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

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# PREFACE

The work presented in this thesis was performed by the author at the Centre for Virus Reseach, 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 homo-oligomers 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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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:

Bernhard, O. K., Sheil, M. M. and Cunningham, A. L. (2004): Lateral membrane protein associations of CD4 in lymphoid cells detected by cross-linking and mass spectrometry. *Biochemistry*, **43**, 256-264.

### CHAPTER 6:

Bernhard, O. K., Wilkinson, J., Sheil, M. M. and Cunningham, A. L.: DC-SIGN forms tetramers on monocyte-derived dendritic cells which increases its affinity for mannan and HIV gp120. *Submitted to J. Immunol. (Dec 2003).*

## LIST OF SELECTED PRESENTATIONS OF THIS WORK

Bernhard, O. K., Burgess, J. A., Hochgrebe, T., Sheil, M. M. and Cunningham, A. L.: "Mass Spectrometry Analysis of CD4-Associated Proteins Using Affinity Chromatography and Affinity Tag-Mediated Purification of Tryptic Peptides". Presented by OKB at the 27th Lorne Conference on Protein Structure and Function. Lorne, Victoria, February 2002.

Bernhard, O. K., Sheil, M. M. and Cunningham, A. L.: "Investigating CD4-Associated Molecules Using Affinity Chromatography And Mass Spectrometry". Presented by OKB at the ASHM Conference, Sydney, NSW, October, 2002.

Bernhard, O. K., Sheil, M. M. and Cunningham, A. L.: "Evaluation of Cross-linking followed by mass spectrometry analysis for the detection of membrane protein associations". Presented by OKB at the 28th Lorne Conference on Protein Structure and Function. Lorne, Victoria, February, 2003 And at Lorne Proteomics Symposium preceding the conference as invited oral presentation.

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Bernhard, O. K., Wilkinson, J., Dunstan, K. J., Sheil, M. M. and Cunningham, A. L.: "Investigating Interactions between C-Type Lectin Receptors and CD4 on the Surface of Dendritic Cells". Presented by OKB at the ASHM Conference, Cairns, QLD, October, 2003.

Bernhard, O. K., Wilkinson, J., Sheil, M. M. and Cunningham, A. L.: “Identification of High-Mass Oligomers of DC-SIGN on Monocyte-Derived Dendritic Cells and Preferential Recognition by Mannan and HIV-gp120”. To be presented by OKB at the 11th Conference on Retroviruses and Opportunistic Infections (CROI), San Fransisco, U. S. A., February, 2004.

# ABBREVIATIONS

| | |
|---|---|
| AIDS | acquired immunodeficiency syndrome |
| AP | adaptor protein |
| APC | antigen presenting cell |
| BIAM | *N*-(biotinoyl)-*N*'-(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-*O*,-*O'*-bis(2-amino-ethyl)-*N*, *N*, *N'*, *N'*, -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 ionisiation |
| 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 | *tat* 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 |
| $V_H$ | immunoglobulin variable region heavy |
| $V_L$ | immunoglobulin variable region light |
| ZAP | zeta associated protein |

## AMINO ACID ABREVIATIONS AND RESIDUE MASS

| Amino acid | Single Letter code | Three letter code | Residue average mass | Residue monoisotopic mass |
|---|---|---|---|---|
| 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 |