

**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*.

### **CHAPTER 5:**

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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