identification of cross-linked peptides could provide much needed spatial information on the location of cross-linking. This could reveal the epitopes engaged in the protein interaction and assist in determining the quaternary structure of cell surface receptors.

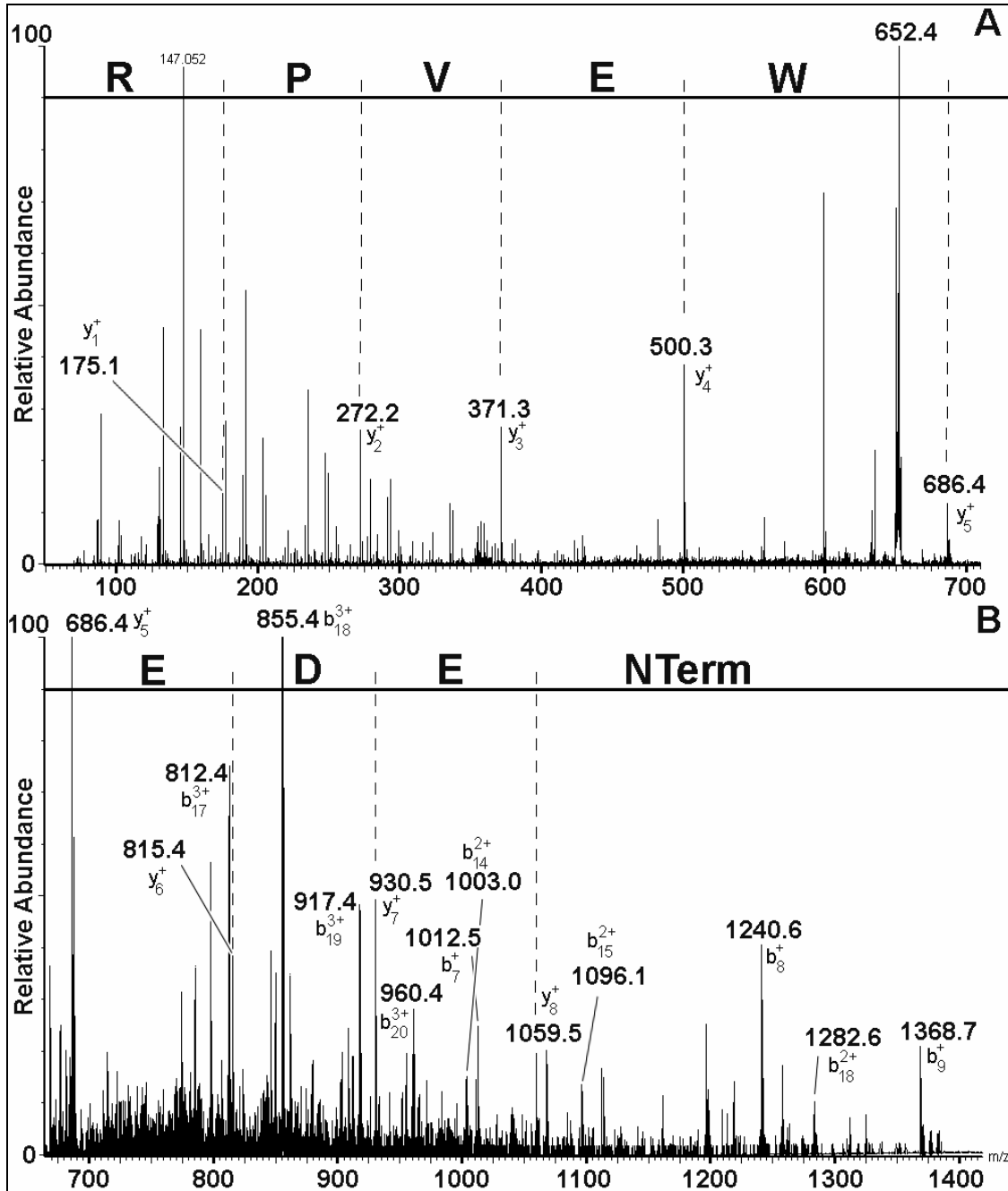
Biologically, novel interactions of CD4 were identified and the oligomerisation state of DC-SIGN was revealed on MDDCs. Whereas the interactions described for CD4 have been thoroughly described, the oligomerisation of DC-SIGN provides a basis for several future studies. First, ligands can be designed that take into account the quarternary structure and they could serve as potential blocking agents for initial virus entry. Secondly, the complexes could be immobilised and binding affinities for different viral strains such as X4 or R5; T-tropic or M-tropic or trimeric gp120 compared. This could reveal preferential binding of individual strains, accounting for phenotypic differences in infecting DCs additional to preference for CXCR-4 or CCR-5. Lastly, it will be very interesting to determine how ligand binding to quarternary DC-SIGN results in endocytosis of the complex and how binding of HIV alters the endocytic pathway to escape the lysosomal pathway, mediating transfer to T-lymphocytes and enabling the establishment of an infection in the peripheral lymph nodes.

## **CHAPTER EIGHT:**

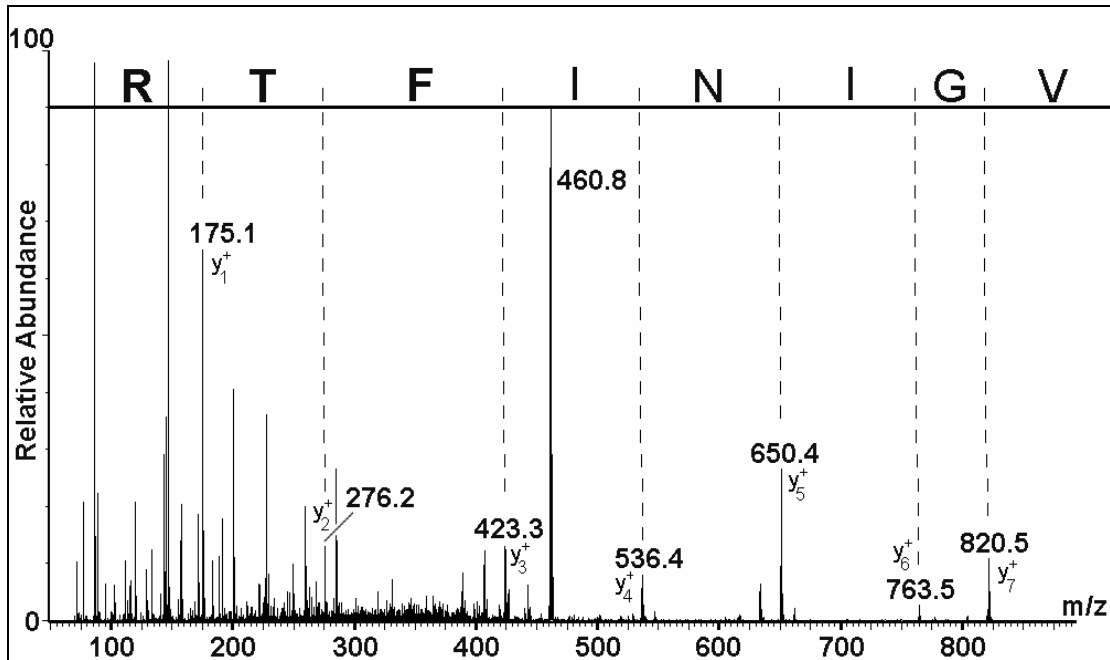
### **APPENDIX**

### 8.1 Appendix to chapter 3

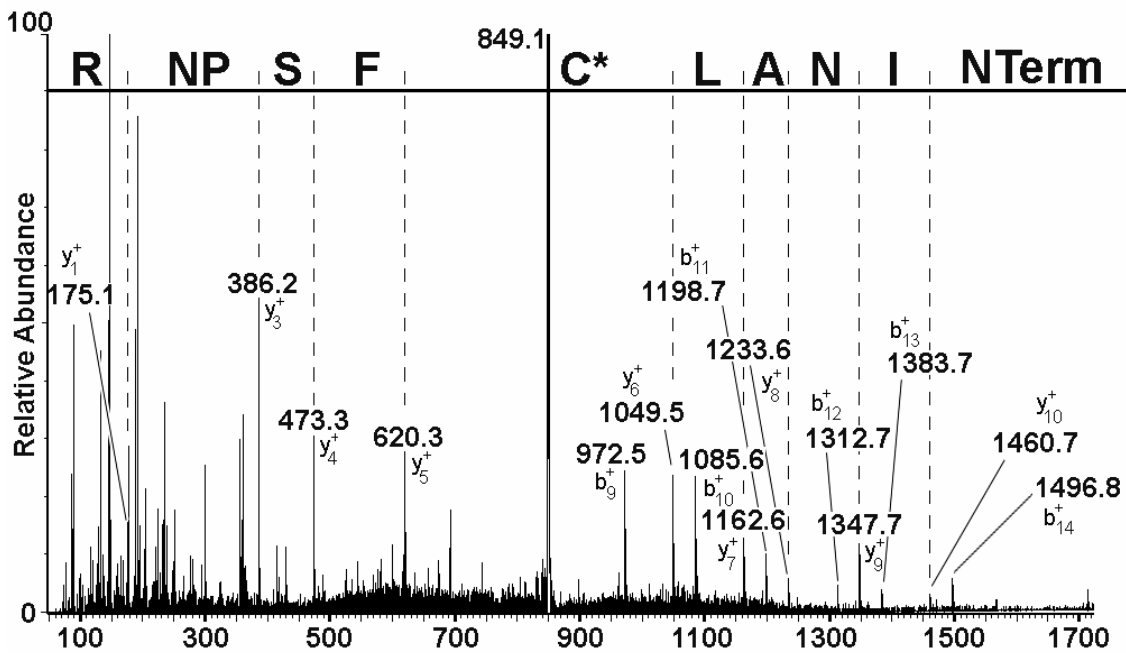
Figures 8.1 to 8.3 show key MS/MS spectra leading to the identification of p56lck, avidin and the guanine nucleotide binding protein.



**Figure 8.1:** Identification of proteins via tandem MS spectrometry: A tandem MS spectrum from a peptide with a precursor m/z of 652.4 (5+, peptide mass 3259.5) is shown with Figure 8.1A comprising of the m/z region up to 700 and Figure 8.1B representing the region from 700 to 1400. The full sequence is LSRPC\*QTQKPQKPWWEDEWEVPR and identified it as derived from p56lck.



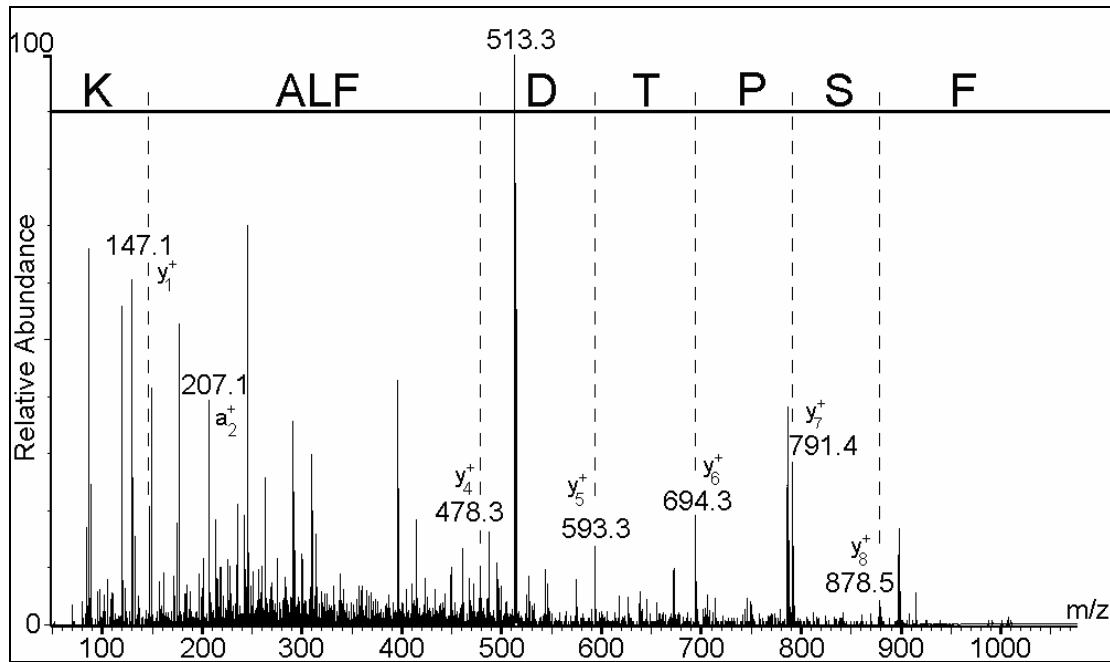
**Figure 8.2:** Identification of proteins via tandem MS spectrometry: A tandem MS spectrum from a peptide with a precursor  $m/z$  of 460.8 (2+, peptide mass 919.6). The full sequence is VGINIFTR and identified it as derived from avidin.



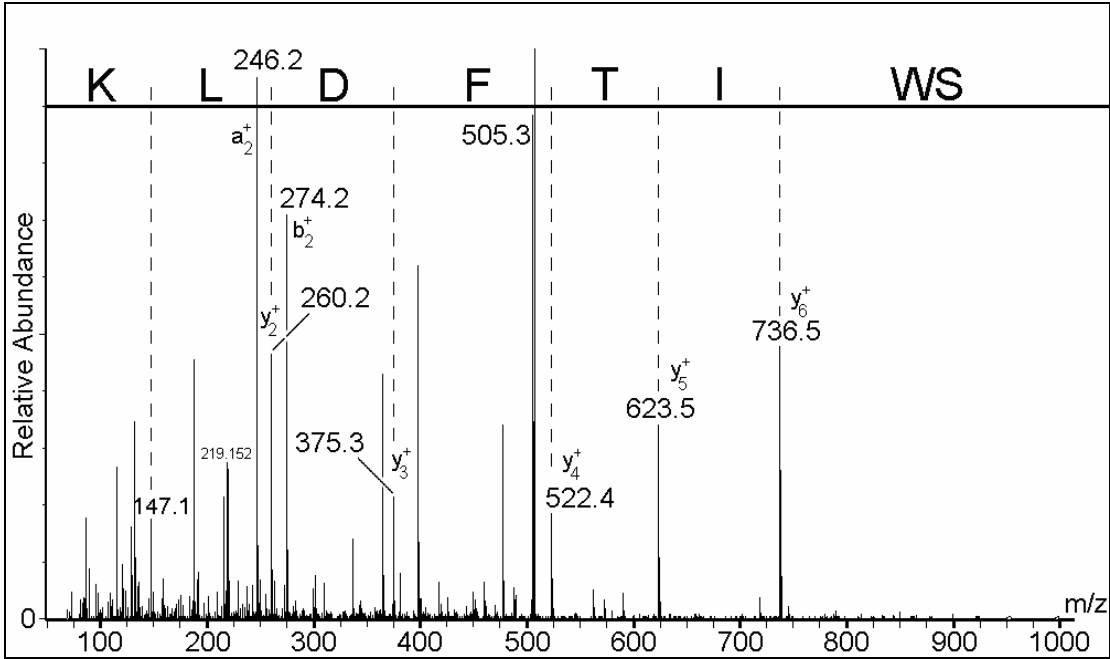
**Figure 8.3:** Identification of proteins via tandem MS spectrometry: A tandem MS spectrum from a peptide with a precursor  $m/z$  of 849.1 (3+, peptide mass 2545.2). The full sequence is HLYTLDGGDIINALC\*FSPNR and identified it as derived from avidin.

## 8.2 Appendix to chapter 4

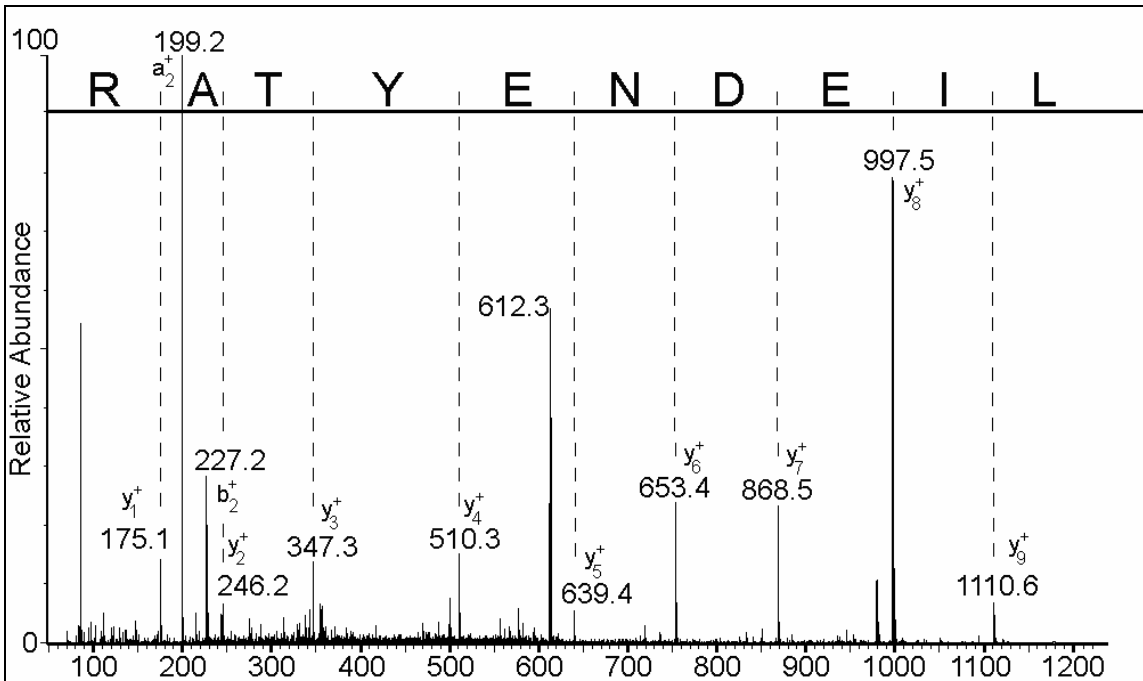
Figures 8.4 to 8.6 show key MS/MS spectra leading to the identification of CD4, p56lck and the leucine-rich protein.



**Figure 8.4:** Identification of proteins via tandem MS spectrometry: A tandem MS spectrum from a peptide with a precursor m/z of 513.3 (2+, peptide mass 1025.5) is shown. The full sequence is FSPTDFLAK and identified it as derived from the leucine-rich protein.



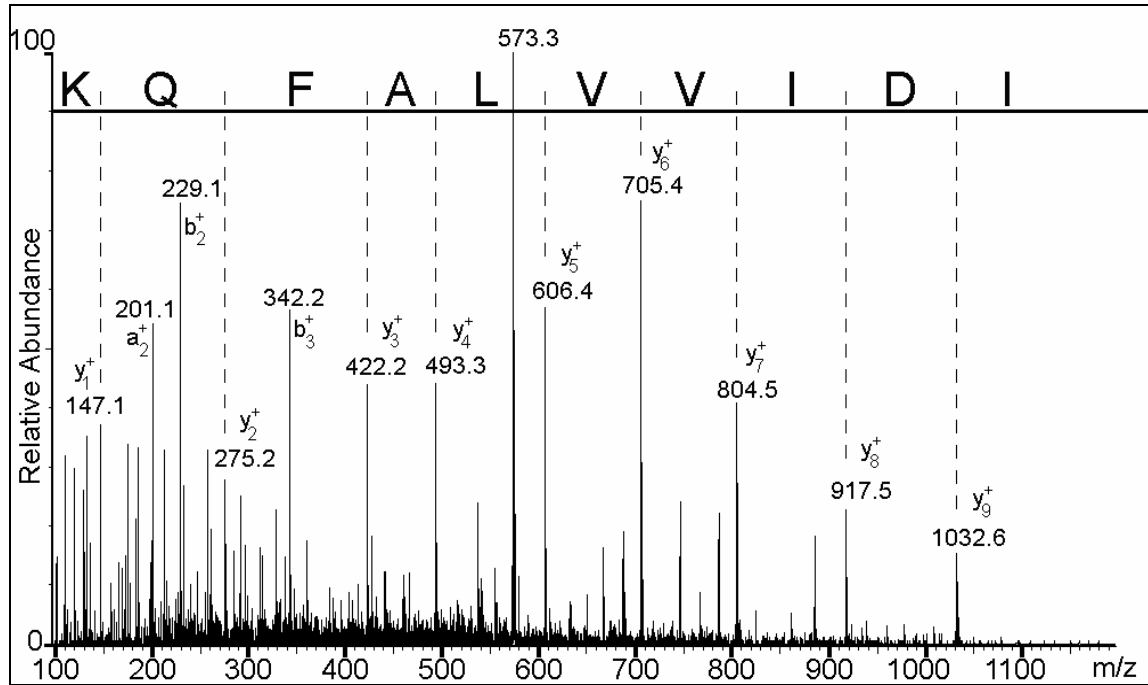
**Figure 8.5:** Identification of proteins via tandem MS spectrometry: A tandem MS spectrum from a peptide with a precursor  $m/z$  of 505.3 ( $2+$ , peptide mass 1009.5) is shown. The full sequence is SWITFDLK and identified it as derived from CD4.



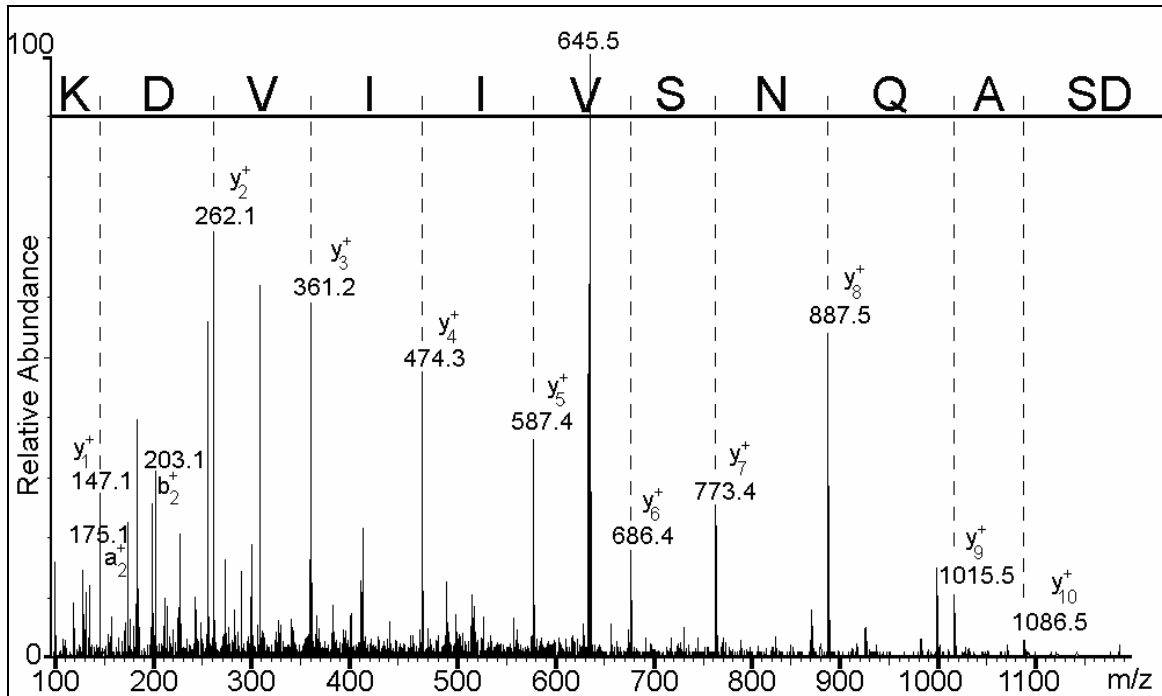
**Figure 8.6:** Identification of proteins via tandem MS spectrometry: A tandem MS spectrum from a peptide with a precursor  $m/z$  of 612.3 ( $2+$ , peptide mass 1223.6) is shown. The full sequence is LIEDNEYTAR and identified it as derived from p56lck.

### 8.3 Appendix to chapter 5

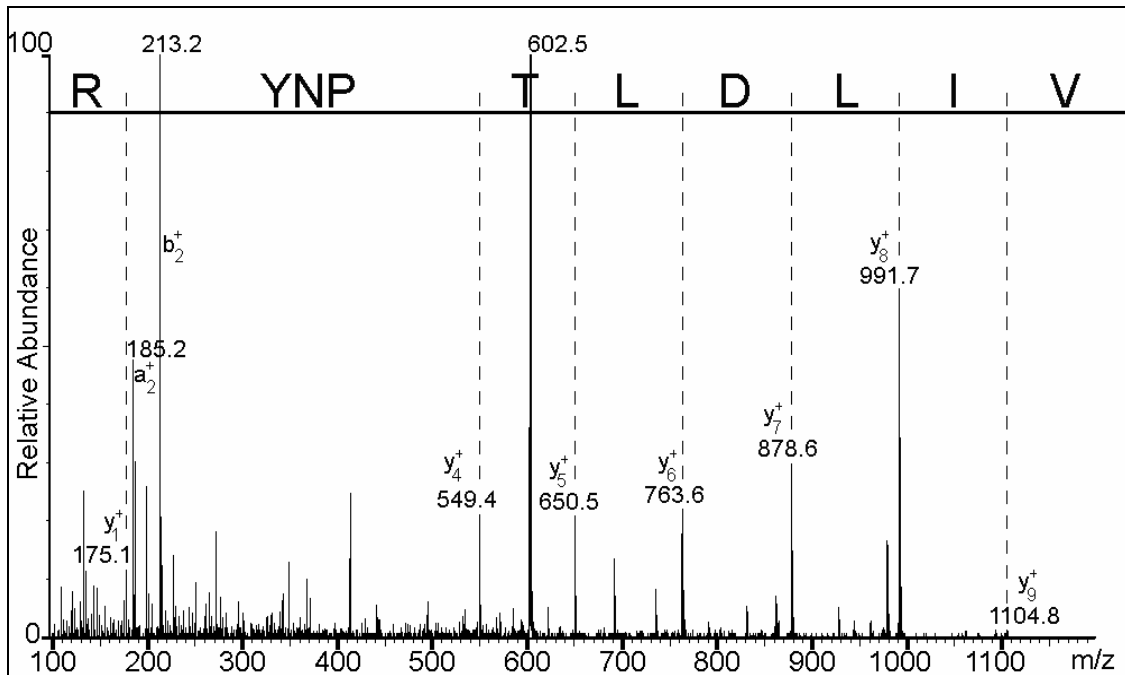
Figures 8.7 to 8.10 show key MS/MS spectra leading to the identification of CD4, p56lck, CD71 and CD98. Table 8.1 lists all proteins and peptides that were identified in the study and table 8.2 provides a list of all gel-slices analysed and the proteins identified therein.



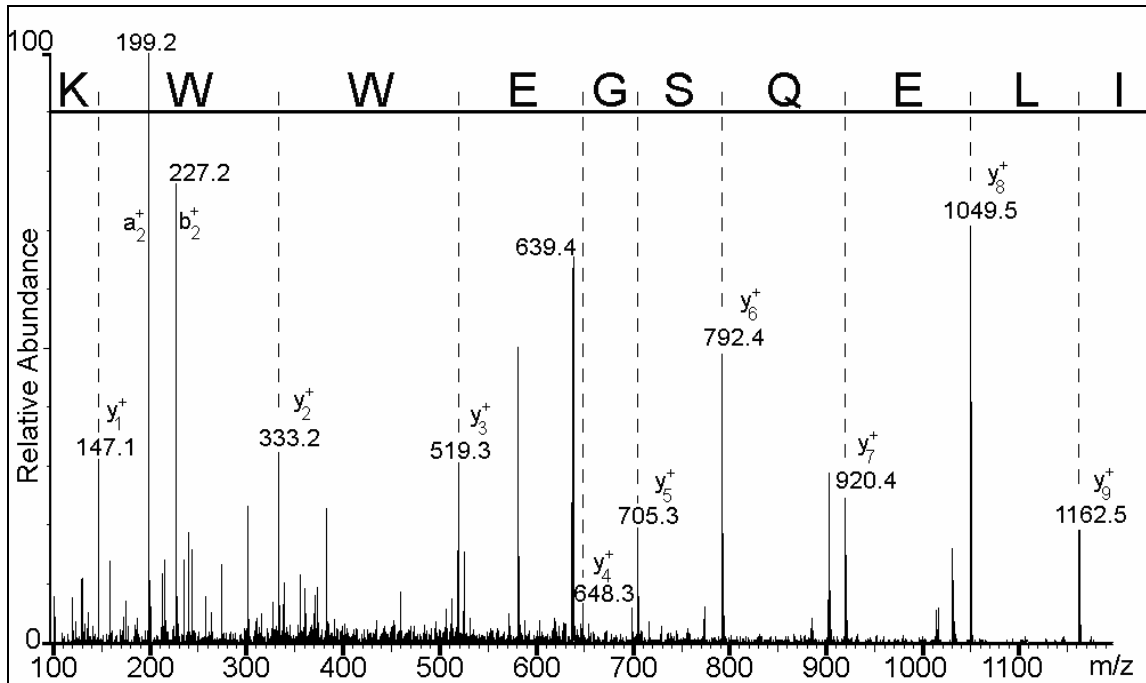
**Figure 8.7:** Identification of proteins that were cross-linked to CD4 via tandem MS spectrometry: A tandem MS spectrum from a peptide with a precursor  $m/z$  of 573.3 (2+, peptide mass 1145.6) is shown. The full sequence is IDIVVLAQK and identified it as derived from CD4.



**Figure 8.8:** Identification of proteins that were cross-linked to CD4 via tandem MS spectrometry: A tandem MS spectrum from a peptide with a precursor m/z of 644.9 (2+, peptide mass 1288.8) is shown. The full sequence is DSAQNSVIIVDK and identified it as derived from CD71.



**Figure 8.9:** Identification of proteins that were cross-linked to CD4 via tandem MS spectrometry: A tandem MS spectrum from a peptide with a precursor m/z of 602.5 (2+, peptide mass 1204.0) is shown. The full sequence is VILDLPNYR and identified it as derived from CD98.



**Figure 8.10:** Identification of proteins that were cross-linked to CD4 via tandem MS spectrometry: A tandem MS spectrum from a peptide with a precursor m/z of 638.4 (2+, peptide mass 1275.8) is shown. The full sequence is ILEQSGEWWK and identified it as derived from lck.

**Table 8.1** Proteins and peptides that were identified in the study presented in chapter 5

Mass (Da)	Protein	Position	MASCOT Score	Sequence
1957.0	CD4	215-231	60	EGEQVEFSFPLAFTVEK
1532.7	CD4	232-244	70	LTSGELWWAER
1530.8	CD4	85-97	ND	SLWDGNFPLIK
1353.7	CD4	308-318	23	LHQEVNLVVM*R
1352.6	CD4	34-46	ND	GDTVELTCTASQK
1177.7	CD4	61-71	71	ILGNQGSFLTK
1145.7	CD4	97-206	67	IDIVVLAFAQK
1009.5	CD4	250-257	31	SWITFDLK
945.5	CD4	48-54	21	SIQFHWK
720.4	CD4	338-343	16	LM*LSLK
1203.7	CD98	203-212	42	VILDLTPNYR
1672.8	CD71	395-409	65	GFVEPDHYVVVGAQR
1616.8	CD71	601-613	36	LTHDVELNLDYER
1561.7	CD71	40-53	55	LAVDEENADNNTK
1468.8	CD71	326-339	52	SSGLPNIPVQTISR
1433.8	CD71	496-508	65	VSASPLLYTLIEK
1314.7	CD71	429-439	50	LAQM*FSDM*VLK
1298.7	CD71	429-439	59	LAQM*FSDMVLK
1298.7	CD71	429-439	59	LAQMFSDM*VLK
1288.7	CD71	194-205	64	DSAQNSVIIVDK

1227.6	CD71	135-145	41	LDSTDFSTIK
1226.7	CD71	614-623	43	YNSQLLSFVR
1204.6	CD71	146-155	36	LLNENSYVPR
1203.7	CD71	589-600	12	AAAEVAGQFVIK
1095.5	CD71	656-665	40	LTTDFGNAEK
1084.6	CD71	478-486	58	AFTYINLDK
1016.7	CD71	386-394	32	ILNIFGVIK
986.6	CD71	232-240	17	LVHANFGTK
958.6	CD71	419-428	41	SGVGTALLLK
936.5	CD71	487-495	56	AVLGTSNFK
1244.6	Annexin II	135-144	44	TNQELQEINR
1222.6	Annexin II	104-114	41	TPAQYDASELK
1841.9	Lck	276-292	62	QGSM*SPDAFLAEANLM*K
1464.7	Lck	118-129	50	ANSLEPEPWFFK
1456.7	Lck	154-167	58	ESESTAGSFSLSVR
1352.7	Lck	184-195	49	NLDNGGFYSR
1281.7	Lck	196-206	20	ITFPGLHELVR
1275.6	Lck	89-98	58	ILEQSGEWWK
1223.6	Lck	387-396	51	LIEDNEYTAR
862.5	Lck	379-386	41	IADFGLAR
1354.6	CD45	972-982	ND	NSNVIPYDYNR
1320.7	CD45	651-660 <sup>#</sup>	61	LFLAEFQSIPR
1280.7	CD45	292-303	ND	TLILDVPPGVEK

\* indicates oxidises methionine

<sup>#</sup> The detected peptide conflicts with the sequence of human CD45 from the SWISS-PROT database inasmuch as a leucine residue was detected at position 650 instead of a proline residue behind an arginine at position 649. The human CD45 sequence (NP\_563579) from the NCBI database ([http://www.ncbi.nlm.nih.gov/htbin-post/Entrez/query\\_old?](http://www.ncbi.nlm.nih.gov/htbin-post/Entrez/query_old?)) however shows a leucine residue in position 650 as does the sequence of murine CD45. We concluded that either the sequence of the human CD45 is incorrectly held in the SWISS-PROT database or that there is a polymorphism, which in our cell line produces the detected peptide. The MASCOT score for this peptide represents the match with the rat CD45 derived peptide.

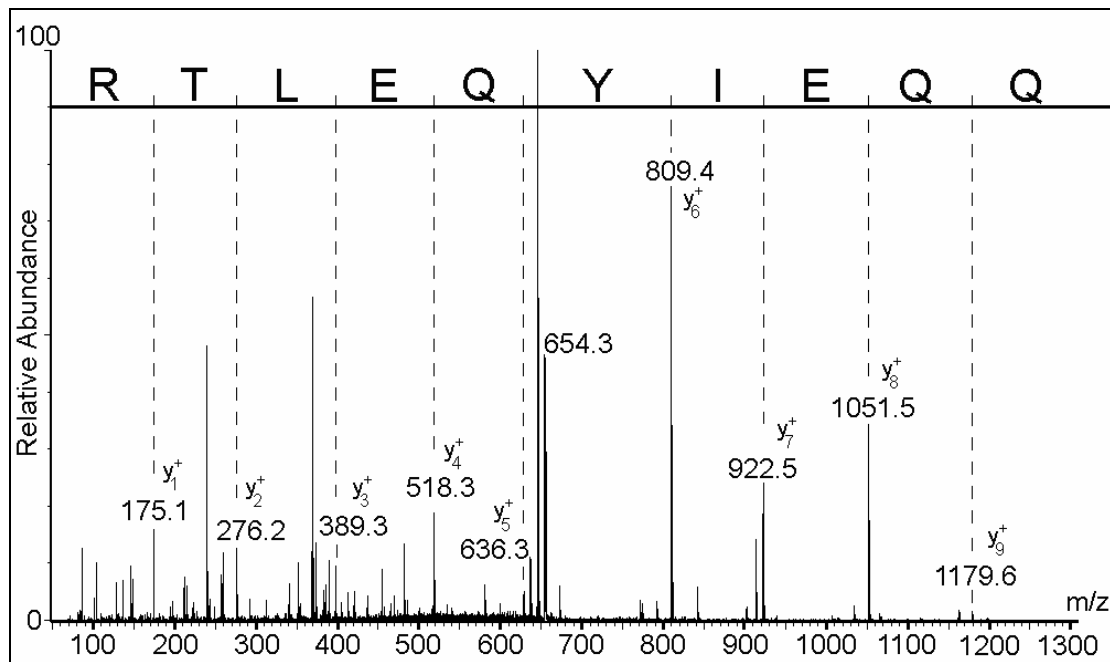
**Table 8.2** List of gel slices excised and proteins detected therein

MW range (kDa)	Experiment #	Proteins detected
> 300	1	CD4, CD71, CD45, lck
200 – 300	1	CD4, CD71, CD45, CD98, lck
150 – 200	1	CD4
45 – 60	1	CD4
> 250	2	CD71, CD45, CD98
50 – 70	2 <sup>#</sup>	CD4
>150	3	CD45, CD71, CD4
75 – 150	3	CD4, CD71, CD98
40 – 75	3	CD4, lck
15 – 40	3	Annexin II

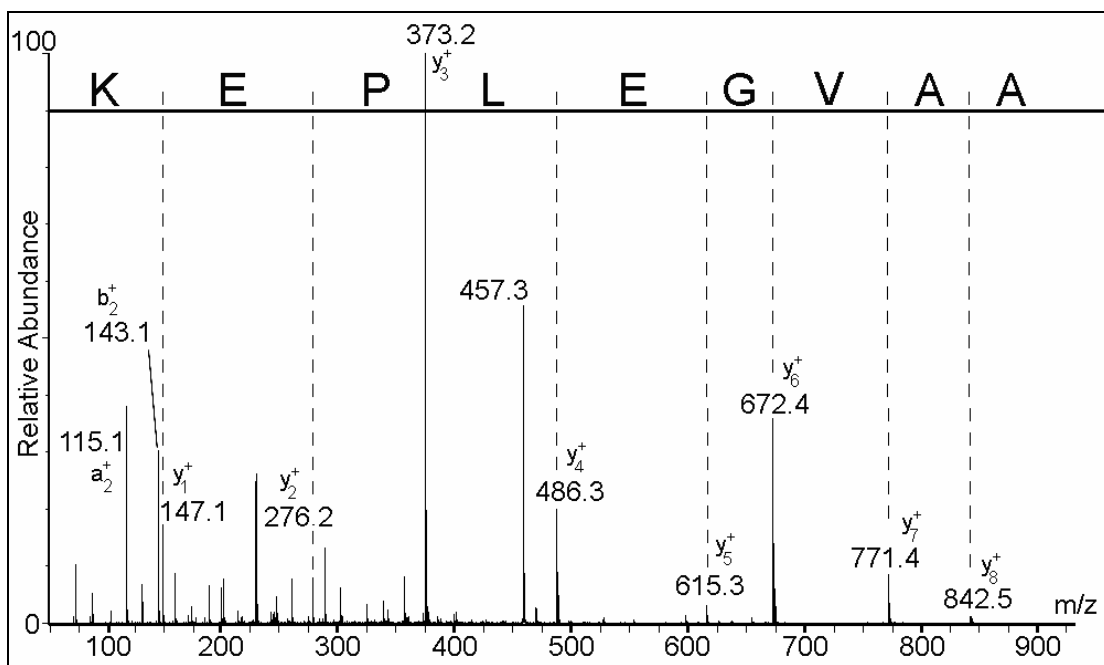
<sup>#</sup> Because of technical problems with the instrument the other excised gel-slices did not yield any and the successfully analysed gel-slices showed only low peptide abundance.

#### 8.4 Appendix to chapter 6

Figures 8.11 and 8.12 show two representative MS/MS spectra leading to the identification of DC-SIGN.



**Figure 8.11:** Identification of proteins via tandem MS spectrometry: A tandem MS spectrum from a peptide with a precursor  $m/z$  of 654.3 (2+, peptide mass 1307.6) is shown. The full sequence is QQEIQELTR and identified it as derived from DC-SIGN.



**Figure 8.12:** Identification of proteins via tandem MS spectrometry: A tandem MS spectrum from a peptide with a precursor m/z of 457.3 (2+, peptide mass 913.5) is shown. The full sequence is AAVGELPEK and identified it as derived from DC-SIGN.

## **CHAPTER NINE:**

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