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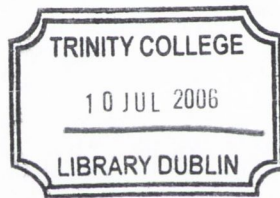# *Characterization of Peptide Mimics to Antigens Chaperoned by Hsp70 in MDA-MB-231 Breast Tumour Cells*

Thesis submitted to the University of Dublin,
Trinity College,
For the Degree of Doctor of Philosophy

by

Blanca Esther Arnaiz Villanueva
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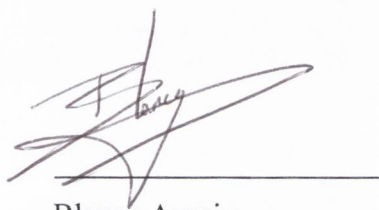Moyne Institute of Preventive Medicine,
Trinity College,
Dublin

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FOR MY PARENTS

Table of Contents

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A	adenosine triphosphate or alanine
Aa	amino acid
ABTS	2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid)
ADCC	antibody-dependent cell-mediated cytotoxicity
ADP	adenosine diphosphate
Ag	antigen
α-GalCer	α-galactosyloceramide
APC	antigen presenting cell
APL	acute promyelocytic leukaemia
ATCC	American Type Culture Collection
ATP	adenosine triphosphate
ATPase	ATP hydrolase
BAC	bacterial artificial chromosome
BAGE	B antigen
BiP	Ig chain-binding protein
BLAST	Basic Local Alignment Search Tool
brc-abl	breast cancer- Abelson murine leukemia
BSA	Bovine Serum Albumin
C	cysteine
CD	cluster of differentiation
CDC27	cell division cycle protein 27
CDK	cyclin-dependent Kinase
cDNA	complementary DNA
CEA	carcinoembryonic antigen
CLIP	class II-associated invariant-chain peptide
CML	chronic myelogenous leukaemia
CT7	Cancer-testis antigen 7
cTAGE-1	cutaneous T-cell lymphoma-associated antigen isoform 1
CTL	cytotoxic T-lymphocyte
D	aspartic acid
Da	Dalton

DAB	diaminobenzidine
DAPI	4',6-diamidino-2-phenylindole
dATP	deoxyadenosine triphosphate
DC	dendritic cell
DC-CK	dendritic cell chemokine
DEC 205	dendritic and epithelial cells 205
DETOX	detoxified Freund´s adjuvant
DMEM	Dulbbecco´s modified eagle medium
DMSO	Dimethyl Sulfoxide
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
DTT	dithiotreitol
E	glutamic acid
EDTA	ethylenediaminetetra-acetic acid
EGF	epidermal growth factor
ELISA	enzyme-linked immunosorbant assay
ER	endoplasmic reticulum
ESTs	expressed sequence tags
EU	endotoxin units
ExPASy	expert protein analysis aystem
F	phenylanine
FasL	Fas ligand
FCS	foetal calf serum
FGFR	fibroblast growth factor receptor
FITC	fluorescein isothiocyanate
FSC	forward scatter
G	guanidine triphosphate
g	gravitational force
GAGE	G antigen
GM-CSF	granulocyte-macrophage colony-stimulating factor
Gnt-V	N-acetylglucosaminyl-transferase V
gp 75	glycoprotein 75
gp100	glycoprotein 100
Grp	glucose regulated proteins

GSSs	genome survey sequences
H	histidine
H&E	haematoxylin and eosin
HBSS	Hank´s balanced salts solution
HDI	high dose interferon
Hepes	N-2-hydroxyethyl-piperazine-N'-2-ethanesulphonic acid
HLA	human leukocyte antigen
HPB	Health Protection Branch
HPV	human papilloma virus
hr	human recombinant
HRH	human RNA helicases
HRP	horse radish peroxidase
Hsc	heat shock cognate
Hsp	heat shock protein
HspPC	heat shock protein-peptide complex
I	isoleucine
iDC	immature dendritic cells
IFN	interferon
Ig	immunoglobulin
IL	interleukin
K	lysine
KIRs	killer inhibitory receptors
KLH	keyhole limpet hemocyanin
L	leucine
LAK	lymphokine-activated killer
LEF	lymphoid enhancer binding factor 1
LL	lower left
LMP	Low molecular mass protein
LPS	lipopolysaccharide
LR	lower right
MAGE	melanoma-associated antigen
MALDI-ToF	matrix assisted laser desorption ionization time of flight mass spectrometry
MART-1	melanoma antigen recognised by T cells

mDC	mature dendritic cells
MECL1	Multicatalytic endopeptidase complex subunit 1
METH-A	methylcholanthrene A
MHC	major histocompatibility complex
MIC	MHC class I chain related antigen
MIP-1α	macrophage inflammatory protein 1α
MITAP	Mitosin-associated protein
MLR	monomeric laminin receptor
mRNA	messenger RNA
MTS	3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide
N	asparagine
NADH	Dihydronicotinamide Adenine Dinucleotide
NADPH	Dihydronicotinamide Adenine Dinucleotide phosphate
N-CAM	neural-cell adhesion molecule
NCBI	National Center for Biotechnology Information
NCI	National Cancer Institute
NEB	New England Biolabs
NFκB	nuclear factor kappa-B
NK	natural killer
NKG2A	natural killer group 2A
NKRP1A	natural-killer-cell receptor protein 1A
NP-40	nonidet P-40
NTC	non-tumour cell extract
ORF	open-reading-frame
OS	overall survival
P	proline
PAGE	polyacrylamide gel electrophoresis
PBMC	peripheral blood mononuclear cells
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PE	phycoerythrin
PEG	polyethylene glycol-8000
PerCP	peridinin-chlorophyll-protein

PFN1	profilin 1
PFU	plaque-forming units
PML	promyelocytic leukaemia
psi	pressure per square inch
PSMA	prostate-specific membrane antigen
Q	glutamine
R	arginine
RAGE	receptor for advanced glycosylation end products
RANTES	regulated upon activation, normal T-cell expressed and secreted
RARA	retinoid acid receptor-α
RFS	relapse free survival
RNase	ribonuclease
RPMI	Roswell Park Memorial Institute
RREB-1	RAS- responsive element binding protein 1
RT-PCR	reverse-transcription PCR
S	serine
SCP1	single-stranded DNA-binding protein MSSP1
SDS	sodium dodecyl sulphate
SEREX	serological expression of cDNA expression libraries
SF	splicing factor
snRNP	Small nuclear ribonucleoprotein
SSC	side scatter
SSX2	Synovial sarcoma, X breakpoint 2 protein
STS	Sequence tagged sites
SCP1	synaptonemal complex protein 1
T	threonine or thymidine triphosphate
TAA	tumour-associated antigen
TAP	transporter associated protein
TAP	transporter associated with antigen processing
TC	tumour cell extract
TCA	trichloroacetic acid
TCF	T cell-specific transcription factor 1
TCR	T cell receptor

TGF	transforming growth factor
TIL	tumour infiltrated lymphocyte
TNF	tumour necrosis factor
TPI	triosephosphate isomerase
TPR	tumour potentiating region
TRAIL	TNF-related apoptosis-inducing ligand
TRP	tyrosinase-related protein
TSA	tumour specific antigens
UCD	University College Dublin
UL	upper left
UR	upper right
UV	ultra violet
V	valine
VEGFR	vascular endothelial growth factor receptor
WHO	World Health Organisation
Y	tyrosine

Tumour-derived heat shock protein (Hsp) preparations have been shown to elicit anti-tumour immune responses in both mice and man. Analysis of Hsp preparations from tumour cells revealed that the immuno-protective property resides with the peptides complexed with Hsps (Hsp-PCs). Clinical trials are on going using autologous Hsp extracts as a source of antigens to boost immune responses against tumours. The advantage of this vaccine is that no prior knowledge of the tumour antigens associated with Hsps is required. The disadvantages are that it is patient-dependent and requires invasive surgery.

A novel approach to the development of potential tumour vaccines has been the isolation of peptide mimics to epitopes of known tumour specific antigens. Using a unique two-step phage display bio-panning technique, Bond and James (unpublished) generated a number of synthetic peptides mimics of antigens associated with heat shock protein 70 (Hsp70-PC) from the breast tumour cells MDA-MB-231 in an attempt to overcome the disadvantages mentioned above.

This study focuses on the Hsp70-PC mimic peptide, named DSP and the Hsp70-PC recogniser peptide, TMG. To determine if the mimic peptide possessed biological properties similar to antigens associated with Hsp70, the immuno-stimulatory activity of the peptide was examined by testing its ability to stimulate CD14⁻ PBMCs *in vitro*. The results indicated that both the mimic peptide DSP and the recogniser peptide TMG could stimulate CD14⁻ PBMCs to produce IFN-γ but only when presented by dendritic cells and following two rounds of stimulation. T-cell stimulation *in vitro* assay was used to demonstrate that the DSP and TMG peptides resemble antigens present in breast cells and more specifically, antigens associated with Hsp70.

Cytochemistry and histochemistry techniques revealed that the DSP and TMG peptides interact with cellular components of tumour and non-tumour cell lines in a confluency-dependent fashion. The same pattern of peptide binding was observed in breast ductal carcinoma tissue sections. Furthermore maximum peptide binding correlated with cells showing high proliferative rates within the tumour, thus encouraging further investigations of the use of these peptides as potential vectors for tumour drug targeting.

A number of approaches were taken to identify the cellular targets of peptide binding. *Ex vivo* cross-linking of proteins to biotinylated peptides indicated that both peptides interact with a protein of 70kDa in size. Mass spectrometry identification of the crosslinked proteins yielded inconclusive results, however, Hsp70 was identified among the spectra. Another approach taken was the screening of a breast tumour cDNA expression library for DSP or TMG-binding proteins. A number of interesting clones were identified by this approach however in most cases the peptides interacted with out-of-frame translation products of the cDNAs.

In this study a method for the validation of Hsp70 peptide mimics has been established and future work will investigate its usefulness in tumour treatment applications.

Presentations

Poster presentations:

B. Arnaiz, L. Golden-Mason, Y. Buggy, G. McEntee, O. Traynor, J. Hegarty, C. O'Farrelly and T. Maguire. (2000) ETS transcription factors in normal and tumour-bearing human liver. **The Irish Association for Cancer Research (IACR) /Irish Society of Medical Oncology (ISMO) Joint Cancer Meeting. 2000, Galway, Ireland.**

B. Arnaiz, L. Estebas-Madrigal, D. Doherty, T. James and U. Bond. (2004) Heat shock protein 70 and peptide mimics as tumour vaccines. **EORTC-NCI-AACR Symposium – Molecular Targets and Cancer Therapeutics, Geneva, Switzerland**.

B. Arnaiz, and U. Bond. (2004). The search for a cancer vaccine. **HRB Annual Symposium-A selection of Irish Health Reseach, Dublin, Ireland**: Oral presentation (by U. Bond).

Oral presentation:

B. Arnaiz, L. Estebas-Madrigal, D. Doherty, T. James and U. Bond. (2004) Structural mimics of HSP70 associated peptides from breast tumour cells can prime T cells to respond to tumour antigens. **Irish Society for Immunology and Ulster Immunology Group Joint Meeting, Maynooth, Ireland**

Publicatons:

B. Arnaiz, L. Estebas-Madrigal, D. Doherty, T. James and U. Bond. (2004). A Structural Mimic of Heat Shock Protein 70-peptide complexes from Breast Tumour Cells can Prime T Cells to Respond to Breast Cell Antigens. (*Submitted*).

B. Arnaiz, L. Estebas-Madrigal, D. Doherty, T. James and U. Bond. (2004) Heat shock protein 70 and peptide mimics as tumour vaccines. EORTC-NCI-AACR Symposium –

Molecular Targets and Cancer Therapeutics, Geneva, Switzerland. European Journal of Cancer **2**:79.

Figures:

Tables:

Chapter 5

GENERAL INTRODUCTION

1 GENERAL INTRODUCTION

With over 10 million people diagnosed with cancer each year (a figure which is estimated to rise to 15 million by 2020) (http:www.who.int/cancer/en/., 2005) the importance of scientific research into this disease and methods of treating it is unquestionable. Cancer is a molecular disease caused by the acquisition of transforming mutations within the cell which result in inappropriate and uncontrolled cellular proliferation and/or a diminished rate of cell depletion (via apoptosis). Surgery is currently the primary method for treatment of malignant disease but may not be possible or entirely effective in the eradication of cancerous growths, with the occurrence of metastases often limiting its success. Chemo- and/or radiotherapy are commonly required but have well documented associated toxicities, rendering them difficult treatment regimens to administer to patients with already severely compromised health. In the search for more specific and less toxic cancer treatment strategies, cancer immunotherapy has emerged as promising candidates.

1.1 MOLECULAR ASPECTS OF TUMOUR DEVELOPMENT

Cancer can be described as the hyper proliferation of cells, unresponsive to cell-cell or cell-matrix growth inhibition contact signals and is generated by accumulation of mutations and/or chromosome rearrangements. The following sections describe some of the changes that are observed in cancer cells.

1.1.1   Cell senescence and cell death

Cancer cells undergo a higher number of cell divisions than normal cells. This is due to a breakdown in the homeostasis between cell proliferation, and differentiation. As tumour cells progress from differentiated to dedifferentiated, the proliferation rate increases. This is seen in the much shorter doubling times of metastases compared with those of primary tumours. Cell senescence involves telomere shortening and in adult somatic cells, telomerase declines with the consequence that telomere length progressively shortens about 4Kb at each round of DNA synthesis, disrupting cell function sufficiently to cause senescence. In cancer cells, telomerase is frequently reactivated by unknown mechanisms although not to the levels seen in germ cells. This

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extends their lifespan by preventing further loss of telomeric repeats but does not lengthen those repeats to the original levels. Hence a degree of chromosome instability remains.

Increased cell proliferation rates, differentiation blockade, loss of differentiated cell feature, and delayed senescence are some of the properties that can be found in cancer. Moreover, cell death is also altered in cancer cells. There are two types of cell death; necrosis and apoptosis. Necrosis occurs in areas of the tumour with a shortage of blood supply, nevertheless malignant tumours secrete angiogenic factors to provide themselves with a supply of nutrients and oxygen and a means of invasion to distant tissues during cancer progression. Apoptosis, also called programmed cell death or cell suicide, occurs in tissue remodelling and is induced by DNA damage, withdrawal of growth stimulatory signals or death-promoting signals. In cancer cells, apoptosis is decreased by alterations of the relative proportions of proapoptotic and antiapoptotic proteins, therefore cell survival is promoted (Jaattela 2004).

1.1.2 DNA damage promotes a cancerous state

DNA damage can be produced by chemicals and short wavelength radiation. Genotoxic carcinogens such as polycyclic aromatic hydrocarbons, aromatic amines, nitrosamines and alkylating agents, form covalent adducts with bases of DNA. Aduct formation distorts the DNA structure and if this is not repaired, an inappropriate base is introduced into the new strand. Ionising radiation, γ-rays and X-rays generate single and double-stranded breaks in DNA resulting in deletions and chromosome rearrangements. UV light generates covalent links between adjacent pyrimidines resulting in cyclobutane dimers that are read as thymines during DNA replication and can result in a point or double tandem mutations. Ionising radiation can also form reactive oxygen species, such as superoxide, hydroxyl radicals and hydrogen peroxide, which can alter the DNA, decrease DNA repair by damaging essential proteins and activate signal transduction pathways (Bertram 2000).

1.1.3 DNA mutations and chromosome rearrangements

Mutations in the genetic material, either within a gene or within the promoter region of a gene can result in qualitative or quantitative changes of proteins involved in

regulatory pathways such as membrane receptors, signal transduction and gene transcription. The genes coding for these proteins are named oncogenes and their overexpression or activation promotes cell growth. This same effect can be facilitated by retrovirus infection and the provision of the host with one more copy of an oncogene homologue. Moreover, cases of gene amplification due to defective start signals at DNA replication forks have been detected in advanced cancers (e.g. c-erbB2 oncogene in breast and cervix cancers). Mutations in coding regions can also generate loss or malfunction of inhibitory protein products involved in pathways regulating cell proliferation and death, for example the Rb gene in retinoblastoma, lung, prostate and breast cancers and p53 in ovarian, pancreatic and lung cancer among others. The genes coding for these proteins are named tumour suppressors and their inactivation gives rise to cell proliferation. Carcinogenic DNA viruses usually code for tumour suppressor binding proteins that inactivate the function of the tumour suppressor protein. Chromosome rearrangements can result in the chromosomal translocation of an oncogene placing it under a strong promoter leading to overexpression of its gene product. An example of this is found in Burkitt´s lymphoma where the c-myc gene on chromosome 8 is translocated next to a strong immunoglobulin promoter on chromosome 14. Additionally chromosomal translocations can result in the formation of fusion proteins with altered biological activity as in the case of retinoid acid receptor-α (RARA) in acute promyelocytic leukaemia (APL). APL is characterised by a reciprocal chromosome translocation such that RARA gene from chromosome 17 is translocated next to the promyelocytic leukaemia gene (PML) on chromosome 15. The fusion protein has a RARA segment that has lost the N-terminal region including part of the transactivation domain contributing to block promyelocyte differentiation and their resultant accumulation in the blood. (Loktionov 2004).

1.2 TUMOUR IMMUNOLOGY

1.2.1 Immunesurveillance. Mechanisms of escape

The alterations in chromosome structure, gene activity and cellular metabolism leads to the expression of unique proteins on the cell surface of tumour cells that are not present in normal cells or at least not the same quantities (tumour associated antigens, TAAs). In the 1950s, Brunet described the ability of the immune system to detect and

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eliminate cancer cells as immune surveillance. Although its existence has been questioned it is now clear that the immune system has an important role in the control of tumours as both cytotoxic T lymphocytes (CTL) and antibodies specific for TAAs have been found in patients with cancer. These CTLs can lyse autologous and HLA-matched tumour cells *in vitro* (Mocellin, Mandruzzato et al. 2004). Unfortunately, in the majority of cases the immune response mounted against tumour cells *in vivo* is ineffective and does not clear the body of the cancer cells. There are several mechanisms by which tumour cells escape immune surveillance. Tumour cells can be recognized as 'self' and therefore tolerated. The localisation of the tumour, the lack of potent antigens with inadequate affinity for host MHC molecules, the lack of antigen presenting and co-stimulatory molecules, insufficient numbers of antigen-specific T cells in the reportoire (Houghton and Guevara-Patino 2004) as well as disrupted processing and presentation of TAAs (eg, by disruption of TAP1 in the proteosome) can limit the effectiveness of the immune response against tumour cells. Tumour cells have been shown to down regulate the expression of MHC class I molecules thereby reducing their recognition by CTLs. However this phenotype increases their susceptibility to NK cells (see below). Tumours can suppress immunity in a local or generalised way by the expression of molecules such as GasL, CTL–inhibiting serpins (Todryk 2002), IL-10 which reduces dendritic cell (DC) development and activity and TGF-β which tends to suppress inflammatory T-cell responses and cell-mediated immunity. Tumours can initially express antigens to which the immune system responds but lose them by antibody-induced internalization or antigenic variation. When tumours are attacked by cells responding to a particular antigen, any tumour cell that does not express that antigen will have a selective advantage (immune selection of antigen-loss variants) and thus continue to proliferate. For instance, colon cancers lose the expression of a particular MHC class I molecule, this occurs perhaps through immunoselection by T cells specific for a peptide presented by that MHC class I molecule.

1.2.2 T lymphocytes can recognise certain types of tumours

Despite the limitations of immune responses to tumour cells, a number of tumour-specific antigens, such as melanoma antigens, renal cell carcinoma antigen and MUC-1 (mucin-1) have been shown to be recognised by T lymphocytes from patients with certain tumours. Malignant melanoma and renal cell carcinoma, unlike other tumours,

occasionally, even at quite advanced stages of the disease, can show spontaneous remission. Melanomas express at least five different antigens that are recognised by CTLs. Interestingly, none of these antigens are derived from the mutant proto-oncogenes or tumour suppressor genes that are likely to be responsible for the initial transformation of the cell into a cancer cell, although a few are the products of mutant genes. The rest are from the MAGE family that represent early developmental antigens re-expressed in the process of tumorigenesis, also expressed in testis and differentiation antigens specific to the melanocyte lineage from which melanomas arise, such as, gp100, MART1, and gp75 (Van Der Bruggen, Zhang et al. 2002).

The following section provides a general description of the mechanism of how an immune response can be mounted against a tumour cell.

1.2.3 Antigen presentation

Tumour-associated antigens (TAAs) are presented to T-cells by macrophages or other antigen–presenting cells in close association with major histocompatability complex (MHC) proteins. There are two major MHC-type molecules designated as class I and class II. Class I molecules consists of two covalently attached proteins referred to as the heavy chain and the light chain. Additionally, these molecules associate non-covalently with a protein called beta-2 microglobulin. Humans synthesise three different types of class I molecules designated HLA-A, HLA-B, and HLA-C. (HLA refers to human leukocyte antigen as the molecules were first studied on leukocytes). These differ only in their heavy chain, all sharing the same type of beta-2 microglobulin. The genes encoding the different heavy chains are clustered on chromosome 6 in the major histocompatibility complex (MHC).

MHC class I molecules bind peptides in an intracellular location and deliver them to the cell surface, where antigen recognition by T cells can occur. MHC I molecules bind peptides from proteins degraded in the cytoplasm of the cell. Peptides produced by the proteasome are transported into the endoplasmic reticulum by a heterodimeric ATP-binding protein called TAP (Transporter Associated with Antigen Processing) where they are then available for binding by partially folded MHC class I molecules. By binding stably to peptides from proteins degraded in the cytosol, MHC class I molecules serve to display fragments of normal cell constituents, fragments of molecules encoded

by intracellular parasites and fragments of proteins encoded by mutated genes in cancer cells (Moron, Dadaglio et al. 2004).

Class II molecules are normally expressed only on certain types of cells such as DCs, macrophages and B lymphocytes that specialise in processing and presenting extracellular antigens to T lymphocytes. Human class II molecules also designated HLA-D, are prevented from binding to peptides in the endoplasmic reticulum (ER) by their early association with the invariant-chain polypeptide in the ER, which fills and blocks their peptide-binding groove. Instead, the human class II molecules are targeted by the invariant chain polypeptide to an acidic endocytic compartment where, in the presence of active proteases, in particular the cathepsins S or L, the invariant chain is digested and the CLIP (class II-associated invariant-chain peptide) peptide remains bound to the MHC class II molecules. The specialised MHC class II-like molecule, DM, catalyses the release of CLIP and the binding of antigenic peptides. In this way, MHC class II molecules bind peptides from proteins that are degraded in endocytic vesicles, thereby capturing peptides from pathogens that enter the vesicular system of macrophages and DCs or peptides from the specific antigens internalised by the immunoglobulin receptors of B cells (Watts 2004). In this same manner, tumour antigens associated with heat shock proteins are released from necrotic tumour cells that once internalised by macrophages and DCs are presented on MHC class II molecules to CD4$^+$ T cells. There is also a phenomenon termed cross-priming, the exact mechanism of which has not yet elucidated, by which heat shock proteins associated with tumour antigens can be presented on MHC class I molecules to CD8$^+$ T cells.

1.2.4 Antigen presenting cells

Naïve T lymphocytes are primed by antigens loaded onto MHC molecules on the surface of antigen presenting cells (APCs) such as macrophages, B cells and dendritic cells (DCs) in lymph nodes. These proliferate and circulate until encountering the same antigen in association with MHC molecules on cell surfaces other than APCs. So far, DCs have been found to function exclusively as APCs, whereas macrophages and B cells can be activated by CD4$^+$ T cells to execute other functions. Only APCs express on their cell surface the co-stimulatory molecules necessary to activate naïve T lymphocytes. Immature DCs express an immature phenotype associated with low levels of MHC molecules and lack of co-stimulatory B7 molecules. They emerge from their

myeloid progenitors in the bone marrow and migrate via the blood vessels to their peripheral location. Immature DCs are found in most surface epithelia such as Langerhans´ cells of the skin and solid organs such as the heart and kidneys. They are not yet equipped to stimulate naïve T lymphocytes, however they take up antigens very actively both via receptor (e.g. DEC 205 and CD91) mediated endocytosis and macropinocytosis. Once they are stimulated by infection or tumour environment (TNF-α) to migrate to the local lymph nodes, they express the mature phenotype. These mature DCs no longer take up antigens but the levels of MHC molecules on their surface rise and persist for longer, thus, presenting peptides acquired from the infection or tumour (Granucci, Zanoni et al. 2003). Mature DCs also express high levels of adhesion and co-stimulatory B7 molecules, and secrete a chemokine known as DC-CK, that specifically attracts naïve T lymphocytes in the lymph node.

1.2.5 Effector αβ – T cells

T cells develop in the thymus and migrate via blood to the peripheral lymphoid organs, recirculating between blood and peripheral lymphoid tissue until they encounter antigen. Naïve T cells are induced to proliferate and differentiate into cells capable of contributing to the removal of pathogens. These cells are termed armed effector T cells as they can act rapidly upon encountering specific antigen on other cells, thus participating in an adaptive immune response. The cells on which armed effector T cells act are termed target cells.

The first encounter of naïve T cells with antigen on a professional antigen-presenting cell triggers a primary immune response and also generates immunological memory, providing protection from subsequent challenge by the same pathogen. Like naïve T cells, memory T cells are quiescent and require activation by professional antigen-preseting cells to regenerate effector T cells.

There are three functional classes of effector T cells that detect peptide antigens derived from different types of pathogens: CTLs, T_H1 cells and T_H2 cells. Peptides derived from pathogens that multiply within the cytoplasm of the cell are carried to the cell surface by MHC class I molecules and presented to $CD8^+$ T cells, which differentiate into CTLs that kill infected target cells. Peptide antigens derived from pathogens multiplying in intracellular vesicles and those derived from ingested extracellular bacteria and toxin are carried to the cell surface by MHC class II molecules

and presented to CD4$^+$ T cells that can differentiate into two types of effector T cells; T_H1 and T_H2 cells. Pathogens that accumulate in large numbers inside macrophage vesicles tend to stimulate the differentiation of T_H1 cells, while extracellular antigens tend to stimulate the production of T_H2 cells. T_H1 cells activate the microbicidal properties of macrophages and induce B cells to make IgG antibodies that are very effective at opsonising extracellular pathogens for uptake by phagocytic cells. T_H2 cells initiate the humoral immune response by activating naïve antigen-specific B cells to produce IgM antibodies, and may subsequently stimulate the production of different isotypes, including IgA and IgE, as well as neutralizing and/or weakly opsonising subtypes of IgG.

1.2.6 Cytokine production by the effector $\alpha\beta$ – T cells

Cytokines are the principal mediators of T-cell effector actions which are directed at specialised cells that express receptors for these effector molecules. The main cytokine released by CTLs cells is interferon γ (IFN-γ). IFN-γ can also activate Natural Killer (NK) cells and promote the expression of MHC class I and II on macrophages and DCs and inhibits T_H2 cells production. T_H1 cells and T_H2 cells release different but overlapping sets of cytokines which define their distinct actions in immunity. T_H1 cells secrete IFN-γ, which is the main macrophage and dendritic cell-activating cytokine, and lymphotoxin (LT-α or TNF-β), which activates macrophages, inhibits B cells and is directly cytotoxic for some cells.

1.2.7 CTLs

CTLs recognise surface markers on other cells in the body that label those cells for destruction. Targets of CTLs may include: virus-infected cells, cells infected with intracellular bacterial or protozoal parasites, allografts such as transplanted kidney, heart, lungs, etc. and cancer cells. There is also evidence that CTLs are active in some autoimmune disorders.

Most CTLs belong to the CD8$^+$ subset of T cells and use the $\alpha\beta$ T-cell receptor for antigen (TCR$\alpha\beta^+$), thus recognise antigens nestled in the groove of class I histocompatibility (MHC) molecules. A brief encounter with the antigen/MHC for which their TCR is specific causes them to undergo through several rounds of mitosis

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("clonal expansion") followed by differentiation into CTLs. Their differentiation includes forming a large number of modified lysosomes loaded with proteins: perforin and several types of granzyme. Most of these CTLs will die (of apoptosis), but some will become memory cells: long-lived cells poised to respond to the antigen if it should reappear. Virtually every cell in the body expresses class I MHC molecules, so CD8$^+$ CTLs are not limited in the targets they can attack.

1.2.8 Target recognition by CTLs

The TCRαβ$^+$ is very similar to immunoglobulin in both structure and in the way in which variability is introduced into the antigen-binding site. TCRαβ$^+$ on most T cells is made up of two transmembrane polypeptides designated alpha and beta and like antibodies, each polypeptide has an N-terminal variable region with 3 hypervariable regions. The hypervariable regions of the two chains cooperate to form a single binding site for the epitope. The binding site for the peptide on an MHC molecule lies in a cleft between two α helices, thus, the CTL receptor recognises a ligand that has a region of high variability. As a consequence of the binding of T cell receptor to a combined peptide:MHC ligand, T cells manifest MHC-restricted antigen recognition, such that a given T cell is specific for a specific peptide bound to a specific MHC molecule.

1.2.9 Mechanisms of killing by CTLs

1.2.9.1 Perforin/granzyme Killing

CTLs have cytoplasmic granules that contain the proteins perforin and granzymes. When the CTL binds to its target, the contents of the granules are discharged by exocytosis. Perforin molecules insert themselves into the plasma membrane of target cells and this enables granzymes to enter the cell. Granzymes are serine proteases that once inside the cell proceed to cleave the precursors of caspases thus activating them to cause the cell to self-destruct by apoptosis. The two cells are attached tightly at a small patch of plasma membrane. Special adhesion molecules hold them together. The granules are discharged only at that small portion of the plasma membrane.

1.2.9.2 FasL/Fas killing

CTLs express on their surface a transmembrane protein, the death activator designated Fas ligand (FasL). Most potential CTL targets express a receptor for FasL designated Fas. When cytotoxic T cells recognise (bind to) their target, they produce more FasL at their surface. This binds with the Fas on the surface of the target cell leading to its death by apoptosis.

1.2.10 Natural Killer cells or NK cells

Natural Killer (NK) cells are non-T, non-B lymphoid cells that can target antibody coated cells by antibody-dependent cell-mediated cytotoxicity (ADCC), virus infected cells in the early days of infection, and tumour cells. The mechanism of attack is analogous to that of CTLs, which involves the release of perforin and granzymes from cytoplasmic granules. Among the cytokines known to increase NK cells activity are IFN-α, IFN-β and IL-12 which are produced in early viral infection. IL12, also produced in response to tumour necrosis, in combination with TNF-α, can also elicit the secretion of large amounts of IFN-γ by NK cells (Wu and Lanier 2003). IFN-γ has important anti-tumour activity. There are inhibitory receptors on the surface of NK cells that recognise distinct HLA-B and HLA-C alleles. These receptors are members of the immunoglobulin gene superfamily and are called p58 and p70, or killer inhibitory receptors (KIRs) (Yokoyama and Scalzo 2002). Furthermore, NK cells express a heterodimer of two C-type lectin molecules, called CD94:NKG2A which is also a NK inhibitory receptor that recognise distinct HLA-E alleles. A common feature of all the inhibitory NK receptors is the presence of a tyrosine-inhibitory motif in their cytoplasmic domains. The binding of inhibitory receptors to MHC class I molecules inhibits NK activity, therefore syngeneic cells expressing normal levels of MHC class I molecules are protected from attack by NK cells. However, tumour cells lack or show low level expression of MHC class I molecules making them susceptible to NK cell attack.

1.2.11 Natural Killer T (NKT) cells

NKT cells are also described as NK1.1$^+$TCR$\alpha\beta^+$ cells (Smyth, Crowe et al. 2002). They also express activating and inhibitory receptors typical for NK cells (Brutkiewicz and Sriram 2002). Upon stimulation, NKT cells secrete immunoregulatory cytokines characteristic of both Th1 (pro-inflamatory) and Th2 (anti-inflamatory) responses, including IL-4, IL-10, IL13, TNF and IFN-γ (Godfrey, Hammond et al. 2000; Smyth and Godfrey 2000). Stimulation of NKT cells via IL-12 receptor, preferentially induces IFN-γ production, whereas stimulation via IL-7 receptor preferentially induces IL-4 (and possibly IL-13) production. These cells also possess death-inducing effector molecules such as perforin and TNF-related apoptosis-inducing ligand (TRAIL). NKT cells initiate effective anti-tumour responses when stimulated with exogenous factors such as low-doses of IL-12 or α-galactosyloceramide (α-GalCer) or with endogenous tumour-derived glycolipid antigen (Endog. glycolipid Ag). Their anti-tumour activity results mainly from perforin-dependent cytotoxicity, IFN-γ production and the augmentation of DC, NK and CD8$^+$ T cells function.

1.2.12 $\gamma\delta$ T cells

$\gamma\delta$ T cells are a minor fraction of the T lymphocytes in peripheral blood and can be found mainly in intestine, skin, tongue, esophagus, trachea, lungs and genital epithelia. $\gamma\delta$ tumour infiltrated lymphocytes (TILs) recognise antigens over-expressed at the tumour site. To reach the tumour site, both circulating and resident $\gamma\delta$ T cells express receptors to chemokines such as, macrophage inflammatory protein 1α (MIP-1α) and 1β (MIP-1β), and the Regulated upon Activation, Normal T-cell Expressed and Secreted protein (RANTES) (Roth, Diacovo et al. 1998). In response to these chemokines, $\gamma\delta$ T cells could potentially extravasate, migrate through the extracellular matrix and reach the tumour site. To extravasate and/or recirculate, $\gamma\delta$ T cells express adhesion molecules such as the neural-cell adhesion molecule (N-CAM/CD56) and use it to bind to endothelial cells and subendothelial matrix (Zocchi and Poggi 1993) . To transmigrate across endothelial cells they express the natural-killer-cell receptor protein 1A (NKRP1A) which might function as an adhesion molecule. The expression of NKRP1A is regulated by IL-12, therefore release of IL-12 at the level of the damage

tissue or tumour might modulate γδ T cell re-circulation (Poggi, Zocchi et al. 1999). γδ T cells display a unique reportoire of TCR-antigen specificity. Among them are nonpeptide antigens shared by microbial and mammalian cells; in particular, phosphorylated thymidine related products that could be involved in nucleic acid synthesis and repair in damage or tumour cells (Constant, Davodeau et al. 1994). γδ T cells also recognise proteins such as some belonging to the heat shock protein family (Kaufmann and Kabelitz 1991; Hayday 2000) and the human MHC-class-I-related molecules MICA and MICB expressed under the control of the heat-shock- responsive promoters by intestinal epithelia and epithelial tumours, including carcinomas of the lung, kidney and colon (Groh, Steinle et al. 1998; Groh, Rhinehart et al. 1999). γδ T-cells also recognise CD1c expressed mainly on APCs which also express MICA and MICB and the monomeric laminin receptor (MLR) expressed on cancer cells and which is involved in tumour invasion and metastasis (Ferrarini, Heltai et al. 1996). Apart from TCRγδ$^+$, the natural-killer-cell receptor NKG2D is also expressed on γδ T cells and interacts with MICA and MICB (Groh, Rhinehart et al. 1999; Das, Groh et al. 2001). Another NK-like receptor also expressed on γδ T cells is the killer inhibitory receptor (KIR), which interact with MHC class I molecules and activates γδ T cells against tumour cells showing low levels of MHC class I molecules, as it is often the case during cancer progression (Fisch, Moris et al. 2000) . By contrast to αβ T cells, γδ T cells can recognise antigens with no requirement for antigen processing or presentation (Chien, Jores et al. 1996; Crowley, Fahrer et al. 2000).

Antitumour effector functions of γδ T cells include perforin-mediated cytotoxicity, up-regulated expression of Fas ligand (FasL) following TCRγδ$^+$ engagement, and secretion of IFNγ (Hayday 2000).

1.3 TAAs: CLASSIFICATION AND DISCOVERY/IDENTIFICATION

The presence of unique markers on tumour cells provides an opportunity, through the characterisation of TAAs, for the development of tumour vaccines. Additionally, tumour specific markers can be exploited to specifically target drugs to the tumour.

Several types of tumour associated antigens have been described to date that can be classified into specific groups (Eisenbach, Bar-Haim et al. 2000). Tissue specific antigens, such as melanoma specific antigens, Melan-A/MART-1, tyrosinase and gp100, and carcinoembyonic antigen (CEA), are expressed in both normal and tumour cells.

Another group of TAAs consists of antigens derived from proteins normally expressed during ontogeny but not, or at low levels in fully differentiated cells apart from testis. This group includes the activated or derepressed family of genes, MAGE, BAGE, and GAGE in melanoma, RAGE in renal carcinoma and NY-ESO-1. A third group includes unregulated gene products found in a wide range of tumours and associated with tumorigenesis such as wild-type p53 and Her-2/neu in breast cancers. Another group includes those antigens derived from oncogenic viruses as the E7 oncoprotein of human papilloma virus (HPV) 16, which is expressed in almost all cervical carcinomas, as well as the latent membrane protein (LMP) 1 of Epstein-Barr virus, consistently expressed in a number of nasopharyngeal carcinomas and Burkitt's lymphomas. Finally, TAAs can be derived from ubiquitously expressed proteins that are mutated in tumour cells such as MUC-1. Such mutations have been found in genes encoding proteins required for cell cycle regulation, such as cyclin-dependent Kinase (CDK) 4, c-ras and genes encoding proteins required for apoptosis such as β–catenin genes and, the CASP-8 gene that encodes for the protease caspase 8. Many of these mutated proteins are the product of chromosomal translocations such as the myeloid leukaemia- associated brc-abl protein.

Due to the cytotoxic activity of CD8$^+$ T cells, a number of MHC class -I-restricted antigens have been identified by employment of *in vivo* sensitised tumor-reactive T lymphocytes from cancer patients. These lymphocytes are used to screen an autologous tumor cDNA expression library for the target antigen (Boon, Cerottini et al. 1994; Morton and Barth 1996; Rosenberg 1999; Wang and Rosenberg 1999). This approach has led to the identification of TAAs, such as tyrosinase, MART-1, gp100, TRP-1/gp75, TRP-2, MAGE-1, NY-ESO-1, CDK4, β-catenin and caspase 8 (Wang 2002). For the identification of MHC class II-restricted antigens such as TPI, LDFP, CDC27 and fibronectin, some of the techniques used are peptide elution from the cell surface of tumour cells (Halder, Pawelec et al. 1997), biochemical characterisation of tumour cell lysates (Halder, Pawelec et al. 1997; Pieper, Christian et al. 1999) and the genetic targeting expression (GTE) system that allowed the capture of expressed proteins by DCs and their presentation on MHC class II molecules (Wang and Rosenberg 1999; Wang et al. 1999). For identification of antibody targets, cDNA microarrays and serological analysis of recombinant random peptides or cDNA expression libraries derived from human tumour cells with autologous serum (SEREX) have been used,

leading to the identification of several antigens such as tyrosinase, MAGE, NY-ESO-1, SSX2, SCP1, cTAGE-1 and CT7.

Analysis of the antigenic epitopes recognised by T cells reveals that these epitopes are generated by a number of different mechanisms (Wang 2002); these include alterations in transcription, and alternative splicing and protein degradation patterns. At a transcription/splicing level, the T-cell epitopes may be generated from aberrant mRNA or the incomplete removal of introns as in the case of the Gnt-V gene and gp100-intron4 epitopes. In other cases, epitopes arises from the translation of alternative open reading frames (ORF) in a protein (Jager, Chen et al. 1998; Wang, Johnston et al. 1998; Rimoldi, Rubio-Godoy et al. 2000). Their biological significance requires more studies and the mechanism by which alternative ORF is translated remains unclear. At the proteolytic level, the interferon-γ inducible immunoproteosome, which contains the additional subunits LMP2, LMP7 and MECL1, changes the cleavage profiles of antigenic peptides that will be presented on MHC class I molecules to T-cells on the cell surface (Dupont 2002). After proteosomic degradation, intracellular proteins are incorporated with the help of Hsp70 to the ER where they can be assembled to MHC class I or II molecules.

1.4 VACCINE DEVELOPMENT FOR SOLID TUMOURS

The presence of unique TAAs capable of being recognised by CTLs, in addition to the anti-tumour activity of immune cells such as NK, NKT and γδ cells, has led to the pursuit of strategies for the development of vaccines against tumours. Below is a summary of the types of approaches that have been developed and a description of clinical trials to test the effectiveness of such vaccines.

1.4.1 Whole cell

Autologous whole tumour cells inactivated by radiation treatment contain a large reportoire of TAAs that can be potentially targeted by the immune system. The major drawback in developing tumour vaccines is the lack of knowledge about the immunogenicity of identified antigens and the limited availability of tumour source, One approach which eliminates the need for knowledge of potential TAAs on the surface of tumour cells is to use a "whole cell" approach using cultured allogeneic

tumour cell lines (Morton and Barth 1996) prepared from solid tumours. This approach has been applied to the treatment of colorectal (Habal, Gupta et al. 2001) melanoma (Berd, Maguire et al. 1990; Eton, Kharkevitch et al. 1998; Hsueh, Nathanson et al. 1999; Leong, Enders-Zohr et al. 1999; Dillman, Wiemann et al. 2003; Vilella, Benitez et al. 2003) and prostate cancer (Eaton, Perry et al. 2002) in phase II clinical trials and renal-cell carcinoma in a phase I clinical trial (Fenton, Steis et al. 1996), and colorectal and colon cancer in phase III clinical trials (Hoover, Brandhorst et al. 1993; Vermorken, Claessen et al. 1999; Harris, Ryan et al. 2000). Two modifications have been introduced in order to make a more immunogenic vaccine; aptenisation of autologous tumour cells by reaction with dinitrophenyl employed in melanoma phase II clinical trials (Berd 2001; Berd, Sato et al. 2001; Lotem, Peretz et al. 2002; Berd, Sato et al. 2004; Lotem, Shiloni et al. 2004) and construction of both autologous and allogeneic tumour cells able to secrete cytokines such as GM-CSF and IL-2 to recruit and activate APCs at the vaccination site. Allogeneic tumour cells secreting IL-2 were employed in melanoma (Belli, Arienti et al. 1997; Arienti, Belli et al. 1999; Osanto, Schiphorst et al. 2000; Maio, Fonsatti et al. 2002), neuroblastoma (Bowman, Grossmann et al. 1998) and colorectal (Sobol, Shawler et al. 1999) phase I or II clinical trials. Allogeneic tumour cells able to secrete GM-CSF were employed in non-small-cell lung carcinoma (Nemunaitis, Sterman et al. 2004), colon carcinoma, renal-cell carcinoma or sarcoma cancers in phase I-II clinical trials (Mahvi, Shi et al. 2002). The use of this type of vaccine could be beneficial as adjuvant therapy after surgery as supported by a phase III randomised study that showed higher 5-year disease-free survival in patients with resectable renal-cell carcinoma who were given the vaccine than in those that were not treated with the vaccine (Jocham, Richter et al. 2004).

1.4.2 Tumour-lysate

Tumour cell lines lysed mechanically or enzymatically can elicit an effective immune response (Mitchell 1998). Viral lysates from allogeneic tumours have been employed for cervical cancer in phase III clinical trials and melanoma in phase II and III clinical trials (Batliwalla, Bateman et al. 1998; Wallack, Sivanandham et al. 1998; Hersey, Coates et al. 2002). Phase III clinical trials in an adjuvant setting has not confirmed the efficacy of mechanical lysate from allogeneic melanoma cells as vaccine, although it has shown an improved disease-free survival on the basis of the HLA

pattern of the patients. 83% of patients with a favourable HLA pattern had 5-year disease-free survival versus 59% for patients with an unfavourable HLA pattern. The vaccine was administered as two intramuscular injections of cell lysate from two cell lines mixed with DETOX (detoxified Freund´s adjuvant, containing mycobacterial cell wall skeleton plus monophosphoryl lipid A) per vaccination. This vaccine is termed melanoma theraccine or Melacine® vaccine (Corixa, USA) and its commercialisation has been approved by the Canadian Health Protection Branch (HPB) after phase III results. The vaccines were delivered as four 6-month cycles, each cycle consisting of 10 vaccinations along 24 weeks. (Sosman, Unger et al. 2002).

1.4.3 Shed-antigens

This vaccine is made by shedding the surface material of selected tumour-cell lines into a culture medium (Bystryn, Henn et al. 1992). It provides a broad array of epitopes from TAAs that are released from HLA molecules on the surface of cancer cells, but exclude irrelevant allogeneic antigens or immunosuppressive factors inside malignant cells. Only studies with allogeneic shed antigens from melanoma have been reported so far (Schultz, Oratz et al. 1995; Reynolds, Albrecht et al. 2003; Reynolds, Zeleniuch-Jacquotte et al. 2003). In patients with resected stage III melanoma, a phase III trial in the adjuvant setting showed a significant benefit for disease-free survival. 40 µg vaccine or placebo (human albumin) was injected into the extremities and aluminium hydroxide was used as adjuvant. There was no local or systemic toxicity (Bystryn, Zeleniuch-Jacquotte et al. 2001) .

1.4.4 Antigen-defined vaccines

1.4.4.1 Tumour associated antigens: carbohydrates

Carbohydrates such as glycophingolipids (eg, gangliosides) and glycoproteins (eg, globoH, sialyl-Tn, Tn-alpha) are either overexpressed by tumour cells (eg, gangliosides) or are originated from altered glycosylation of mucin during tumour progression (Dennis, Granovsky et al. 1999). Patients with humoral response against the vaccine showed a better clinical outcome than did non-responders. A phase III trial studied the efficacy of 1-year vaccination with high dose interferon alfa-2b (HDI) versus

ganglioside GM2 in combination with keyhole limpet hemocyanin (KLH) and QS-21 (GMK) in 774 patients with resected stage IIb/III cutaneous melanoma. HDI was associated with a treatment benefit in all subsets of patients with zero to equal or more than four positive nodes, but the greatest benefit was observed in the node-negative subset. Antibody responses were detected to GM2 and they were associated with a trend toward improved relapse free survival (RFS) and overall survival (OS). This trial demonstrated a significant benefit of HDI versus GMK in terms of RFS and OS in melanoma patients at high risk of recurrence (Marenholz, Zirra et al. 2001)

Other carbohydrates tested are sialyl-Tn for miscellaneous carcinomas (mainly breast and ovarian) in a phase I or II clinical trials (MacLean, Reddish et al. 1996; Sandmaier, Oparin et al. 1999; Holmberg, Oparin et al. 2000); Thomsen-Friedenreich-a (TF-a) for ovarian cancer in a phase I clinical trial (MacLean, Bowen-Yacyshyn et al. 1992) ; ganglioside GD2 for melanoma in a phase I clinical trial (Ragupathi, Livingston et al. 2003); glangioside GD2 and GM2 for miscellaneous (mainly melanoma and sarcoma) cancer in a phase I clinical trial (Chapman, Morrisey et al. 2000) Lewis carbohydrate antigen for ovarian cancer in a phase I clinical trial (Sabbatini, Kudryashov et al. 2000).

1.4.4.2 Tumour-associated antigens: recombinant proteins

Recombinant technology allows a relatively easy production of recombinant proteins and it does not require knowledge of the sequence of the epitope that will be presented to T cells. In the randomised trials performed, patients that respond to this type of vaccine show a survival advantage. The different recombinant TAAs tested are: the Chorionic gonadotrophin b for colorectal cancer in a phase II clinical trial (Moulton, Yoshihara et al. 2002); CO17-1A for colorectal cancer or pancreatic cancer in a phase I clinical trial (Staib, Birebent et al. 2001; Neidhart, Allen et al. 2004); mucin 1 for non-small-cell lung carcinoma or miscellaneous carcinomas in a phase I clinical trial (Goydos, Elder et al. 1996; Palmer, Parker et al. 2001) ; mannan mucin 1 for cancers such as breast, colon, and gastric in a phase I clinical trial (Karanikas, Hwang et al. 1997; Karanikas, Thynne et al. 2001); MAGE3 for colorectal and pancreatic cancer in a phase I clinical trial (Marchand, Punt et al. 2003); and epidermal growth factor for non-small-cell lung carcinoma in a pilot study (Gonzalez, Crombet et al. 2003).

1.4.4.3 Peptide-based vaccines

The majority of peptide derived TAAs studied to date are presented on MHC class I molecules to CTL. However, increasing numbers of recently discovered TAA epitopes are presented by MHC class II to helper CD4 T lymphocytes. Peptide vaccines can only be used in patients expressing the appropriate HLA molecule (Van Der Bruggen, Zhang et al. 2002). Tolerance to TAA epitopes can be broken by the substitution of amino acids that are essential for interaction in the binding pocket of either MHC molecules or T-cell receptors (Parmiani, Castelli et al. 2002). Peptides that bind to MHC class I molecules are 8-10 amino acids long and lie in an elongated conformation along the groove. Peptides binding to a given allelic variant of an MHC molecule have been shown to have the same or very similar amino acid residues at two or three specific positions along the peptide sequence called anchor residues. Both their position and identity can vary depending on the particular MHC class I allele that is binding the peptide. Most synthetic peptides of suitable length that contain these anchor residues will bind the appropriate MHC class I molecule, in most cases irrespective of the sequence of the peptide at other positions. Peptides that bind to MHC class II molecules are at least 13 amino acids long and can be much longer and lie in an extended conformation along the groove. The binding pockets of MHC class II molecules are more permissive in their accommodation of different amino acids than are those of the MHC class I molecule, making it more difficult to define anchor residues and predict which peptides will be able to bind particular MHC class II molecules. The comprehensive database SYFPEITHI allows the search for the most probable HLA class I or II that can associate with a given peptide (http://www.uni-tuebingen.de/uni/kxi/database.html).

Studies have shown a survival advantage for those immune responders to this type of vaccine that employed; mucin 1_{177-92} for breast tumour in a phase I clinical trial, $E7_{11-20,86-93}$ for cervical cancer in a phase I-II clinical trial (van Driel, Ressing et al. 1999); cyclophilin $B_{84-92,91-99}$ in small-cell lung and non-small-cell lung carcinoma in a phase I clinical trial (Gohara, Imai et al. 2002); $GP100_{209-17,154-62,280-88}$ (Salgaller, Marincola et al. 1996; Rosenberg, Yang et al. 1998; Lee, Wang et al. 1999; Rosenberg, Yang et al. 1999; Lee, Wang et al. 2001; Slingluff, Yamshchikov et al. 2001), $MAGE3_{168-76}$ (Marchand, van Baren et al. 1999; Weber, Hua et al. 1999), $MART1_{26-35}$ (Wang, Bade et al. 1999; Cebon, Jager et al. 2003), $tyrosinase_{192-200,\ 206-14,243-51,368-76}$ (Scheibenbogen,

Schadendorf et al. 2003), for melanoma in phase I or II clinical trials; RAS_{5-17} for colorectal, non-small-cell lung, and pancreatic cancer in phase I or II clinical trials (Khleif, Abrams et al. 1999; Gjertsen, Buanes et al. 2001); $NY\text{-}ESO1_{157-67,157-65,155-63}$ for breast, melanoma and ovarian cancer in phase I clinical trials (Jager, Gnjatic et al. 2000); ERBB2 peptides for breast and, ovarian or non-small cell lung carcinoma tumours or prostate cancer in phase I clinical trials (Disis, Schiffman et al. 2000; Knutson, Schiffman et al. 2001; Disis, Gooley et al. 2002; Disis, Rinn et al. 2002; Murray, Gillogly et al. 2002; Salazar, Fikes et al. 2003).

1.4.4.4 Recombinant vaccines

Viral infections can result in the presentation of viral peptides on the surface of infected cells via MHC class I and II molecules. This feature can be used to deliver TAAs into the antigen presenting pathway for immunisation (vectors). Adenoviruses are good vectors as they infect and activate APCs directly (Bonnet, Tartaglia et al. 2000). The use of Avipox virus decreases the production of neutralising antibodies against the viral particle and can express a transgene for 2-3 weeks before undergoing replication. Recombinant virus can also be designed to express adjuvant molecules (eg, B7.1). Vaccinia virus encoding E6 and E7 (Adams, Borysiewicz et al. 2001; Baldwin, van der Burg et al. 2003; Davidson, Boswell et al. 2003) or CEA (Conry, Khazaeli et al. 1999; Conry, Allen et al. 2000), peptides tyrosinase, MART1, and GPP100 with B7.1 and B7.2 (Zajac, Oertli et al. 2003), or mucin (Rochlitz, Figlin et al. 2003) has been tested on cervical or vulval carcinoma, and melanoma in phase I or I-II clinical trials. The avipox virus has been studied encoding CEA and B7.1 on colorectal cancer (Horig, Lee et al. 2000) and p53 on colorectal cancer (van der Burg, Menon et al. 2002) in phase I or I-II clinical trials. Adenovirus encoding GP100 or MART1 has been tested on melanoma patients (Rosenberg, Zhai et al. 1998).

Naked DNA vaccines consisting of DNA encoding a TAA, inserted into a bacterial plasmid under a strong promoter, can be expressed at high rates in eukaryotic cells and in large quantities (Kumar and Sercarz 1996). The plasmid can also be engineered to express adjuvants (eg, cytokines and costimulatory molecules) to increase the immune response against the TAA. DNA plasmids encoding CEA (104), or GP100 (105), or PSMA with or without B7.2 (107), or peptides $tyrosinase_{1-17,207-16}$ (106) have been tested on colorectal cancer, melanoma, prostate and melanoma, respectively, on phase I

clinical trials. Moreover, DNA vaccines contain nucleotide sequences such as oligo-CpG that can act as an adjuvant (Krieg 2002). This type of vaccine in the clinical setting is still at an early stage.

1.4.4.5 Anti-idiotype vaccines

Anti-idiotype vaccines are produced through immunisation with mouse antibodies specific to TAAs. The idiotype is recognised by the variable region of the induced autologous antibodies (Perelson 1989). The variable region of the induced antibody, also called anti-idiotype, should resemble the original TAA and can be used instead of the natural TAAs as tumour vaccines. The advantage is that it requires low amounts vaccine preparation, allows for vaccination against non-protein antigens that are difficult to clone (eg.carbohydrates) and is effective in breaking immune tolerance to TAAs (Bhattacharya-Chatterjee, Chatterjee et al. 2000). The use of humanised antibodies might avoid the generation of blocking antibodies against mouse immunoglobulin by the host (Losman, Qu et al. 1999). Some clinical benefit has been reported in the small number of vaccinated patients. Anti-idiotype monoclonal antibody mimicking CEA (Foon, John et al. 1997), CO17-1A (Samonigg, Wilders-Truschnig et al. 1999; Birebent, Koido et al. 2001), CD55 (Maxwell-Armstrong, Durrant et al. 2001), or SAF-m (Quan, Dean et al. 1997), ganglioside GD2 (Foon, Lutzky et al. 2000), ganglioside GM3 (Alfonso, Diaz et al. 2002), or CA125 (Wagner, Kohler et al. 2001) have been tested for colorectal cancer, melanoma in the case of ganglioside, and ovarian cancer, respectively, in clinical trials I or I-II.

1.4.4.6 Dendritic-cell vaccines

Danger signals such as tissue necrosis promote changes in DCs that enhance antigen presentation. The use of TAAs loaded on DCs requires the knowledge of the sequences of the relevant TAAs or epitopes and of the HLA type of the patient. Loading on DCs of tumour cell lysates or expressing the mRNA encoding the TAA in DCs could overcome this limitations (Nair, Morse et al. 2002). Hybrids of cancer cells and DCs with the characteristics of APCs such as the ability to process endogenous TAAs have been developed (Phan, Errington et al. 2003). Strong preclinical evidence suggests the use of DCs for vaccination as beneficial, however, a lack of an objective tumour response has

been observed in 12 of 35 trials. Melanoma studies on clinical trials I and II have been pursued with dendritic cells pulsed with MART1 (Wagner, Kohler et al. 2001), or MAGE3 (Thurner, Haendle et al. 1999), or the combination of MAGE1, MAGE3, MART1, GP100, and tyrosinase (Mackensen, Herbst et al. 2000), or autologous tumour (Nagayama, Sato et al. 2003), or shed peptides from autologous tumour (Smithers, O'Connell et al. 2003). A variety of other tumours have been tested in clinical trials with dentritic cell pulsed with TAAs. For a review see (Morse, Deng et al. 1999; Nair, Hull et al. 1999; Tjoa, Lodge et al. 1999; Brossart, Wirths et al. 2000; Lodge, Jones et al. 2000; Iwashita, Tahara et al. 2003; Su, Dannull et al. 2003). The labour-intensive and time-consuming methods of preparation for these vaccines is a major drawback. Phase III clinical trials in patients suffering from advanced prostate carcinoma are presently ongoing.

1.4.4.7 New targets

The ideal TAAs for use as a cancer vaccine would possess properties such as the ability for maximum immunostimulation and maximum tumour specificity. The use of xenogeneic homologues of TAAs have been shown to be able to break immunotolerance to weakly immunogenic self-antigens and to control tumour growth in humans (Fong, Brockstedt et al. 2001). Fusion proteins derived from chromosomal translocations that take place during cancer progression are good potential new cancer vaccine targets for two reasons; they are non-self antigens, therefore avoiding tolerance and they are tumour specific. Moreover, peptides from tumour antigens such as NY-ESO-1 known to contain overlapping $CD8^+$ and $CD4^+$ T cell epitopes that are able to bind to MHC class I and II molecules have been synthesised in order to achieve activation of helper T lymphocytes and CTL (Zeng, Li et al. 2002).

Most of the TAAs employed so far (eg. GP100, melanoma antigen family, and CEA) are not necessary for the survival of malignant cells, therefore their use as vaccines tends to select for tumour variants that downregulate their expression (Khong and Restifo 2002). The antiapoptotic protein survivin and the ribonucleoprotein telomerase are antigens that cancer cells need for survival, and vaccines developed against these proteins have been tested in pre-clinical and clinical studies. Similarly, immunisation against antigens expressed by tumour associated-endothelial cells in order to block blood supply and induce tumour necrosis may provide a more rational approach

to generating a universal vaccine against tumour cells. (Niethammer, Xiang et al. 2002).

1.5 HEAT SHOCK PROTEINS

1.5.1 Classification

Heat shock proteins were first identified as a set of proteins induced in *Drosophila* by elevated temperatures (Boczkowski, Nair et al. 1996). They are ubiquitous in nature, and show a high degree of sequence conservation between species. They are traditionally classified into families based on their apparent molecular weight for example Hsp110, Hsp90, Hsp70, Hsp60. A second group consist of proteins that are induced by glucose deprivation and include glucose regulated proteins (grp) with molecular weights of 34, 47, 56, 75, 78, 94, and 174 kDa, and a third group consists of a heterogenous class of low (12 to 43kDa) molecular mass Hsps. Hsps carry out three essential biological roles in the cell; chaperone activity, which prevents the misaggregation of denatured proteins and assists folding of nascent and denatured proteins into their native conformations or into their multimeric form. This function includes chaperone transport of other proteins between intracellular compartments, and facilitation of changes into specific conformations which is exploited by a variety of physiological processes regulating cell function, including the control of the cell cycle, the processing of steroid and Vitamin D receptors, and antigen presentation. The archetypal Hsp with chaperone activity are Hsp70, Hsp90 and Hsp60. A second essential activity of Hsps is the regulation of cellular redox state, best illustrated by Hsp32, known as heme oxygenase-1. Finally, the third biochemical role of hsp is the regulation of protein turnover: the small molecular weight protein, ubiquitin, while expressed in unstressed cells is up-regulated by heat shock; this protein also serves as a molecular tag to mark proteins for degradation by proteosomes. Several Hsps posses ATPase activity which is essential for their cellular functions.

1.5.2 Hsp70 and Hsp96. Immunological functions

1.5.2.1 Endoplasmin, or GP96

Also known as grp100, grp94 or Erp99 is a member of the Hsp90 family. It contains two ATP-binding cassettes and displays magnesium-dependent ATPase activity (Nestle, Alijagic et al. 1998). This protein also has a peptide-binding domain, but peptide-binding is not ATP-dependent. There is no membrane anchor sequence in gp96 however the protein has been found on the plasma membrane (Barratt-Boyes, Kao et al. 1998). Gp96 is found in the ER, where is known to be involved in the assembly of immunoglobulins (Igs) and MHC class II molecules together with grp78, an Ig heavy chain binding protein (BiP) (Przepiorka, Srivastava 1998). In addition, gp96 relays peptides from the transporter associated with antigen processing (TAP) complex to MHC class I molecules; TAP-independent peptides also bind to gp96 (Janeway 1992; Ibrahim, Chain et al. 1995), and immunodominant peptides have been identified in gp96-peptide complexes (HspPC-96) (Matzinger 1994). Thus, HspPC-96 from tumour cells is likely to include a diverse array of peptides as well as larger polypeptides that serve as tumour antigens.

1.5.2.2 Heat shock protein-70 (Hsp70)

Hsp70 family members are most abundant in the cytoplasmic compartment of a cell (Medzhitov and Janeway 1997). The exception to this is grp75, a mitochondrial Hsp that participates in transporting functional mitochondrial proteins and transferring proteins and peptides for proteolysis (Fearon 1997). Hsp70 proteins have an ATP-binding cassette at the N-terminus and display ATPase activity. There is a peptide-binding domain at the C-terminus, and peptide binding is ATP dependent. The HSP70 peptide-binding domain is large and accommodates longer peptides than does gp96. Hsp70 family members play a major role in the protection and folding of nascent proteins through binding to hydrophobic residues as the protein comes off the ribosome (Reid 1998).

In the cytoplasm, Hsp70 is thought to process and shuttle peptides to TAP for subsequent transfer to the ER, although Hsp70 might shuttle TAP-independent peptides as well (Albert, Sauter et al. 1998; Colaco 1998). Two Hsp70 proteins have also been

found on the cytoplasmic membrane: Hsp72, the inducible family member, is a target for lymphokine-activated killer (LAK) cells, and grp75 is a target for γδ-receptor T cells (Morimoto, Kline et al. 1997). It remains unclear whether, in this capacity, Hsp70 presents antigen in an MHC-nonrestricted fashion on the plasma membrane. Additionally, Hsp70 can serve as the antigen itself (Tamura, Peng et al. 1997).

1.5.3 Role in danger theory

The danger theory suggests that the mechanism by which a cell dies will dictate whether an immune response is initiated (Burnet 1970; Matzinger 1994; Ridge, Fuchs et al. 1996; Rowse, Tempero et al. 1998). Necrotic cell death and lysis often occurs during viral or bacterial infection, tissue damage and acute inflammation. The upregulation of Hsp in response to cell stress was initially described as a means of cytoprotection, where unfolded cell proteins are bound and protected from stress-induced damage (Morimoto and Santoro 1998). This response to stress is used by a wide range of microorganisms, and it has been postulated that a response to released Hsp (constitutive or induced) may be an evolutionary remnant from primitive ancestral immunity (Srivastava, Menoret et al. 1998). It has been shown that when certain tumour cells were killed by a non-apoptotic (necrotic) mechanism (mediated by thymidine kinase and ganciclovir) they expressed raised levels of inducible Hsp70 and other Hsps (Melcher, Todryk et al. 1998). Such killing *in vivo* gave rise to immune protection against subsequent tumour challenge. It has also been found that exposure of DCs to necrotic but not apoptotic tumour cells resulted in the upregulation of maturation-specific markers, costimulatory molecules, and the capacity to induce antigen-specific CD4 and CD8 T cells (Sauter, Albert et al. 2000).

Signals such as cytokines or Hsps expressed by cells undergoing damage and necrotic death (Gallucci, Lolkema et al. 1999; Gallucci and Matzinger 2001) as it is the case of tumour cells dying by hypoxia or chemotherapy treatments activate DCs. The danger model (Matzinger 1994) is based on the premise that the immune system has evolved to detect danger by employing professional APCs as sentinels of tissue distress. Antigen-specific T cell and B cell responses are initiated by DCs that capture antigens that are secreted or shed by tumour cells or after cell lysis. The activated antigen-specific CD8 T cells differentiate into CTLs and lyse the tumour cells. (Smyth, Godfrey et al. 2001). It is tempting to suggest that viral-, stress- or inflammation-mediated Hsp

expression may have a potentiating role in self antigen transfer to APCs and presentation to CD8$^+$ T cells (cross-priming) during autoimmunity, graft rejection or necrosis

1.5.4 Hsp96 and Hsp70 as targets for cancer therapy

An interesting novel experimental vaccine approach exploits the role of Hsps in immune responses. Hsp have been shown to have an intimate association with the immune system. In mice, the inducible mouse Hsp70 is encoded on Chromosome 17, in the H-2 histocompatibility locus whereas the constitutive Hsc70 is on the Chromosome 9. By analogy with the localisation of other immunologically important genes in the H-2 locus (TAP1, TAP, LMP2, LMP7, TNF alpha, TNF betha, MHC-I, MCH-II and MHC-III), it is tempting to suggest that the inducible Hsp70 genes co segregates with immunological functions (Botzler, Li et al. 1998; Jolly and Morimoto 2000 Multhoff 2002). Hsp are also known to be the major antigens recognised by the immune system in a number of pathologic states, including bacterial infection, autoimmune diseases, inflammation, neurodegenerative diseases and cancer (Srivastava 2002; van Eden, van der Zee et al. 2005). Cytosolic chaperones can be exposed on the cell surface by stress-induced transport mechanisms to signal damage of the cell and produce removal by the immune system. The detection of a subset of the Hsp on the surface of certain tumour cells and their capacity to activate a immune response that results in elimination by the immune system, has brought the investigation of the use of Hsp as tumour vaccines. NK cells (Srivastava, DeLeo et al. 1986; Palladino, Srivastava et al. 1987; Udono and Srivastava 1994; Botzler, Li et al. 1998; Multhoff 2002) and γδ T cells can directly recognise and respond to various stress proteins expressed by tumour cells (Mahvi, Carper et al. 1993; Laad, Thomas et al. 1999; Thomas, Samant et al. 2000).

In 1980, Dr P. Srivastava began a series of experiments on mice immunised with whole-cell lysates from a METH-A induced tumours from a syngenic host. Prior immunisation with these whole cell promoted tumour rejection upon subsequent challenge with METH-A induced tumours. Further fractionation of the lysates eventually identified Hsps (notably Hsp70 and Hsp96) as the agents responsible for the antitumour protection (Srivastava, Menoret et al. 1998). The underlying principle of this therapy is that one of the physiological activities of heat-shock proteins is to act as intracellular chaperones for proteosome-processed unstable mutated proteins. Therefore

the Hsp-peptide complexes (referred to as Hsp-PCs), contain a wide reportoire of potential antigenic peptides. Subsequent studies indicated that it is the associated peptides that are the molecules responsible for the generation of antitumour protection (Peng, Menoret et al. 1997). Immunogenic peptides isolated from Hsp preparations, when complexed with another carrier, the mouse serum albumin, were not able to induce immunogenic peptide-specific CD8$^+$ T lymphocytes *in vivo*, suggesting that Hsp can be considered as chaperone molecules with adjuvant properties. Further work has shown receptors on the surface of APCs with strong binding affinity for Hsps (CD91) (Binder, Han et al. 2000), thus, Hsp-PCs can be internalised into the antigen processing pathway by receptor mediated endocytosis, where the peptides are stripped off the Hsp, mounted onto MHC class I and II, and then, represented on the APCs. The APC then home to the lymphatic organs where they prime specifically CD8$^+$ T lymphocytes responsible for the rejection of the tumour. Hsp interaction with APCs also leads to the translocation of NFκB into the nucleus of the APC, followed by secretion of a number of inflammatory cytokines and expression of antigen-presenting costimulatory molecules raising not only adaptive but also innate immune responses (Srivastava and Amato 2001).

1.5.5 Heat-shock protein vaccines

Hsp-PCs can be isolated from tumour cells and used as polyvalent, autologous cancer-vaccine preparations with undefined TAAs, thus removing the need to identify the epitopes of the TAAs that are recognised by the CTLs. Encouraging results from pilot studies in humans have been reported. The use of Hsp-PC as prophylactic or therapeutic vaccines was shown to be successful across different species (Xenopus, mice and rats), different tumour types (fibrosarcomas, thymomas, prostate cancers, lung carcinomas, melanomas and colonic cancers), and also different means of generation (UV-induced tumours and spontaneous tumours) (Menoret and Chandawarkar 1998; Srivastava, Menoret et al. 1998). Antigenics, a company co-funded in 1994 by Dr Srivastava, makes personalised cancer vaccines by extracting Hsps from resected tumours. The advantage of this type of vaccine is there is no need of previous knowledge of tumour antigens, however the current drawback is that it is only applicable to resectable tumours as 4g tissue is required for the Hsp-PC preparation, and also is a patient-specific vaccine which raises the cost of it.

Hsp-based vaccines have been studied in early phase clinical trials, using Hsp glycoprotein 96, for various types of malignancies including melanoma, renal carcinoma, gastric cancer, pancreatic cancer, low-grade lymphoma, colorectal cancer and chronic myelogenous leukaemia (CML), in the latest case Hsp70 instead of gp96 was employed (http://www.antigenics.com/trials/results/). All showed minimal toxicity and potential efficacy. In phase I pilot study, of 28 patients with metastatic melanoma, two had a complete response for 559 and 703 days and three had stable disease for 153, 191 and 272, respectively. Specific T cell response was detected in 11 of 28 patients. No treatment-related toxicity was observed. In phase II trials, of 39 patients with metastatic melanoma, two responded with complete disease regression, three with disease stabilisation of more than five months, and four remained disease-free for up to 22 months. Approximately half of the patients demonstrated a significant increase in melanoma-specific T cells and no toxicity associated with the treatment was observed (Belli, Testori et al. 2002). In phase II trials from 61 patients with kidney cancer who received at least one dose of vaccine, 21 patients responded clinically. Patients whose cancers progressed during vaccination also received immunotherapy with interleukin 2. Median progression-free survival was 18 weeks, and 25 weeks for patients who also received IL-2 (Assikis, Daliani et al. 2003). In a phase I pilot study, 15 patients with gastric cancer underwent surgery prior to vaccination. The mean disease-free survival rate is 7+ months and the mean overall survival rate 16+ months. Tumour-specific immune responses were observed in 73% of patients and no toxicity associated with the vaccine was observed (Hertkorn, Lehr et al. 2002). In phase I pilot study with 10 patients with nonmetastatic pancreatic cancer, the median overall survival was 2.5 years. One patient was disease-free after five years and two other patients were disease free after 2.1 years after treatment. No toxicity associated with the treatment was observed (Maki, Lewis et al. 2003). A phase I pilot study with 29 liver metastatic colorectal cancer patients after radical resection, elicited a significant increase in CD8+T cell response against colon cancer. Occurrence of immune response led to better tumour-free survival. As expected, patients with good prognosis had a significantly better clinical outcome than those with poor prognosis. No relevant toxicity was observed (Mazzaferro, Coppa et al. 2003). A phase I pilot study with 8 CML patients was performed in combination with the adjuvant Gleevec (imatinib mesylate, Novartis). In the study, responses were observed in seven of the eight chronic phase CML patients. Vaccination was associated with an increased production of interferon gamma (indicator

of T-cell activation). No serious toxicity was observed (Li, Quiao et al. 2003). Phase III studies for melanoma and renal cell carcinoma are ongoing.

1.6 PREFACE TO THIS THESIS

In order to explore the nature of the immune responses to antigens associated with Hsps and to overcome some of the drawbacks associated with using TAAs from tumour cells, a series of experiments were carried out to generate synthetic peptides mimics of Hsp70-PCs (Arnaiz, et al, submitted for publication). The underlying principle used here is very similar to the approach used to generate anti-idiotype vaccines in which a mirror-image antibody can be made for any given antigen. In this particular case, a potential mirror-image peptide (mimic) was produced for a particular antigen in a pool of Hsp70-PCs using a two step-reverse phage bio-panning approach. The use of mimics of Hsp70-PCs as a source of antigens (mimotopes) removes the problems associated with self recognition and provides a means for designing peptides with improved immunostimulatory activity. Additionally, the use of mimics would solve the limitations in the availability of resectable tumours and requires no prior knowledge of the peptides associated with Hsps. The hypothesis put forward by Drs. U. Bond and T.C. James is that phage display bio-panning in which Hsp70-PCs from a tumour source are used as a bait, could be first used to identify "Recogniser" molecules. By removing phage that recognise Hsp70, these Recognisers would have the property of binding to or recognising the "PEPTIDE-COMPLEX" portion of the Hsp70-PCs which is the portion of Hsp70-PC that is responsible for antigenic activity. The identified recogniser phage could then act as bait in a reverse phage bio-panning to identify potential "Mimic" peptides. In principle, the mimic peptides should possess properties of the original bait material, Hsp70-PCs, such as the ability to stimulate T-cell responses against a syngenic tumour.

Below, is a brief description of the methodology used to isolate Recogniser and Mimic peptides of Hsp70-PCs and a summary of the types of peptides that were obtained from phage display reverse bio-panning experiment. The exact methodology and results of the bio-panning experiment are outlined in the paper entitled "A Structural Mimic of Heat Shock Protein 70-Peptide Complexes from Breast Tumour Cells can Prime T Cells to Respond to Breast Cell Antigens', Blanca Arnaiz, Laura Madrigal Estebas, Derek G. Doherty, Tharappel C. James and Ursula Bond (submitted for publication).

1.6.1 Generation of recogniser and mimic peptides of Hsp70-PC

Protein fractions enriched in Hsp70-PC were isolated from MDA-MB-231 cells using ADP-agarose affinity chromatography as previously described (Srivastava and Amato 2001). The Hsp70-PC fraction was used as a bait to biopan a random peptide M13 display library to isolate recogniser phage as outlined in Figure 1.1. Four rounds of biopanning were performed. Additionally, a round of subtraction biopanning, using Hsp70 depleted of associated peptides as bait was performed, to exclude those phages that recognise just the Hsp70 portion of the Hsp70-PCs.

Three separate biopanning experiments were performed using Hsp70-PCs, from the human breast ductal tumour cell line MDA-MB-231, as a bait. Approximately 400-1000 phages were retained after the fourth rounds of panning. The phage particles enriched after the fourth round were plated at low density to allow isolation of single phage clones. A total of twenty four phage clones were selected at random for further analysis. DNA was isolated from the phages and was sequenced in order to identify the peptide displayed by each particular phage clone. Each peptide sequence was named according to the first three amino acid in the sequence. As shown in Table 1.1, a wide variety of peptide sequences were identified in each biopanning experiment, some of which were common in two of the three biopannings. In one case, the phage displaying the TMG peptide was selected in all three biopanning (Table 1.1).

To identify phage that can interact with the recogniser peptides, and thus, represent potential mimics of the original Hsp70-PCs, a reverse biopanning was carried out using synthetic recogniser peptides as bait. Peptides containing the sequences represented by the NNY, IER and TMG phage were synthesised and used as the bait in a 'reverse' biopanning experiment. Following four rounds of biopannings, a selection of phage clones were sequenced for each peptide bait. The peptide sequences in the enriched phages are shown in Table 1.2. Both IER and NNY peptides selected a number of phage with different peptide sequences within. Theoretically, these phages should have sequences that 'mimic' the bait used to identify the recogniser peptides. Both IER and NNY peptides selected a phage displaying the peptide SVS in a high proportion. However, subsequent literature searches revealed that this peptide has previously been identified in a number of biopanning experiments using unrelated baits (Wu and Lin 2001; Hou, Dove et al. 2004). Despite the number of other unique mimic peptides

12-MER PEPTIDE
M13 PHAGE LIBRARY

BAIT

Phage elution and
amplification

4 rounds

RECOGNISERS

Peptide sequencing, synthesization
and immobilization.

4 rounds

MIMIC

Phage elution
and amplification

RECOGNISER
PEPTIDE

Peptide sequencing and
synthesization.

MIMIC PEPTIDES
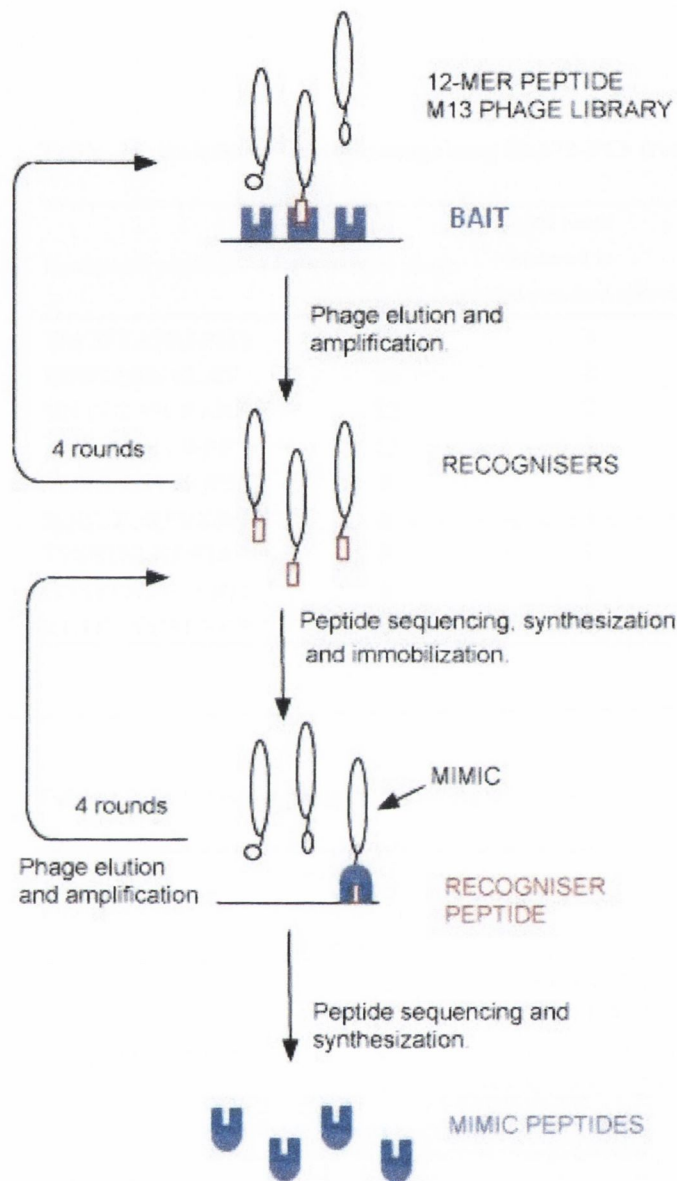
Fig 1.1 Outline of the bio-panning procedure

A 12-mer M13 library was bio-panned using Hsp70PC/HSP70 as bait. The isolated phage were sequenced to identify the displayed Hsp70-PC-"Recogniser" peptides. Peptides containing the amino acids displayed by the Recogniser phage were synthesised and used in a reverse bio-panning to the isolate mimic peptides of Hsp70-PCs.

selected by the NNY and IER peptides, the selection of the SVS peptide casts doubt on the authenticity of these two recognisers. It is possible that SVS peptide recognises the peptide bond which is common among all baits. Therefore, these peptides were not examined further. Unlike the NNY and IER peptides, the TMG peptide enriched a single phage displaying the sequence DSP (Table 1.2). Due to the high specificity of the DSP peptide, TMG/DSP 'recogniser/mimic' pair was chosen for further analysis.

1.6.2 Objectives of study

The aim of this study was to examine the biological properties of the recogniser and mimic peptides of Hsp70-PCs in an effort to validate the use of the two step-reverse bio-panning approach as a method of isolating mimic peptides that can act as antigens to stimulate immune responses against tumour cells.

The prime objectives of this study were as follows:

1)   To examine the ability of DSP to activate human dendritic cells to produce IL-12, a cytokine of great importance in the regulation of antitumour immunity.

2)   To generate an *in vitro* system to test the T-cell stimulatory activity of mimic peptides by monitoring the production of IFN-γ.

3)   To determine if mimic peptides resemble antigens present in MDA-MB-231 tumour cell extracts and specifically if they resemble antigens associated with Hsp70 in these cells.

4)   To examine the capacity of recogniser and mimic peptides to selectively bind to different types of normal and tumour cell lines and human breast ductal normal and tumour tissue sections.

5)   To identify the binding partners of DSP and TMG in the breast tumour cell line MDA-MB-231.

It is hoped that the information resulting from this study will enable advances in the field of novel approaches for the identification of molecules for their use in tumour treatment.

MATERIALS AND METHODS

Germany), and were cultured in 8ml supplemented RPMI medium containing hrIL-4 and hrGM-CSF (see section 2.1.7).

2.1.3.1 Monocyte enrichment by plastic adherence method

Approximately 6×10^7 PBMCs were suspended in 8ml complete RPMI medium, and incubated in two 25cm^2-tissue (BD Bioscience,UK) culture (TC) flask at 37°C and $5\%CO_2$ in a water jacketed incubator. After 2hr incubation, the medium containing non-adherent cells was decanted, 4ml RPMI medium was added to each flask and they were shaken. The now floating adherent population was centrifuged for 1.550 g for 7min and resuspended in 500µl supplemented RPMI medium for counting using the EB/AO staining method described in section 2.1.4.

2.1.3.2 Monocyte enrichment by the CD14 retention method (CD14 MicroBeads, Miltenyi Biotech, UK)

Approximately 6×10^7 or 7×10^8 PBMCs were suspended in either 60µl (10^9 cell/ml) or 7ml (10^8 cell/ml) of PBS containing 0.5% BSA, 2mM EDTA PBS (buffer A) and incubated with 20µl or 100µl respectively of monoclonal mouse anti-human CD14 antibody coated magnetic microbeads for 15min at 6-12°C. Then cells were washed twice with 500µl or 25ml buffer A and centrifuged at 300g or 1,550g for 10min with the brake off. Then resuspended in 500µl or 5ml buffer A. The microbeads-labeled CD14$^+$ PBMCs were applied through a LS$^+$/VS$^+$ column (Miltenyi Biotech, Bergisch Gladbach, Germany) which was placed in a magnetic field. The columns were washed once with 3ml buffer A prior to the application of the cells. The flow-through was collected as the negative fraction or CD14$^-$ PBMCs. The column was then washed three times with 3ml buffer A and the washes mixed with the flow-through. Then the column was removed from the magnetic field, 5ml buffer A was flushed through with the aid of a plunger and the eluted cells collected as the positive fraction or CD14$^+$ PBMCs. Both positive and negative fractions were counted using the EB/AO staining method described in section 2.1.4. The negative fraction was stored in DMSO:FCS (10:90 v/v) at -80° C for subsequent use.

2.1.3.3 Monocyte enrichment by the CD14 depletion method (Monocyte isolation kit, Miltenyi Biotech)

Approximately 6×10^7 PBMCs were resuspended in 60μl buffer A, and incubated with 20μl FcR blocking reagent containing human Ig and 20μl MACS anti-hapten microbeads cocktail containing monoclonal hapten-conjugated antibodies specific for CD3 (mouse IgG2a), CD7 (mouse IgG2a), CD19 (mouse IgG1), CD45RA (mouse IgG1), CD56 (mouse IgG1) and anti-IgE (mouse IgG2a) antibodies for 15min at 6-12°C. The microbeads were washed twice with 500μl buffer A, centrifuged at 300g for 10min (brake off) and, then resuspended in 500μl buffer A. The cells were applied to LS$^+$/VS$^+$ column (Miltenyi Biotech, Bergisch Gladbach, Germany) which was placed in a magnetic field. The columns were washed once with 3ml buffer A prior to the application of the cells. The flow-through was collected as the positive fraction or CD14$^+$ PBMCs, the column was washed three times with 3ml buffer A and the washes mixed with the flow-through. Then the column was removed from the magnetic field, 5ml buffer A was flushed through with the aid of a plunger and the eluted cells collected as the negative fraction or CD14$^-$ PBMCs. Both positive and negative fractions were counted using the EB/AO staining method described in section 2.1.4. The negative fraction was stored in DMSO:FCS (10:90 v/v) at –80° C for subsequent use.

2.1.4 Cell staining by the Ethidium Bromide/Acridine Orange method

Cells were diluted into 0.02% (v:v) ethanol, 1% (w:v) ethidium bromide, 0.3% (w:v) acridine orange PBS (ethidium bromide/acridine orange or EB/AO) and counted on a haemocytometer slide using 10x magnification lenses in a fluorescence microscope that covers the 450-515nm emission light spectrum. The toxicity of the ethidium bromide kills the cells rapidly, therefore the cells were counted immediately after addition of EB/AO. Live cells fluoresce green (with acridine orange) and dead cells fluoresce orange (with ethidium bromide). The viable cell counts were calculated from the counts obtained in a field of view of dimensions 1mm x 1mm, in which the distance between the haemocytometer slide and coverslip is 0.1mm (ie 0.1mm^3).

2.1.5 Flow cytometric staining

The cell suspension is aspirated by the Becton Dickinson Flow Cytometer and forced to flow in single file past two lasers. The fluorochrome conjugated to the antibodies is excited and when it returns to their lower energy state it emits photons of light (fluorescence) of a particular wavelength (colour) that is focused to a photomultiplier detector that converts optical signals into electronic signals and can be recorded and analysed on a computer. Six detectors measure six distinct characteristics of each cell, such as size (forward scatter or FSC), cytoplasmic granularity (side scatter or SSC) and the intensities of four fluorescent dyes which light emission happens at four different wavelengths. The six different characteristics of each cell can be graphically presented by the computer as a dot plot, in which each cell appears as a dot on a plot of the magnitude of one parameter plotted against the magnitude of another parameter.

Approximately 10^5 cells were centrifuged at 1,550rpm for 10min and re-suspended in 50µl 0.07% BSA, 0.02% sodium azide, PBS (PBA buffer). Cells were then incubated with 5µl fluorochrome-conjugated monoclonal mouse IgG1 specific for human cell surface markers. In replica tubes they are incubated with 5µl of fluorochrome-conjugated IgG1 without a particular specificity of recognition, to determine the background fluorescence (isotype control). After 15min incubation in the dark, 2ml PBA buffer was added, the contents mixed and centrifuged at 1,550rpm for 10min. The supernatant was discarded and the pellets re-suspended in 2ml PBA buffer for analysis by flow cytometry (Becton Dickinson Flow Cytometer Systems, San Diego). All the antibodies were obtained from Becton-Dickinson Flow Cytometry Systems (Oxford,UK).

Flow cytometric data was analyzed using CellQuest software (Becton Dickinson Flow Cytometry Systems; San Diego). Electronic gating was used to select the monocytes on the basis of forward and side scatter parameters (size and granularity, respectively). The isotype control samples were used to set the background threshold parameters for each fluorochrome in the CellQuest software.

2.1.6 Cryopreservation of PBMCs or CD14⁻ PBMCs

PBMCs or CD14⁻ PBMCs isolated from approximately 250ml blood and stored at a cell density of 10^6 cells/ml in 5ml DMSO:FCS (10:90 v/v) at $-80°$ C were defrosted as

follows: cells were defrosted rapidly by holding vials under hot tap water and suspended in a large volume (300ml) of RPMI-1460 medium. Cells were washed twice by centrifugation at 1,550g for 10min, resuspended in the appropriated buffer and counted using the EB/AO staining method described in section 2.1.4.

2.1.7 Generation of immature dendritic cells

The generation of immature dendritic cells was performed using a modification of published procedures (Zarling, Johnson et al. 1999). Monocytes were cultured for 6 days in supplemented RPMI medium (see above) containing 60 ng/mL human recombinant GM-CSF (R&D Systems, UK) and 150 ng/mL human recombinant IL-4 (R&D Systems, UK) at 37°C and 5% CO_2 in a water jacketed incubator. Medium and cytokines were replaced every 2 days.

2.1.8 Cell extracts and Purification of Hsp70 and Hsp70-PC

To prepare tumour cell extracts, the cells were harvested by trypsinisation, washed twice in ice-cold PBS and the cell pellets resuspended in 1ml PBS. Cells were lysed by 5 freeze/thaw cycles followed by sonication (15 sec. 3x energy setting 5; Sanyo Probe) and the removal of the insoluble material by centrifugation (20,500g for 30 min). Supernatants were aliquoted and stored at –20°C.

The Hsp70 and Hsp70-PC were purified from MDA-MB-231 cells as follows. Approximately 10^8 MDA-MB-231 cells were harvested using a cell scraper to detach cells from the TC-flask surface. Following two cold PBS washes, the cells were homogenised in 10 mL hypotonic lysis buffer (Peng, Menoret et al. 1997) in an all glass homogeniser. The homogenate was then sonicated (as described above) followed by 3 rounds of freeze/thaw cycles. The insoluble material was removed by centrifugation (20,500g for 30 min). The cleared supernatant was passed through a 5 micron cellulose acetate filter. Hsp70 and Hsp70-PC in the filtrate were affinity purified on ATP- or ADP-agarose columns, respectively, using either 3 mM ATP (for Hsp70) or ADP (for Hsp70-PC) in 10mM Hepes (n-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid) elution buffer, pH 7 containing 3mM $MgCl_2$, 20mM NaCl and 15 mM 2-mercaptoethanol (Peng, Menoret et al. 1997). Both eluates were concentrated through ultra-filtration using 10,000 MW cut off for Hsp70 and 3,000 MW cut off for Hsp70-

Following the incubation, 200 ng/mL biotinylated anti-hrIFN-γ antibody was added and the incubation continued for 2 hours (DuoSet human IFN-gamma, R&D Systems, cat No DY285). Levels of IFN-γ were detected using streptavidin-horseradish peroxidase (SA-HRP, 1:2,500) in PBS and its substrate p-nitrophenyl phosphate according to manufacturer's instructions. Absorbance readings were taken at 405 nm.

To detect IL-12p40 production by iDCs, a similar protocol as for IFN-γ was used (DuoSet human IL-12p40, R&D Systems, cat No DY1240). The concentrations of the different antibodies used are 4 μg/mL anti-hrIL-12 and 175 ng/mL biotinylated anti-hrIL-12 antibodies. All the antibodies and standards used were purchased from R&D Systems.

2.1.11 M13 Phage ELISA

Biotinylated DSP or TMG peptides (200 pmoles) were immobilized onto 200 μg streptavidin-coated magnetic beads (Dynal Co, Norway) according to manufacturer instructions. The beads were retained with a magnet and excess of peptide washed with PBS. Following blocking with 0.5% BSA, 0.1 mM D-biotin in PBS, the beads were incubated for three hours with M13 phage clones (10^{11} pfu) displaying either TMG or DSP peptide. Unbound phage was removed by washing with PBS containing 0.05% TWEEN-20. 100 μL of HRP conjugated anti-M13 antibody (anti-M13-HRP, Roche, UK; 1:2,500) in PBS containing 0.5% BSA was added and incubated for two hours. After washing the unbound antibody with excess PBS, the beads were transferred to wells of a microtiter plate and the presence of the peptide bound to the beads was measured by absorbance at 405 nm using 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) as a substrate in the presence of H_2O_2. As controls, beads with no immobilised peptide were included.

2.2 CYTOCHEMISTRY

2.2.1 Cell lines

The human breast cancer cell line MDA-MB-231 stock was a generous gift from Dr. Boucher-Hayes, Beaumont Hospital, Dublin, the human non-small cell lung carcinoma cell line NCI-H358 was kindly donated by Dr. G. Atkins, Mircrobiology Dept. Trinity

College, Dublin, and the human breast cancer cell line MCF-7 was a gift from the Biochemistry Dept. Trinity College, Dublin. The cell lines were grown in RPMI-1640 (HEPES modification) supplemented with 10% foetal calf serum (FCS) heat-inactivated (Sigma, UK). The human normal breast cell line MCF-12A was purchased from ATTC-LGC (Teddington, U.K) and grown in supplemented 1:1 mixture of Dulbecco´s modified Eagle's medium (DMEM) and Ham's F12 medium as recommended by the supplier. The human prostate normal cell line BPH-1, immortalised with SV-40 large T-antigen, and the human tumour prostate cell line PC-3 were a generous gift from Dr. D. Zisterer, Biochemistry Dept. Trinity College, Dublin, and was grown in a 1:1 mixture of DMEM and Ham's F12 medium supplemented with 10% FCS (Sigma,UK). HPV-G keratinocyte cells were obtained as a kind gift from C. Mothersill, Dublin Institute of Technology, Ireland. They are immortalised but not transformed human keratinocytes derived from foreskin (Pirisi, Creek et al. 1988). They were grown in 1:1 mixture of Dubach's modified Eagle's medium and Ham's F12 medium supplemented with 10% FCS (Sigma, UK) heat-inactivated and hydrocortisone ($1\mu g/ml$, 95% solution). All media also contained 2 mM L-glutamine, 1x antibiotic/antimycotic solution ($100\mu g/ml$ gentamicin, 100U/ml penicillin, $100\mu g/ml$ streptomycin, 250 ng/ml amphotericin B) and 100 U/mL nystatin suspension (all Sigma, UK), and cells were maintained at $37^{\circ}C$ in a humidified atmosphere of 5% CO_2, in $75cm^2$- or $175cm^2$ cell culture flasks (Falcon, UK).

Cells were typically sub-cultured at a 1:3 ratio as follows: sub-confluent monolayers were washed once with PBS and incubated with 0.25% (w/v) trypsin, 0.03% (w/v) EDTA at $37^{\circ}C$ until detachment was evident (5-15 minutes) at which point complete medium was added to terminate trypsination. Cells were then pelleted by centrifugation at 2,000 g for 5 minutes at $4^{\circ}C$. The pellet was resuspended in the adequate medium and 1/3 delivered back into fresh cell culture flasks (Falcon, UK).

2.2.2 Trypan blue exclusion assay

Cells were enumerated before seeding flat bottom 96-multiwell (Nunc, UK) plates or chamber-glass slides (Falcon,USA) as follows; sub-confluent monolayers were washed once with PBS and incubated with 0.25% (w/v) trypsin,0.03%(w/v) EDTA at $37^{\circ}C$ until detachment was evident (5-15 minutes) at which point medium was added to terminate trypsination. Cells were then pelleted and resuspended in complete media. An

aliquot of the cell suspension was diluted 1/4 into trypan blue solution (Sigma, UK) and viable cells counted on a haemocytometer slide. Trypan blue is excluded from live cells which are seen as refulgent spheres under phase 1 setting and 100x magnification lenses in a Nikon ATC-1 version2 microscope. Dead cells are stained blue with the dye.

2.2.3 Binding of DSP and TMG Peptides to Human Tumour Cells

NCI-H358, PC3, BPH-1, Keratinocytes, MCF-7, MDA-MB- 231 and MCF-12A cells were seeded at three different concentrations 10^4, $5x10^4$ and $2x10^5$ cell/ml (100µl) into a flat bottom 96-multiwell (Nunc, UK) plate in triplicates and 500µl of $4-8x10^4$ cells/ml were seeded in duplicates into each of the eight chamber-glass slide (Falcon,USA). After 48 h incubation at $37^{\circ}C$, in a humidified atmosphere of 5% CO_2, cells reached 10-25%, 50-80% and 90-100% confluent, respectively. Then they were fixed with paraformaldehyde (3.7%) in PBS (137 mM NaCl, 2.7 mM KCl and 10 mM phosphate buffer solution, pH 7.4) washed three times with PBS, blocked with 1% casein, 10% H_2O_2 in PBS for 45 minutes and incubated with different concentrations of biotinylated TMG, DSP and WHK peptides in a PBS solution containing 1% casein (100µl) for two hours. The wells were then washed six times with PBS, fixed with paraformaldehyde (3.7%) PBS, and again washed six times with PBS. Washes were performed filling the wells or chambers gently with PBS and tapping the liquid off onto tissue paper. From this point on two different protocols were followed for two types of detection systems.

2.2.3.1 Colorimetric detection

Peptide binding was detected by incubation with 100 µl of a solution containing 1% casein, PBS and ExtrAvidin conjugated with horseradish peroxidase (HRP) (Sigma, UK) (1:1000). After 1 hour incubation at room temperature and following three washes with PBS, 100 µl of a solution containing 50mM Tris-HCl pH 7.4, 0.15M NaCl, 1mg/ml 3,3'-diaminobenzidine (DAB) and 0.03% H_2O_2 was added to each well. The reaction was stopped by adding a drop of glycerol after 10-30 minutes. Plates were examined by light inverted microscopy using a Nikon eclipse TE300 light inverted microscope and 200 x magnification lenses.

2.2.3.2 Fluorescence detection

Peptide binding was also detected by incubation with 100µl of a solution containing Streptavidin-Cy3 (Sigma, UK) (1:5000), 0.5M NaCl and 10mM Hepes for 1 hour at room temperature. The slides were separated from the plastic chambers attached to them following manufacturer instructions (Falcon, USA). After overnight incubation of the slides in a solution containing 10mM Hepes, 0.5M NaCl to reduce non-specific binding of Streptavidin-Cy3, the slides were washed in PBS once and then nucleus were stained by incubation with PBS containing 5ng/ml 4´,6-diamidino-2-phenylindole (DAPI) (Sigma,UK) for 15 min, washed and mounted with VECTASHIELD Mounting Medium (Vector, USA). Slides were examined using a Nikon eclipse E400 microscope and appropriate filters (DAPI; DAPI filter, excitation 340-380nm, Cy-3; G.2A filter, excitation 510-560 nm).

2.2.4 Validation of the binding of the peptides to the cell lines

MDA-MB- 231 was seeded at $5x10^4$ cell/ml (100µl) into a flat bottom 96-multiwell (Nunc, UK) and treated as described in the sections 2.2.3 and 2.2.3.1 for the colourimetric detection with the following modifications.

2.2.4.1 Effect of biotinylated peptide concentration

Cells were treated in triplicates with 130µM, 90 µM, 65 µM, 40 µM, 20 µM and 1 µM biotinylated peptides. Duplicate samples were additionally incubated with 0.05% NP-40 PBS for 10 min previously to the blocking step.

2.2.4.2 Effect of d-biotin

Cells were incubated with d-biotin 400µM (Sigma, UK) for 2 hours following the blocking step or was added in the incubation of the cells with biotinylated DSP and TMG peptides at the same concentration (130µM). Cells were permeabilised by incubation with 0.05% NP-40 PBS for 10 min previously to the blocking step. Duplicate samples treated without d-biotin were used as positive controls.

2.2.4.3 Effect of non-biotinylated peptide

After fixation, cells were incubated with 0.05% NP-40 PBS for 10 min previously to the blocking step. Cells were then treated in triplicates with 400µM non-biotinylated peptides or no peptide for 2hr, washed six times with PBS and incubated with 40µM biotinylated peptides for 2 hr.

2.2.5 Metabolic activity of cell lines

2.2.5.1 Cell growth curve

Four flat bottom 96-multiwell (Nunc, UK) were seeded in quadruplet wells with NCI-H358, BPH-1, keratinocytes, MCF-7, MDA-MB- 231 and MCF-12A cells at a concentration of 10^5 cells/ml in 100 µl medium and cultured for 48 hr as described in the section 2.2.1. At time points 12hr, 24hr, 36hr and 48hr, absorbance readings at 492 nm were taken using one plate for each time point. Three hours and thirty minutes prior to each time point, 20µl of the reagent CellTiter Aqueous One Solution (Promega, UK) was added to each well to measure metabolic activity. Wells with medium without cells were used as blanks. Trypan Blue solution (Sigma, UK) was used for cell counting.

2.2.5.2 Effect of the peptides on cell growth

Ten flat bottom 96-multiwell (Nunc, UK) were seeded with BPH-1, keratinocytes, MCF-7 and MDA-MB- 231 cells in 100 µl medium and cultured for 132 hr as described in the section 2.2.1 Each cell line was seeded in duplicate plates. The concentrations of the cells at the time of seeding were 10^4 cell/ml (MCF-7 and keratinocytes), $2x10^4$ cell/ml (MDA-MB-231) and $4x10^3$ cell/ml (BPH-1). Non-biotinylated peptides DSP, TMG and WHK at concentration of 0, 650fM, 650pM, 650nM and 65µM were added in quadruplet wells for each time point and cell line, 84hr after seeding and the cells incubated for a further 24hrs. Three hours and thirty minutes prior to reading the absorbance at 492 nm, 20µl of the reagent CellTiter 96 Aqueous One Solution (Promega, UK) was added to each well. Cell metabolism was also measured at time points 60hr, 84hr, 108hr and 132hr in the absence of added peptide.

Wells with medium without cells were used as blanks. In order to subtract the absorbance due to floating cells, media was replaced 20hr after seeding.

2.3 HISTOCHEMISTRY

2.3.1 Tissue samples

Human normal breast tissue and ductal tumour breast tissue samples were donated by Dr. Elaine Kay, Pathology Dept., Beaumont Hospital, Dublin. The tissue blocks were paraffin-embedded and the sectioning as well as the hematoxilin and eosin staining was performed by Dr. B. Sheahan, Veterinary Dept., UCD, Dublin.

2.3.2 Routine histology

Coded H&E stained tumour sections were examined blind by light microscopy and relevant histological details were noted such as: tumour cell morphology, invasion of surrounding tissues by tumour cells and mitotic cells.

2.3.3 Binding of DSP and TMG Peptides to human breast tissue, normal and tumour

Human breast tissue sections from tumour and normal tissue from four different patients were tested in three independent experiments. Staining with the fluorescent Cy3-labelled streptavidin was used to detect peptide bound to cells of the tissue sections as described above in the section 2.2.3.2.

Paraffin embedded tumours were dewaxed with 3 x 10 min washes in 100%, 95%, 70% ethanol for 5 min each followed by dH$_2$O and placed in PBS. Antigen retrieval was performed by immersing slides in boiling 10mM citric acid, pH6 buffer in a pressure cooker (Prestige, UK) for 3 min at 121°C and 15 psi followed by 20 min cooling at room temperature. After antigen retrieval, slides were washed twice for 5 min in PBS, sections were outlined using a DAKO Cytomation pen and non-specific binding sites were blocked using 1% casein in PBS at room temperature for 45 min in a humidified chamber. After a brief wash in PBS, endogenous avidin and biotin was blocked using the Vector avidin/biotin blocking kit (Vector laboratories, USA)

according to manufacturer's instructions and slides were again washed briefly in PBS.

Sections were incubated with 40 µM biotinylated DSP, TMG and WHK peptides or with different concentrations of TMG peptide (0, 40, 65, 90, 110 or 130 µM) at room temperature in a humidified chamber for 2 hr. Slides were then washed three times for 10 min with PBS followed with fixation at room temperature for 10 min with 3.7% paraformaldehyde (Sigma, UK) in PBS and washed three times for 5 min with PBS. Tissue autofluorescence was quenched by incubation with sodium borohydride (Sigma, UK) (0.1%) in PBS for 30 min (Clancy and Cauller 1998) followed by three 5 min PBS washes. Peptides were detected with the fluorescence tag Cy3 by incubation with Streptavidin-Cy3 (Sigma, UK) (1:5000) in 0.5mM NaCl, 10mM Hepes. Slides were washed three times with PBS for 10 min and then, mitotic cells were stained by incubation with polyclonal rabbit anti-human ki67 antibody (1:1000) in 0.5% BSA PBS at 4°C overnight, the slides were washed again and incubated with (FITC)-conjugated swine anti-rabbit (DAKO A/S, Denmark) antibody (1:20) in 0.5% BSA in PBS at 4°C overnight. Slides were washed three times with PBS for 10 min, and then nuclei were stained incubating with 5ng/ml DAPI (Sigma, UK) PBS for 15 min and washed as before. Finally, slides were mounted using VECTASHIELD Mounting Medium (Vector, USA). Slides were examined using a Nikon eclipse E400 microscope and appropriate filters (DAPI; DAPI filter, excitation 340-380nm, cy-3; G2A filter, excitation 510-560 nm, FITC; B-2E filter, excitation 450-490nm).

2.4.1 BIOCHEMICAL ASSAYS

2.4.1 Cell lysates preparation for protein extraction

Pellets containing MDA-MB-231 cells are suspended in 500µl-1ml hypotonic buffer containing 25mM Hepes, 5mM $MgCl_2$, 5mM DTT, 5mM EDTA and proteinase inhibitor cocktail (Sigma,UK). After 1 hr incubation at 4 °C for hypotonic shock, remaining whole cells were lysed by 6 freeze/thaw cycles followed by sonication (3cycles of 15 sec. 3x energy setting 5; Sanyo Probe). DNA and RNA molecules were digested by 30min incubation on ice with 25 µg/ml RNase A (Sigma,UK) and 10U DNase I (Roches, UK).

2.4.2 Far-Western analysis of MDA-MB-231 protein extracts

MDA-MB-231 cells were grown in monolayers for 4 days at a 50-65% confluency stage in 7x175 cm^2-tissue culture flasks as described in section 2.2.1. Approximately 5x10^7 MDA-MB-231 viable cells were counted after staining with Trypan Blue as described in section 2.2.2. The monolayers were trypsinated and the detached cells were washed twice in PBS by centrifugation at 2,000 g for 5min at 4^{0}C. Protein extracts were prepared from the cell pellets as described above in section 2.4.1. The cell lysate was centrifuged at 20,500g for 30 min and the supernatant or cytosolic fraction precipitated with trichloro-acetic acid (TCA) as described in section 2.4.6 and stored at -20 ^{0}C. The pellet fraction, containing membrane proteins was extracted by incubation with 80 μl 1xSDS sample buffer (62.5 mM Tris-HCl pH6.8, 1m mercaptoethanol, 2% SDS, 0.2% Bromophenol Blue, 20% glycerol) containing 5M urea at ambient temperature overnight and subsequent vortexing. Insoluble material was pelleted and the supernatant (membrane protein fraction) and the cytosolic fraction (TCA pellet) were pooled in 250μl 50 mM Tris-HCl, pH6.8. Following 1 to 20 dilution in 50 mM Tris-HCl, pH 6.8, the protein content was measured by the method Bradford (Bradford 1976) using BSA as a standard. The protein sample in SDS sample buffer and 1.8-3.6 μg prestained protein marker (6-175kDa) (NEB,UK) were boiled for 10min. 50 μg protein sample was loaded into each of the eight lanes and separated by 12% SDS-PAGE. The proteins were transferred to a nitrocellulose membrane and the membrane was divided in eight pieces, each corresponding to one lane of the gel. The non-specific binding sites of half of the membranes were blocked for 2hr with 5% casein PBS without biotin and the other half with biotin (avidin/biotin blocking kit, Vector laboratories, USA) at the dilution described by manufacturers. The membranes were washed three times with PBS and each of the four membranes sets was incubated with either 65 μM DSP peptide, TMG peptide, WHK peptide in 5% casein PBS or no peptide for 2hr at room temperature. The membranes were washed five times with PBS, incubated for 30 min with ExtrAvidin HRP conjugate (Sigma,UK) (1:10,000) in 5% casein PBS, and washed again five times prior to incubation with SuperSignal Westpico Chemiluminescent Substrate (Pierce,UK) and then exposed to X-ray film.

2.4.3 Far-Western analysis of cross- linked proteins from MDA-MB-231 cells

3×10^6 MDA-MB-231 viable cells were seeded into $16\times175cm^2$-tissue culture flasks and grown for 4 days until they reached 50-65% confluency as described in section 2.2.1. The monolayers were fixed with 3.7% paraformaldehyde in PBS for 15 min and washed three times with PBS, permeabilised with 0.5% NP-40 PBS for 10 min and washed three times again. Non-specific binding sites were blocked by 45min incubation with 1% casein PBS containing biotin (avidin/biotin blocking kit, Vector laboratories, USA) at the dilution described by manufacturers. Cells were washed three times with PBS, and the cells were incubated with either 65 µM biotinylated DSP, TMG in 1% casein PBS or no peptide for 2hr and washed three times again. Then, they were fixed with 3.7% paraformaldehyde in PBS for 15 min and washed three times with PBS. Cells were scraped off the flasks, pelleted at 20,500g for 30 min at 4°C. The supernatant was concentrated using 10,000 dal molecular weight cut off Nanospin Plus filters (Gelman Science, USA) and both pellet and concentrated supernatant were stored at -20 °C for further processing. All the incubations were performed at room temperature unless otherwise specified. Once about 2×10^8 MDA-MB-231 cells were accumulated, protein extracts were prepared from the cell pellets as described in section 2.4.1. The cell lysates were pooled with the corresponding concentrated supernatants, then further divided into three equal parts each, pelleted at 20,500g for 30 min and the supernatants containing cytosolic proteins precipitated with TCA as described in section 2.4.6. The membrane protein samples (pelleted fraction) were extracted by overnight incubation with 100 µl 25mM NH_4^+Ac, pH4 or PBS containing 0.5M urea and 0.01% SDS. The samples were vortexed and the membranes pelleted again at 20,500g for 30 min. The supernatants containing membrane proteins were precipitated with TCA as described in section 2.4.6, resuspended in 40 µl PBS containing 1xTrypsin solution (Sigma,UK) or 25mM NH_4^+Ac, pH4 containing 1.2µg endoproteinase Glu-C (Roche,UK) or PBS, respectively, added to the precipitated cytosolic proteins, and digested for 14 hr at 25 °C. Protein samples treated with endoproteinase Glu-C, trypsin or not treated were mixed with equal volumes of 2x SDS sample buffer. 1.8-3.6 µg prestained protein marker (6-175kDa) (NEB, UK) were boiled for 5 min and the samples and protein markers separated by 7.5% SDS-PAGE. Then proteins were transferred to a nitrocellulose membrane and the non-specific binding sites blocked for 2 hr with 5% casein PBS containing biotin (avidin/biotin blocking kit, Vector laboratories, USA) at

the dilution described by manufacturers. The membrane was washed three times with PBS, incubated with ExtrAvidin HRP conjugate (Sigma,UK) (1:10,000) in 5% casein PBS, and washed again five times with PBS, prior to incubation with SuperSignal Westpico Chemiluminescent Substrate (Pierce,UK) and the membrane exposed to X-ray film for 10min or overnight.

2.4.4 Isolation of DSP *in vitro* binding proteins on streptavidin-coated magnetic beads

MDA-MB-231 cells were grown in monolayers to 50-65% confluency in 27x175 cm^2-tissue culture flasks as described in section 2.2.1. Approximately 4×10^8 MDA-MB-231 viable cells were counted after staining with Trypan Blue as described in section 2.2.2. The monolayers were trypsinated and the detached cells were washed twice in PBS by centrifugation at 2,000 g for 5min at 4^0C. Protein extracts were prepared from the cell pellets as described above in section 2.4.1. The cell lysate were centrifuged at 20,500g for 30 min and the supernatant or cytosolic fraction precipitated with TCA as described in section 2.4.6 stored at -20 ^{0}C. Membrane proteins were extracted from the pellet fraction by incubation with 80 µl 1xSDS sample buffer containing 5M urea at ambient temperature overnight and subsequent vortexing. The membranes were pelleted and the supernatant (membrane fraction) and the cytosolic fraction were pooled and dialysed for 22 hours against 500ml PBS using a 3,500 dal molecular weight cut off Spectra/Por molecularporous membrane (Spectrum, USA). Following 1 to 20 dilution in PBS the protein content was determined by the method of Bradford (Bradford 1976) using BSA as standard.

Approximately 3mg streptavidin-coated magnetic beads (Dynabeads M-280 Streptavidin, Dynal Co, Norway) were pre-washed three times on a magnetic eppendorf holder with 300 µl solution A (0.1M NaOH, 0.05M sodium chloride), once with 300 µl solution B (0.1M sodium chloride) and twice with 250µl PBS to eliminate the bovine serum albumin (BSA) of the buffer (0.1% BSA, 0.02% sodium azide, PBS pH 7.4) in which they are stored.

Approximately 2mg proteins from MDA-MB-231 cells (7mg/ml final concentration) were incubated with 20µg biotinylated DSP peptide on ice rocking for 2hr in duplicate. The beads were incubated with the peptide-cell extract mix for 30min on ice. The unbound fraction was collected, the beads were washed with 1ml PBS ten times, and

every single wash was collected. Next, the beads were incubated with either 200µg DSP or TMG (0.7 mg/ml final concentration in PBS), rocking for 1hr. The washes were precipitated with TCA as outlined in section 2.4.6. One fifth of the unbound fraction, the eluted fraction, the precipitated washes and the beads were mixed or resuspended with SDS sample buffer. The samples and 1-2 µg prestained protein marker (6-175kDa) (NEB, UK) were boiled for 5 min and separated by 12% PAGE. The gel was silver stained as described in the section 2.4.7.

2.4.5 Isolation of DSP and TMG *ex-vivo* binding proteins on streptavidin-coated magnetic beads

Approximately 2.3×10^8 MDA-MB-231 viable cells were grown for 4 days to 50-65% confluency in 75×175 cm^2-tissue culture flasks (3×10^6 cell/flask) as described in section 2.2.1. The monolayers were fixed with 3.7% paraformaldehyde PBS for 15 min and washed three times with PBS, permeabilised with 0.5% NP-40 PBS for 10 min and washed three times again. Non-specific binding sites were blocked by 45min incubation with 1% casein PBS containing biotin (avidin/biotin blocking kit, Vector laboratories, USA) at the dilution described by manufacturers. Cells were washed three times with PBS, equal numbers of monolayers cultures were incubated with either 40 µM biotinylated DSP or TMG peptides for 2hr and washed three times again. The cells were fixed with 3.7% paraformaldehyde PBS for 15 min and washed three times with PBS. Cells were scraped off the flasks, pelleted at 20,500g for 30 min at 4°C and the supernatant was concentrated using 10,000 molecular weight cut off Nanospin Plus filters (Gelman Science, USA) and both cell pellet and concentrated supernatant were stored at -20 °C for further processing. All the incubations were performed at room temperature unless otherwise specified. Once about 9×10^8 MDA-MB-231 cells were accumulated, they were defrosted and protein extracts prepared as described above in section 2.4.1. The cell lysate was pelleted at 20,500g for 30 min and the cytosolic proteins in the supernatants were pooled with the filter-concentrated supernatant and precipitated with TCA as described in section 2.4.6. The membrane proteins were extracted from the pelleted fraction of the cell lysate by overnight incubation with 100 µl 1x SDS sample buffer, 5M urea. The sample was vortexed and the membranes pelleted again at 20,500g for 30 min. The membrane fraction (now in 100ul 2x SDS buffer containing 5M urea) was added to the precipitated cytosolic protein fraction

following re-suspension in, 250 µl PBS and dialysed against 500 ml PBS for 22 hours employing a 3,500 molecular weight cut off Spectra/Por molecularporous membrane (Spectrum, USA). The dialysate was mixed with 2mg streptavidin-coated magnetic beads (Dynabeads M-280 Streptavidin, Dynal Co, Norway), and incubated for 30 minutes at room temperature. The beads had been pre-washed as described in section 2.4.4. The unbound fraction from both samples (treated with TMG and DSP) were collected. The beads were washed then times with 1ml B&W buffer (1mM EDTA, 2M NaCl, 10mM Tris-HCl pH 7.5) and each of the washes collected. The 1st, 7th and 10th washes were precipitated with the TCA as described in section 2.4.6, resuspended in 40µl Laemli sample buffer. One eight (31µl) of the unbound fractions were mixed with equal volumes of 2x SDS sample buffer. Then the beads were mixed with 40 µl of SDS sample buffer. The samples and 1-2 µg prestained protein marker (6-175kDa) (NEB, UK) were boiled for 15 min to allow the protein-protein and protein-peptide cross-links on the beads to break and obtain the eluted fraction (Layh-Schmitt, Podtelejnikov et al. 2000). Proteins were separated in a 10% SDS-PAGE and silver stained using the European Molecular Biology Laboratory (EMBL) silver staining protocol (Shevchenko, Wilm et al. 1996) compatible with protein analysis by mass spectrometry. A band of approximately 70kDa present in both samples was excised and sent to be analysed by mass spectrometry to the Moredun Research Institute (Pentlands Science Park, Bush Penicuik EH26, Scotland, UK).

2.4.6 Protein precipitation by lowering the pH with trichloro-acetic acid

One volume of TCA (sigma, UK) was added to four volumes of the protein samples and incubated on ice for 10min. The proteins were pelleted by centrifugation at 20,500 x g for 5min and the supernatants removed. The pellets were washed with 200µl cold acetone and centrifuged at 20,500g for 5min. The supernatants were removed and the pellets washed with 200µl cold acetone. The pellets were dried in a water bath at 95°C for 5-10min.

2.4.7 Silver staining for acrylamide gels

Proteins were fixed for 5 min by incubation of the gel with 30ml 50% (v/v) acetone, 1.25% (v/v) TCA, 0.015% (v/v) formaldehyde, distilled water. The gel was rinsed three times with 50ml distilled water and washed for 5min in 30ml distilled water. Next, it was soaked for 5min in 50% acetone (v/v) distilled water and then, with 17% (w/v) sodium sulfite for 5min. The gel was rinsed three times with distilled water and coloured for 8 min with 30ml 0.37% formaldehyde, 0.27% (w/v) sodium nitrate in distilled water. After 5 rinses, the gel was developed by incubation with 30ml 0.02% sodium carbonate (w/v), 0.015% formaldehyde (v/v), 0.0043% (w/v) sodium sulphyte in distilled water. Development was stopped by discarding the solution and replacing it with a solution of 50% glacial acetic acid.

2.4.8 Bio panning of the breast tumour cDNA T7Select10-3 library

2.4.8.1 Breast tumour cDNA phage T7Select10-3 library amplification

The breast tumour cDNA T7Select10-3 library (Novagen, UK) containing 10^7 primary recombinants, which had undergone a single round of amplification by the manufacturers, was screened to identify proteins binding to DSP and TMG peptides. In order to reach 10^8 p.f.u./μl, the library was amplified according to the manufacturers instructions. The amplified eluate was purified by overnight precipitation at 4^0C with the addition of 4% (w/v) polyethylene glycol-8000 (PEG), 500mM NaCl final concentrations, 15 min spin at 12,000 g at 4^0C, resuspension of the precipitate with 12ml TE/NaCl buffer (10 mM Tris-HCl, pH 8.0, 1mM EDTA, 1M NaCl) and its separation through a cesium chloride (CsCl):TE gradient (62.6%, 41.7%, 31.3%, 20.86% CsCl) by ultracentrifugation at 100,000g for 60 minutes. The collected purified library (turbid band in the 41.7% CsCl layer) was titred. Phage titres was determined by incubation of serial dilutions of the phage with a log-phase culture of *E. coli*, strain BLT 5615. Melted Top-agar at 50°C was added at the phage/bacteria mixture before plating on LB-agar petri dishes and the plates were incubated at 37°C for at least 4 hours. Each plaque represents a phage clone and is referred to as a plaque forming unit (P.F.U).

2.4.8.2 Screening of a breast tumour cDNA phage display library with DSP and TMG peptides

The T7Select^{TM} System manufacturers instructions were explicitly followed for the preparation of *E. coli* cultures (strain BLT5615), growth of T7 lysates, pre-amplification and purification of the library and the plaque lift assay. However the biopanning protocol was modified as detailed below. 200 pmole biotinylated peptides (TMG, DSP and WHK) was immobilised on 0.5 mg streptavidin magnetic beads and blocked for 1 hour with 0.5%BSA, 0.1mM biotin PBS. The cDNA library (10^8 p.f.u.) was incubated with the beads without peptide and after 1 hour transferred into the tubes containing beads with biotinylated peptide for one hour incubation at ambient temperature. Then they were washed 20 times. The phage that bind the peptides were eluted by incubation with 200 pmole of non-biotinylated peptide (TMG, DSP and WHK, respectively) for one hour. This incubation (first round of bio panning) was repeated 4 times. After each round, the titre of the eluate was determined as described in the section 2.4.8.1. The amplified eluate was purified by overnight precipitation with PEG/NaCl as described in the section 2.4.8.1 at 4^0C, resuspension of the precipitate with 1ml TE/NaCl buffer (10 mM Tris-HCl, pH 8.0, 1mM EDTA, 1M NaCl) and its separation through a 4 ml CsCl:TE gradient (62.6%, 41.7%, 31.3%, 20.86% CsCl) by ultracentrifugation at 100,000g for 60 minutes. The titre of the collected purified library (turbid band in the 41.7% layer) was determined and used for the next round of bio panning. The stringency of these washes was raised with each subsequent round of bio panning (0.05%TWEEN-20, 0.3% TWEEN-20, 0.5% TWEEN-20 and 0.7% TWEEN-20, PBS respectively).Theoretically, after four rounds of bio panning the final phage lysate should be enriched for phage that specifically interact with each of the peptides employed.

2.4.8.3 Selection of T7 clones by plaque lift

The eluates from the fourth rounds of bio panning against DSP and TMG peptides containing 550 p.f.u were plated on LB-agar. After overnight incubation at 37^0C, and 1min chilling at 4^0C, three nitrocellulose discs (Nitropure, Micron Separations Inc, USA) were layered on each agar plate for 1 min. Following lifting, the membrane was dried at 37^0C for 20-30 minutes, then blocked with 5% casein PBS for 30 minutes.

Three plaque lifts were taken from each plate. Each of the membrane lifts was incubated with 0.1% TWEEN-20 PBS (PBST) for 1 hour containing either 50 μM biotinylated DSP peptide, TMG peptide or WHK peptide. They were washed three times with PBST and incubated with ExtrAvidin HRP conjugate (Sigma,UK) (1:10,000) PBS for 30 min prior to incubation with chemiluminescence subtrate (Sigma, UK) and exposed to X-ray film.

2.4.8.4 PCR analyses of recombinant phage DNA

From each of the two agar plates containing the two fourth round eluates, twenty plaques (clones) were chosen and amplified in 3 ml *E.coli BLT5615* cultures for DNA amplification by polymerase chain raction (PCR) and sequence identification. The primers used (0.2 pmole/μl) were T7SelectUp (GGAGCTGTCGTATTCCAGTC) that anneals the vector 42 base pairs upstream of the beginning of the cDNA insert, and T7SelectDown (AACCCCTCAAGACCCGTTTA) that anneals the vector 64 base pairs downstream the end of the cDNA insert. The reaction mix consisted of *Taq* DNA Polymerase 10X Reaction Buffer with $MgCl_2$ (Promega, UK), 0.2 mM deoxyadenosine triphosphate, deoxythymidine triphosphate, deoxyguanidine triphosphate and deoxycytosine triphosphate (Sigma-Genosys, UK), 0.2 pmole/μl T7SelectUP and T7SelectDown primers and pure molecular biology grade water (BDH Chemicals, UK) to a volume of 24μl per reaction tube. One microliter from 3-ml plaque lysates were added to each reaction tube. 5U/μl Taq DNA Polymerase (Promega, UK) was used to catalyse the PCR, which consisted of 40 cycles in a thermal cycler (Gene Amp PCR System 2400) (Perkin Elmer,USA). One PCR cycle was: 94^0C for 50 secs, then 50^0 C for 1 min, then 72^0C for 1min, followed by a final extension at 72^0C for 6 min. 5 μl PCR products were analysed on a 1.8% (w/v) agarose mini-gel containing 0.5μg/ml ethidium bromide, and each gel was documented using a UV-light transilluminator Kodak Image Station (The Medical Supply Company, MSC, USA). The size of the products was estimated by comparing their gel-migration to a 50bp DNA ladder (Roche, UK) separated on the same gel. The amount of the PCR product obtained was guided by rough comparison of the intensity of the band between the samples and the DNA ladder which contains an average of 33ng DNA per band. 20ng of each 100bp PCR product and 10 pmole/μl T7SelectUp primer, in a sealed 1.5-ml eppendorf tube, was sent for sequencing to MWGAG Biotech (Ebersberg, Germany), together with the filled order

form they provide on the company website http://www.mwg-biotech.com/html/i_custom/i_comfortread.shtml. Before sending the PCR products for sequencing they were purified using the GeneElute PCR Clean-Up Kit (Sigma, UK).

2.4.8.5 DNA sequencing, sequence analysis, and data mining

The PCR products were sequenced at MWG Biotech AG at Ebersberg, Germany (http://www.mwgbiotech.com/html/i_information/i_overview.shtml) using Big Dye Terminator chemistry on an ABI PRISM® 3700 DNA Analyser, and the T7SelectUP as the sequencing primer.

Five different computer-based analysis tools were used to analyze the resulting DNA sequences and their corresponding protein sequences. Tool 1: Clustal W (1.82), which is a program designed to look for the best sequence alignments among multiple DNA or protein sequences. In the case of protein alignments it aligns not only identical but also conserved and semi-conserved amino acids. Tool 2: The NEB Cutter V1.0 is a program that finds the sites for all restriction enzymes that cut the DNA sequence and calculates the length of the restriction products (see http://tools.neb.com/NEBcutter/index.php3 for details). Tool 3: BLAST (Basic Local Alignment Search Tool), which is a set of similarity search programs designed to compare (align) the query sequence with the sequences in all available DNA and/or protein databases (see http://www.ncbi.nlm.nih.gov/BLAST/ for details). Tool 4: PubMed (http://www.ncbi.nlm.nih.gov/pubmed) was used to search for protein sequences of interest. Tool 5: CancerGene Database (http://caroll.vjf.cnrs.fr/cancergene/) was used to look for citations from the biomedical literature, which included a connection between proteins and genes obtained from using BLAST program and cancer disease. Tool 6: The 'DNA-protein Translate' tool on the ExPASy (Expert Protein Analysis System) proteomics server of the Swiss Institute of Bioinformatics (http://au.expasy.org/tools/dna.html) was used to obtain the polypeptide sequences that cDNA inserts codify for.

2.4.8.6 Length of the polypeptides expressed by the selected T7 phage clones by western blot

Aproximately 8×10^6 pfu of the bacteriophage T7 clones T6, T8, T11, T12, T13, T18, D3, D4 and D6 were amplified in 100 ml cultures of *E.coli BTL 5615* according to the T7 library (NEB, UK) manufacturer instructions. The phage were extracted by two PEG/NaCl precipitation steps at $4\ ^0$C, one overnight and the other one for 1 hour. After 15min centrifugation at $4\ ^0$C, the pellets containing approximately 10^{15} p.f.u were resuspended in 1.5 ml 100mM Tris-HCl pH7.5, 50mM $MgCl_2$ containing 1x proteinase inhibition cocktail (Sigma, UK). The phage suspension was sonicated (4 cycles of 30 sec. 3x energy setting 5; Sanyo Probe) until clearing. The sample was centrifuged at 20,500g for 1min at 4^0C and the supernatant treated with 10 units of DNase I (Roches,UK) and 1 unit of RNase A (Sigma, UK) for 1hr at $30\ ^0$C. The proteins in the supernatant were precipitated with the TCA as outlined in section 2.4.6 with the elimination of the washes with acetone, and then, the pellets were resuspended in 50 μl 1x SDS sample buffer and pooled with the precipitated supernatant. Following 1 to 8 dilution in 50 mM Tris-HCl, pH6.8, the protein content was determined by the method of Bradford (Bradford 1976) using BSA as standard. 20 μg protein samples, 40 ng T7 Tag Positive Control Extract (Novagen, UK), a 31.1kDa protein that contains the 11 amino acid T7 Tag peptide (present in the major and minor T7 phage coat proteins) in SDS sample buffer and 1.8-3.6 μg prestained protein marker (6-175kDa) (NEB,UK) was separated by 12% SDS-PAGE. The proteins were transferred from the gel to a nitrocellulose membrane at 4^0C overnight. The membrane was blocked with 5% casein PBS for 2 hours at room temperature, washed three times with PBS , 0.1% Tween-20 and treated with T7 Tag antibody HRP conjugate specific for the T7 Tag peptide (1:5000) (NEB, UK) in PBS, 0.1% Tween-20 for 3 hours at room temperature. The membrane was washed ten times with PBS, prior to incubation with SuperSignal Westpico Chemiluminescent Substrate (Pierce, UK) and exposure to X-ray film.

DSP PEPTIDE AS AN IMMUNOLOGICAL MIMIC OF HSP70-PC FROM MDA-MB-231

3.1 INTRODUCTION

A novel approach to the development of potential tumour vaccines has been the isolation of peptide mimics to epitopes of known oncogenes or tumour specific antigens. Biopanning of phage peptide display libraries using antibodies directed against the breast tumour antigen HER-2/neu or the gastric cancer antigen MG7 as bait has been employed to identify the corresponding mimic epitopes (mimotopes) (Xu, Xu et al. 2001; Ashok, David et al. 2003). The selection of mimotopes with high affinity and stringent specificity for the antibody idiotype indicates that the peptides displayed on the phage mimic the antibody-binding domain of HER-2/neu and MG7 antigens. Significantly, the selected 'mimotopes' elicited highly specific humoral immune responses against the peptides and/or the original tumour antigen. Linnermann et al. proposed a method for the selection of peptides that mimic a given tumour antigen-reactive T-cell epitope (Linnemann, Tumenjargal et al. 2001). The libraries employed were combinatorial nonapeptide libraries with a defined amino acid in one of the sequence positions combined with random mixtures of all amino acids in the remaining positions, to determine the essential amino acids of the T cell epitope. The screening consisted of the stimulation of a CD8$^+$ T cell clone specific for the cutaneous T-cell lymphoma (CTCL) cell line, MyLa, with MyLa cells loaded with combinatorial peptide libraries. The selected T-cell 'mimotopes' triggered cytolysis of MyLa cells and induced IFN-γ production and antitumour cytotoxicity by T cells isolated and expanded from CTCL patients. The new approaches for the generation of mimics to epitopes recognised by tumour-specific antibodies or tumour infiltrated lymphocytes (TIL) opens a new field for the development of vaccines without previous knowledge of the tumour antigen.

Since HSP-PCs are a rich source of tumour antigens, TC James and U Bond, (unpublished) put forward a hypothesis that peptide mimics to Hsp-PCs could be prepared using a two-step reverse bio-panning approach. In the first round of bio-panning Hsp70-PCs are used as a bait to identify peptides that "recognise" the PCs associated with Hsp70. Subtraction bio-panning was carried out with Hsp70 depleted of its associated peptides to eliminate peptides that recognise the Hsp70 component of Hsp70-PCs pool. Using a random peptide (12-mer) M13 phage display library to identify peptides binding to Hsp70-PC extracted from the human breast cancer cell line, MDA-MB-231, a number of recogniser peptides were identified. These recogniser peptides are used as the bait in a second (reverse) bio-panning experiment to identify

putative structural mimics of Hsp70-PCs. As outlined in the section "preface to this thesis", one of the recogniser peptides, designated TMG, led to the selection of the single mimic peptide sequence DSP.

In theory, the mimic peptides should posses properties related to the antigenic peptides associated with Hsp70. Therefore, it is essential to show that the mimic peptides functionally mimic these antigenic peptides. One of the goals of this chapter was to establish an *in vitro* assay to determine if the mimic peptides possess any immuno-stimulatory activity similar to Hsp70-PCs and to determine if the mimics functionally resemble the antigens associated with Hsp70. In this chapter, the methodology used to establish a T-cell stimulation assay to test the immune stimulatory activity of the peptide mimics is outlined. The mimic peptides were first loaded *in vitro* onto immature dendritic cells (iDCs) and these were subsequently used to stimulate CD14$^-$ PBMCs from an autologous healthy donors. It was shown that two consecutive stimulations of CD14$^-$PBMCs with iDCs are required to elicit IFN-γ production. Using this two-step stimulation assay it was found that CD14$^-$ PBMCs primed with Hsp70-PC from MDA-MB-231 cells, produced IFN-γ upon subsequent stimulation with the mimic peptide. Furthermore, CD14$^-$ PBMCs primed with the mimic peptide, also produced IFN-γ when challanged with cell extracts from either MDA-MB-231 (tumourigenic) or MCF-12A (non-tumourigenic) breast cell lines. These results suggest that the mimic peptide immunologically resembles peptides present in the protein extracts from breast cell lines and more specifically resemble peptides complexed with Hsp70. Thus, the approach outlined in this chapter for the detection of Hsp70-PC mimics should also prove useful in the identification and validation of tumour-specific peptide mimics with immune modulatory properties.

3.2 RESULTS

3.2.1 Isolation of monocytes by three methods: plastic adherence, CD14$^+$ retention and CD14 depletion method

Abundant literature can be found in which CD14$^+$ PBMCs can be isolated through their propensity of plastic adherence (Buchler, Hajek et al. 2003). The disadvantages of this technique are the low cell recovery and mixed cell populations yielded. A number of new technologies have been developed to isolate CD14$^+$ PBMCs through the use of antibody-coated magnetic beads. In order to chose one method to be employed for the rest of the studies in this chapter, a comparative study for the isolation of CD14$^+$ PBMCs was performed employing the three methods available at the moment; the plastic adherence method, which exploits the ability of CD14$^+$ PBMCs to adhere to the tissue culture flask, the CD14 retention method using CD14 antibody coated magnetic microbeads (CD14 MicroBeads, Miltenyi Biotech, UK) and the CD14 depletion method in which T cell, NK cells, B cells, dendritic cells and basophils are removed using magnetic microbeads conjugated with CD3, CD7, CD19, CD45RA, CD56, and anti-IgE antibodies leaving monocytes (Monocyte isolation kit, Miltenyi Biotech, Bergisch Gladbach, Germany).

Blood from healthy female donors was collected from the Blood Transfusion Service (St. James Hospital, Dublin). PBMCs were isolated by Lymphoprep® density gradient centrifugation. Aproximately 6×10^7 PBMCs (unseparated fraction) isolated from the same donor were separated into CD14$^+$ PBMCs (positive fraction) and CD14$^-$ PBMCs (negative fraction) by the three methods mentioned above. Cells in each fraction were counted by using the EB/AO: see *Materials and Methods* sections 2.1.2, 2.1.3 and 2.1.4. The recovery yield of CD14$^+$ PBMCs was estimated taking into account that 10% of PBMCs from a healthy individual are CD14$^+$ cells. Therefore, 6×10^6 was defined as 100% recovery (Table 3.1). The plastic adherence method is the most economic choice, nevertheless the low separation yield of 15.8% is a major disadvantage. Therefore this method was no longer pursued in any of the experiments described in this chapter. The CD14 depletion method gave the highest cell recovery (78.3%). It was observed during the separation procedure that the cells clumped more in the more concentrated cell suspensions. Therefore, the negative fraction (CD14$^-$ cells) suffered more cell losses than the positive fraction. The low numbers obtained in the

Table 3.1 Cell numbers obtained after separation of CD14$^+$ PBMCs by three different methods

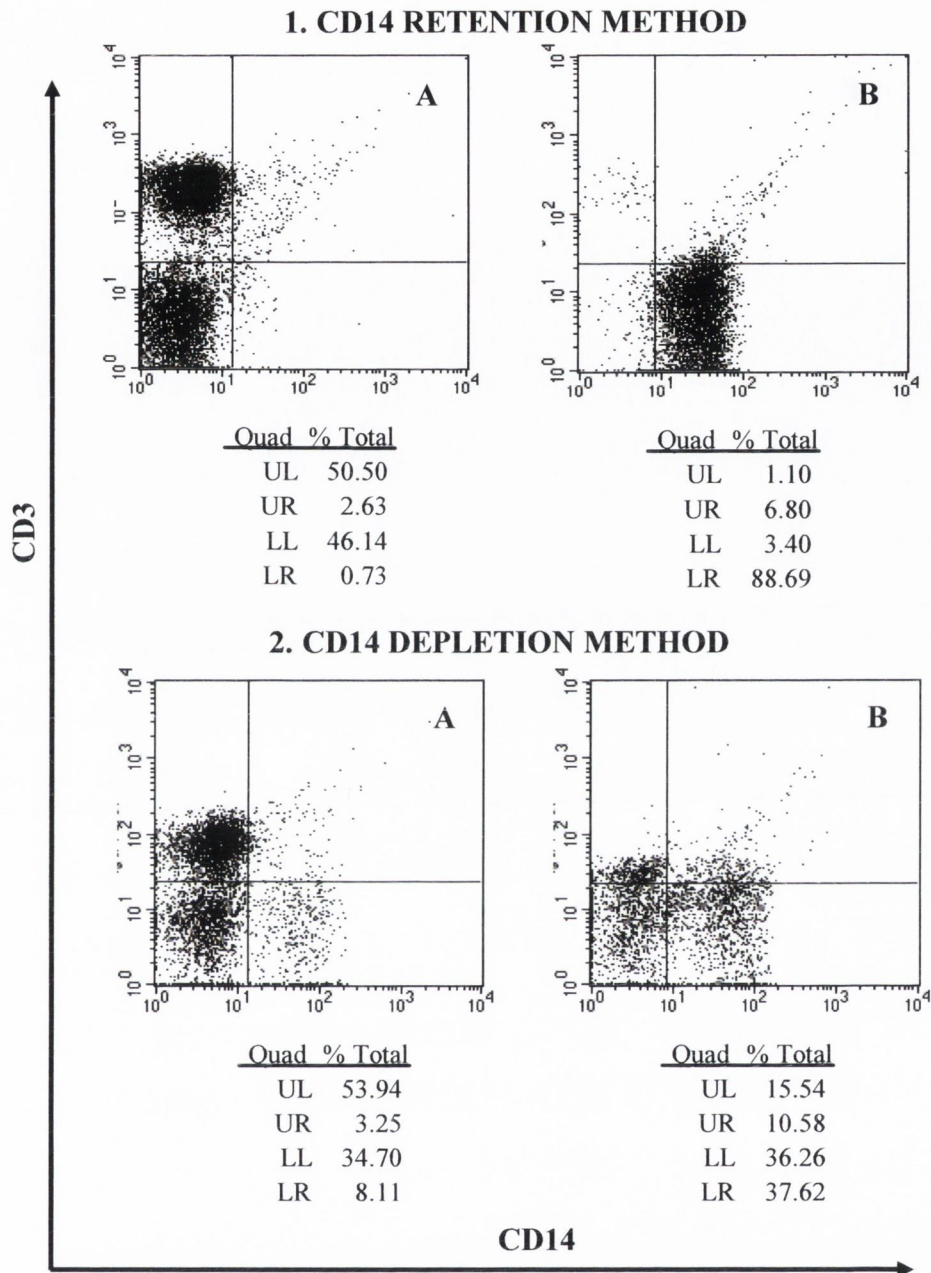| Method	Fraction (cell Number)			Positive fraction
	Unseparated	positive	negative	recovery yield*
Plastic adherence	6x10^7	0.95x10^6	3.5x10^6	15.8%
CD14$^+$ retention	6x10^7	3x10^6	3.3x10^6	50%
CD14$^+$ depletion	6x10^7	4.7x10^6	1.04x10^6	78.33%

*For the calculation of the recovery yield obtained in the positive fractions, it was taken in account that only 10% of the total PBMCs from healthy individuals are CD14$^+$ PBMCs. Therefore, a theoretical number of 6x10^6 was defined as 100% yield.

negative fraction and the higher counts in the positive fraction when using the CD14 depletion method could be due in part to the saturation of the binding capacity of the microbeads used. The CD14 retention method gaved an intermediate recovery of 50%. Next, the question to be addressed was which of the two methods yielded a more pure CD14$^+$ cell population in their positive fraction.

3.2.2 Assessment of the CD14$^+$ cell content of fractions obtained by CD14 retention and CD14 depletion methods by flow cytometry

Further analysis to assess the advantage of using the CD14$^+$ retention (CD14 MicroBeads) or CD14$^+$ depletion (Monocyte isolation kit) methods for the isolation of CD14$^+$ PBMCs were carried out. The phenotype of the cells contained in the positive and negative fractions were monitored by flow cytometry using fluorochrome-conjugated antibodies against the cell surface markers CD3 (present on approximately 70% of PBMCs) and CD14 (approximately 10% of PBMCs such as monocytes and some dendritic cells). The antibody labelling of 10^5 cells from each fraction from the positive and negative selection methods was performed as described in the *Materials and Methods* section 2.1.5. Figure 3.1 shows the percentages of CD14$^+$ and CD3$^+$ cells in both negative (Fig3.1.A) and positive (Fig3.1.B) fractions isolated by the two methods. Using the CD14 retention method, the yield of CD14$^+$ CD3$^-$ cells in the positive fraction was 88.69%. The low level of CD14$^-$ CD3$^+$ cells in this fraction (1.10%) indicates that this method is efficient in separating CD3$^+$ cells from CD14$^+$ cells (Fig.3.1. B; CD14 Retention). The flow through fraction (negative fraction) from the CD14 retention method contained a mixture of CD14$^-$ CD3$^+$ and CD14$^-$CD3$^-$cells (Fig. 3.1 A). Using the CD14 depletion method, the yield of CD14$^+$, CD3$^-$ cells in the positive fraction was 37.62%, (Fig.3.1 B; CD14 Depletion method). A higher percentage of contaminating CD14$^-$CD3$^-$ (36.26%), CD14$^-$CD3$^+$ (15.54%) and CD14$^+$CD3$^+$ (10.58%) cells were also isolated along with the CD14$^+$CD3$^-$ cells by this method (Fig 3.1 B, CD14 DEPLETION METHOD) indicating a less efficient separation. The higher yield of CD14$^+$ CD3$^-$ cells isolated by the CD14 retention method (Fig.3.1.B;CD14 RETENTION METHOD) compared to that obtained by the CD14 depletion method (Fig.3.1.B;CD14 DEPLETION METHOD), indicates that the former method is more efficient and generates a more pure CD14$^+$ cell population. Since the

1. CD14 RETENTION METHOD

Quad	% Total
UL	50.50
UR	2.63
LL	46.14
LR	0.73

Quad	% Total
UL	1.10
UR	6.80
LL	3.40
LR	88.69

2. CD14 DEPLETION METHOD

Quad	% Total
UL	53.94
UR	3.25
LL	34.70
LR	8.11

Quad	% Total
UL	15.54
UR	10.58
LL	36.26
LR	37.62

Fig 3.1 Flow cytometric analysis of CD3$^+$ and CD14$^+$ PBMCs separated by different methods
1; CD14 retention method is a positive selection method for retention of CD14$^+$ PBMCs by incubating with anti-human CD14 antibodies coated magnetic microbeads.2; CD14 depletion method is a negative selection method for depletion of CD14$^+$ PBMCs by magnetic labelling of anti-human CD3$^+$, CD7$^+$, CD19$^+$, CD45RA$^+$, CD56$^+$, and IgE antibodies. A; Negative fraction or CD14$^-$ fraction. B; Positive fraction or CD14$^+$ fraction. Each cell fraction was incubated with fluorochrome-conjugated anti-human CD3 and CD14 antibodies and analysed by flow cytometry. The tables show the percentage of cells present on each quadrant of the dot plots; upper left (UL), upper right (UR), lower left (LL), lower right (LR). Cells in the LR quadrant are CD14$^+$CD3$^-$. Cells in the UL quadrant are CD14$^-$CD3$^+$. Cells in the UR quadrant are CD14$^+$CD3$^+$. Cells in the LL quadrant are CD14$^-$CD3$^-$.

CD14 depletion method led to a poorer yield of CD14$^+$CD3$^-$ cells, optimisation of this particular method was not pursued. Therefore, the CD14 retention method was used for the isolation of CD14$^+$ PBMCs in all the subsequent experiments described in this results chapter.

### 3.2.3 Assessment of CD14$^+$ PBMCs differentiation into iDCs by flow cytometry

The next goal was to achieve the differentiation of the fractionated CD14$^+$ PBMCs population into iDCs. Considering Basu et al (Basu, Binder et al. 2001) discovered the existence of receptors on DCs (CD91) that drives the endocytosis of antigens associated with Hsp70 and their cross-primed representation on MHC class I molecules, it was decided to test whether Hsp70-PC mimics loaded on iDCs can stimulate CD14$^-$PBMCs (which include NK, T- and B-cells). iDCs retain their ability for antigen uptake but lose this ability when they reach maturation. Thus, CD14$^+$ PBMCs were differentiated into iDCs, and to corroborate the success of the process some expected phenotypic features of iDCs such as the lack of CD14 and CD83 expression and increased CD11c expression was monitored.

Approximately 6.5 x 10^6 CD14$^+$ PBMCs isolated by the CD14 retention method were incubated for six days with medium containing IL-4 and GM-CSF as described in the *Materials and Methods* section 2.1.7. Samples were taken at days zero and six of the incubation period. The phenotype of the CD14$^+$ PBMCs differentiated into iDCs was monitored by flow cytometry using fluorochrome-conjugated antibodies against the cell surface markers CD83, CD11c and CD14 as described in the *Materials and Methods* section 2.1.5. CD83 is expressed on matured dendritic cells while CD11c forms the CD11c/CD18 complex on monocytes (CD14$^+$ PBMCs), granulocytes, macrophages, NK, dendritic cells and subsets of T and B lymphocytes. The results in figure 3.2 showed that 96.25% freshly prepared CD14$^+$ cell-enriched PBMCs expressed CD14 at day 0 (Fig3.2. CD14, D.0) and 81.33% expressed low levels of CD11c (Fig3.2. CD11c, D.0). The cells analysed at day six of the incubation period showed downregulation of CD14 and a lack of CD83 expression (Fig3.2. CD14, D.6 and CD83, D.6) and an increased percentage of cells (93.97%) expressing CD11c (Fig3.2. CD11c, D.6). This phenotype correspond to that of monocytes (CD14$^+$ PBMCs) differentiated into iDCs cells described previously (Arrighi, Hauser et al. 1999; Chang, Wright et al. 2000).

Fig 3.2 Flow cytometric analysis of the expression levels of CD11c, CD14 and CD83 by isolated CD14$^+$ PBMC at day zero and day six of incubation with hrGM-CSF (60 ng/mL) and hrIL-4 (150 ng/mL)

Cells are stained with phycoerythrin (PE)-conjugated anti-human CD11c IgG$_1$, FITC-conjugated anti-human CD14 IgG$_1$ and peridinin-chlorophyll-protein (PerCP)-conjugated anti-human CD83 IgG$_1$, and analysed by flow cytometry (red histograms). Black histograms show cells after labelling with fluorochrome-conjugated isotype control antibodies. M1; Marker of positivity for enumeration of cells in the tables below. D0; Day 0, before addition of hrGM-CSF and hrIL-4. D6; Day 6, after addition of hrGM-CSF and hrIL-4.
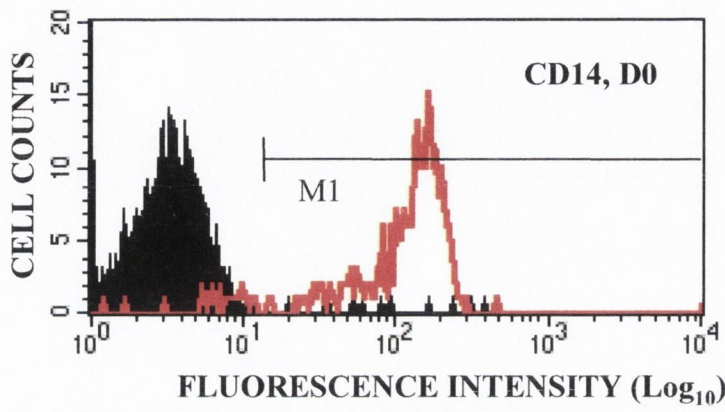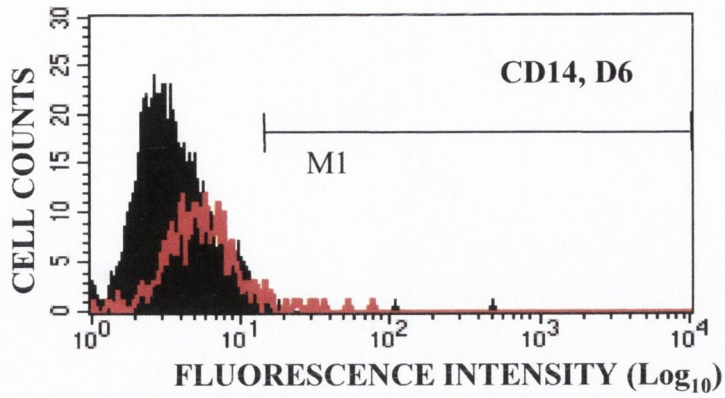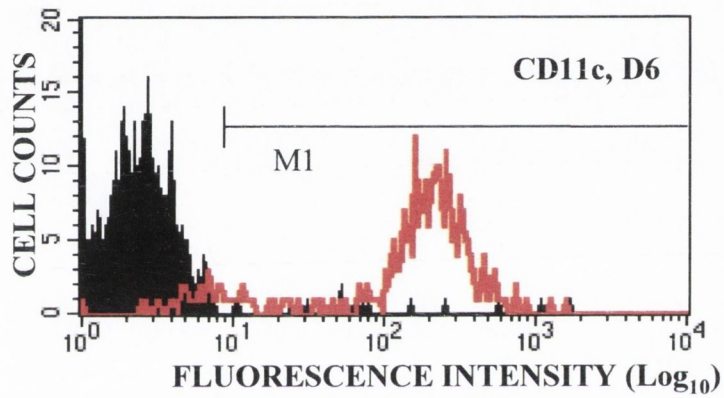
Marker	% Gated		Marker	% Gated	Median
All	100.00		All	100.00	135.16
M1	1.36		M1	96.25	137.00
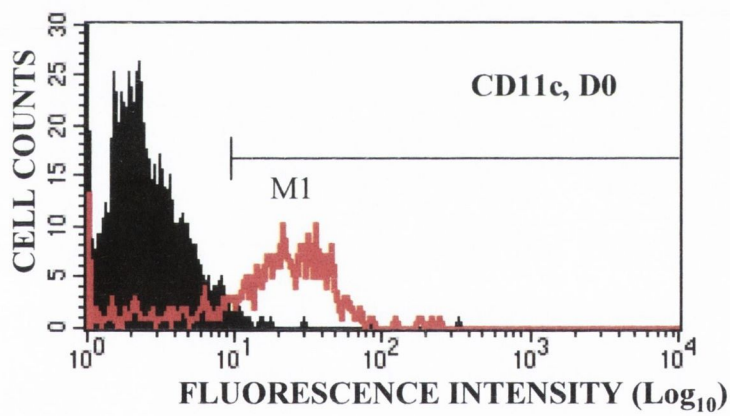
Marker	% Gated		Marker	% Gated	Median
All	100.00		All	100.00	21.48
M1	1.05		M1	81.33	25.25

Marker	% Gated		Marker	% Gated	Median
All	100.00		All	100.00	4.91
M1	0.68		M1	1.94	21.58

Marker	% Gated		Marker	% Gated	Median
All	100.00		All	100.00	191.10
M1	1.63		M1	93.97	196.32
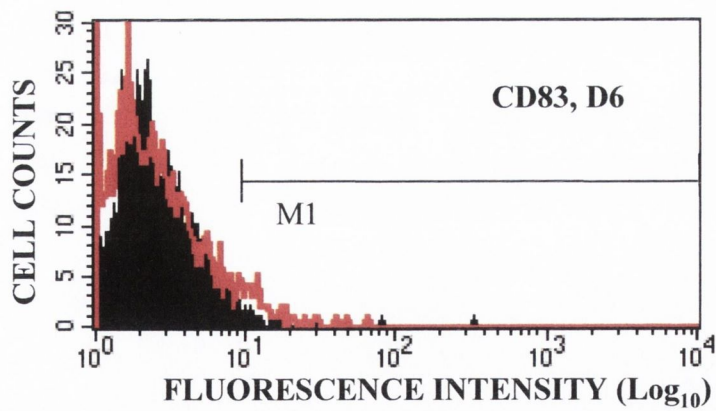
Marker	% Gated		Marker	% Gated	Median
All	100.00		All	100.00	2.00
M1	1.09		M1	3.12	11.65

### 3.2.4 DSP peptide shows specific interaction to TMG phage in ELISA

Prior to testing whether the Hsp70-PC mimic, DSP, can induce immune responses against antigens associated with Hsp70 (Hsp70-PC), the capacity of DSP peptide and the Hsp70-PC recogniser peptide TMG to interact with each other was studied, since DSP was obtained by bio paning using the TMG peptide as a bait .

To determine the specificity of the TMG/DSP interaction, an ELISA test was devised where biotinylated TMG peptide was immobilised on Streptavidin-coated magnetic beads and then incubated with M13 phage displaying the DSP peptide (Fig. 3.3. TMG) as described in *Materials and Methods* section 2.1.11. The TMG-bound DSP phage was quantified by horse-radish peroxidase (HRP)-conjugated anti-M13 antibody according to the manufacturer's instructions. Conversely, immobilisation of biotinylated DSP peptide followed by incubation with an M13 phage displaying the TMG peptide also gave a positive signal (Fig. 3.3.C, DSP). Negative controls containing no biotinylated peptides gave no colour development. (Fig. 3.3.C, TMG negative, DSP negative).

3.2.5 Effect of DSP and TMG peptides on the maturation and activation of iDCs

Having established the specificity of the TMG/DSP interaction, the immune stimulatory activity of these peptides was examined. Firstly, the effects of these peptides on the differentiation of iDCs into mature DCs (mDCs) was studied.

In the peripheral tissues, iDCs effectively capture antigens (Ag) via phagocytosis, macropinocytosis and endocytosis (Mellman 1990; Larsson, Berge et al. 1997). After encountering Ag in environment of inflammatory mediators, iDCs undergo a maturation process characterised by an increased expression of MHC and costimulatory molecules such as CD80 and CD86 (Sallusto, Cella et al. 1995; Cella, Engering et al. 1997). These changes are accompanied by migration to areas of secondary lymphoid organs where they prime naïve T-cells (Inaba, Metlay et al. 1990; Austyn 1992). During their migration, DC undergo further changes of phenotype and function (maturation) including loss of antigen uptake and processing and increase in accessory function. *In vivo* and *in vitro* DC maturation can be induced by toll-like receptor ligands, such as LPS. In the present study, the ability of DSP and TMG to induce differentiation of iDCs into mDCs in a manner similar to LPS was examined.

INTERACTION BETWEEN DSP&TMG PEPTIDES
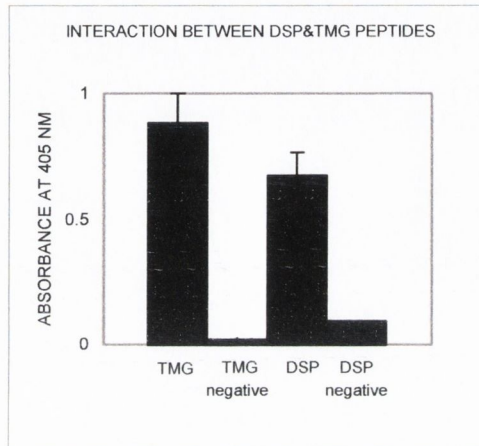
**Fig 3.3   ELISA assay for detection of the interaction between biotinylated TMG and DSP peptides**

Streptavidin magnetic beads coated with biotinylated TMG peptide (TMG) or no peptide (TMG negative) were incubated with the M13 phage clone displaying DSP. Likewise streptavidin magnetic beads coated with biotinylated DSP peptide (DSP) or no peptide (DSP negative) were incubated with M13 phage clone displaying TMG. All beads were then incubated with anti-M13 antibody conjugated with Horse Radish Peroxidase. Interactions were detected by absorbance at 405nm using ABTS/H_2O_2 as substrate.

2x10^5 iDCs were incubated with 10µg/ml LPS and 25µg/ml DSP or TMG, LPS alone or supplemented RPMI-1460 medium alone for 24hr at 37 °C. The cells were incubated with fluorochrome-conjugated antibodies specific for the maturation marker CD83 and the co-stimulatory molecule CD86 as described in the *Materials and Methods* section 2.1.5.

The results in Fig 3.4 show that iDCs incubated with LPS alone (Fig3.3. A&B; blue histogram), LPS and DSP (Fig 3.4. A&B; red histogram) or TMG peptides (Fig 3.4. A&B; green histogram) expressed upregulated levels of CD83 but not CD86 when compared to the expression observed when iDCs are incubated with medium alone (Fig 3.4. A&B; black histogram). The histograms in Fig 3.3 show a nearly exact overlapping for the two samples containing peptides and the one containing only LPS. This suggests the expression of CD83 by DCs is due to LPS, and the contribution of the peptides towards maturation is none or lower than the contribution of LPS. The effect of the peptides in the absence of LPS was not tested and therefore the role for these peptides in DC maturation cannot be excluded. Moreover, none of the stimuli used upregulated the expression of the co-stimulatory molecule CD86 on iDCs (Fig3.4.B). Surprisingly, iDCs incubated with medium alone (Fig3.4.A&B; black histogram) showed some expression of CD83 and CD86 (fluorescence intensity (log$_{10}$): 1-10^2) compared with the isotype control monoclonal antibodies (1-10) (see fig 3.2). As reported previously CD86 expression follows LPS stimulation (Eisendle, Lang et al. 2003) and it requires a longer incubation time than CD83 expression (Cao, Lee et al. 2005). It is possible the 24hr-incubation period of iDCs with LPS was long-enough to up-regulate the expression of the maturation marker CD83 but not the co-stimulatory molecule CD86.

It has been reported previously that IL-12 secreted by iDCs triggers IFN-γ production by T cells, NKT cells and NK cells (*see General Introduction*). IL-12 secretion by iDCs can be elicited by LPS (Jiang, Muckersie et al. 2002). The ability of DSP or TMG to induce IL-12 production by iDCs in an analogous manner to LPS was next tested. iDCs were incubated with or without LPS, and with or without the peptides TMG, DSP or WHK for 24 hours. The supernatants were tested for the presence of IL-12 by ELISA. As shown in figure 3.5, IL-12 secretion by iDCs was detected after stimulation with LPS alone or with a combination of LPS and TMG, WHK or DSP peptides. However, no IL-12 release by iDCs was detected after incubation with DSP, TMG or the control peptide WHK, in the absence of LPS. Together, these data in figures 3.4 and 3.5 suggest that TMG or DSP do not directly induce maturation of DCs.
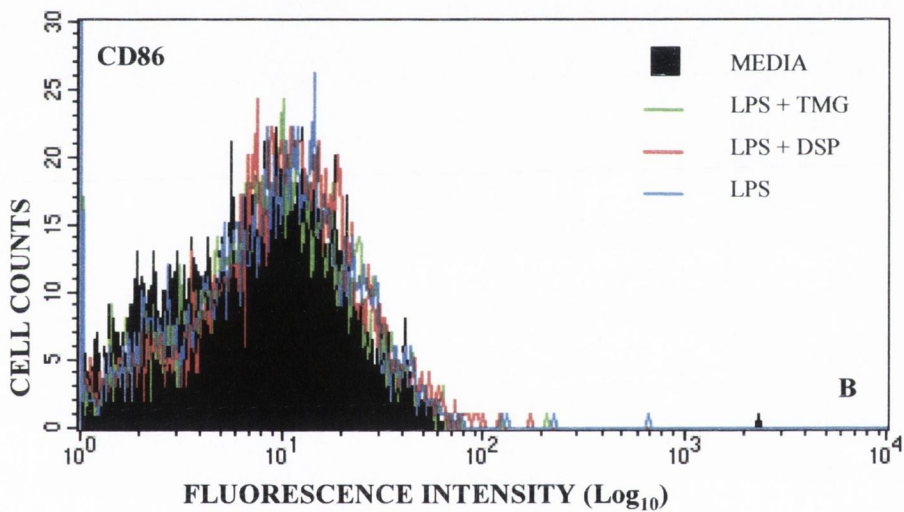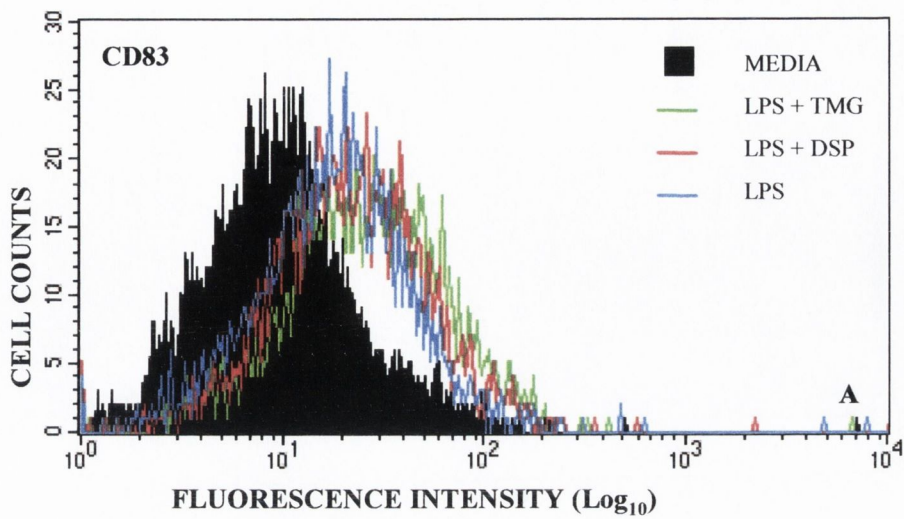
Fig 3.4 Flow cytometry analysis of the expression levels of CD83 and CD86 by iDCs after 24hr-incubation with medium, LPS and TMG peptide, LPS and DSP peptide or LPS alone Each set of cells were labelled with fluorochrome-conjugated anti-human CD83 (**A**) or CD86 (**B**) monoclonal antibodies and each fluorochrome was analysed by flow cytometry.
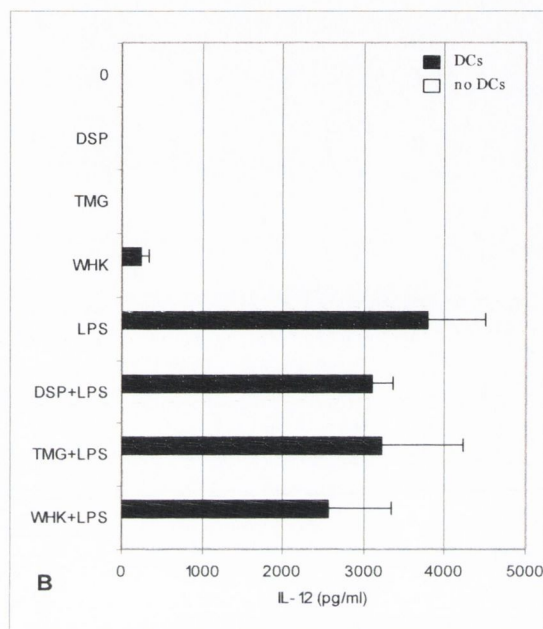
Fig 3.5 Activation of iDCs loaded with DSP or TMG peptides to secrete IL-12 in the presence or absence of LPS

7×10^4 iDCs were incubated with 25µg/ml DSP, WHK, TMG or no peptides with or without LPS (10µg/ml) for 24 hr in 96-well plates. Wells without DCs were included as negative controls. The concentration of IL-12 in the supernatants was tested by ELISA.

3.2.6 DCs pulsed with DSP and TMG peptides can stimulate CD14⁻ PBMCs to produce IFN-γ

The ability of the peptides-pulsed DCs to stimulate CD14⁻ PBMCs to produce γ-interferon was next tested. Naive CD8⁺ T-cells are primed or first stimulated by Ag presented by matured DCs in areas of secondary lymph organs, such as lymph nodes. When primed T-cells circulate to peripheric organs and encounter the same Ag they become effector T cells; they proliferate, produce cytokines, such as IFN-γ and mount a cytotoxic attack against the cell presenting the Ag on MHC class I molecules (e.g. tumour cells). By contrast to αβ T cells (e.g. CD8⁺ T cells), γδ T cells can recognise antigens with no requirement for antigen processing or presentation engaging secretion of IFNγ (Chien, Jores et al. 1996; Crowley, Fahrer et al. 2000).

In order to test whether DSP, TMG and WHK peptides can stimulate T-cells to produce IFN-γ, a two-step *in vitro* stimulation assay was developed. Since 'professional' antigen-presenting cells are required for activation of naïve T cells, an autologous antigen presentation system consisting of iDCs pulsed with DSP, TMG or WHK and CD14⁻ PBMCs prepared from the same donor, was employed. LPS was not included in these experiments to test the immunostimulatory ability of the peptides by themselves. As described above the CD14⁺ cells were isolated from PBMCs of healthy donors by adsorption to magnetic beads coated with anti-CD14 antibody and were induced to differentiate into iDCs. CD14⁻ PBMCs, (which include NK, T- and B-cells), from the same donor were incubated for 48 hours either directly with DSP or TMG peptides in solution or with the irradiated iDCs pre-incubated with peptides (referred to herein as priming or first stimulation). The cells were incubated for further 10 days with hrIL-2, following which, they were re-stimulated with either the respective peptide in solution or a second batch of iDCs, from the same donor, loaded with either TMG or DSP or WHK peptides (second stimulation). After both stimulations the supernatants were assayed for IFN-γ by ELISA as described in *Materials and Methods* section 2.1.10.

The results of this experiment are shown in figure 3.6. CD14⁻ PBMCs responded weakly to a single (first) stimulation with iDCs loaded with either DSP or TMG peptides but not at all to WHK (Fig. 3.6. DCs-white bars). However, when the cells were re-stimulated with iDCs primed with the same peptides (second stimulation),
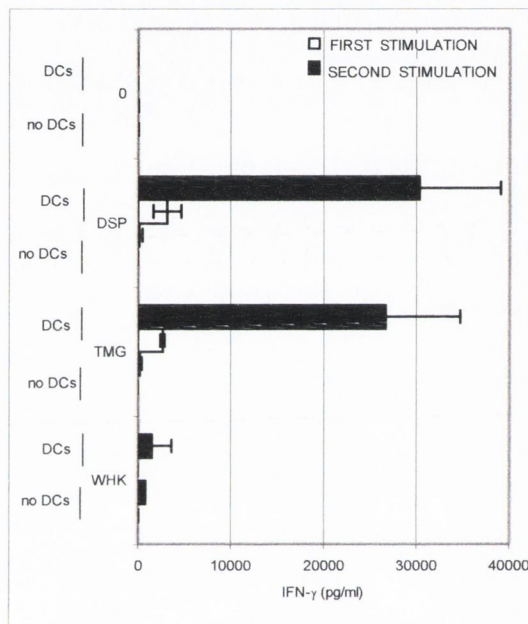
Fig 3.6 IFN-γ secretion by CD14⁻ PBMCs stimulated *in vitro* with peptides in the presence or absence of iDCs as antigen presenting cells

CD14⁺ cells were isolated from PBMCs from healthy donors and differentiated into immature DCs as described in the *Materials and Methods* section 2.1.7. 25µg/ml DSP, WHK, TMG or no peptides were incubated with iDCs for 24hr. Free peptide in solution (no DCs), and the peptide-loaded iDCs were incubated with CD14⁻ PBMCs from the same donor (first stimulation). The DC:CD14⁻ PBMC ratio was 1:10. The cells were cultured with IL-2 (25ng/ml) for 10 days and then, incubated with a second batch of iDCs loaded with the same peptide (second stimulation). The concentration of IFN-γ in the supernatants of the CD14- PBMCs was determined 48 hours following the first or second stimulations.

significantly higher levels of IFN-γ were produced (Fig 3.6. DCs-filled bars). Again, WHK peptide responded weakly, showing IFN-γ levels close to background levels. Peptides alone (without iDCs) (Fig 3.6. no DCs) either in the first or second stimulation did not trigger secretion of IFN-γ, indicating that DC-mediated presentation of the peptides is required for the stimulation of CD14⁻PBMCs. The observation that iDCs pulsed with DSP or TMG were sufficient to prime the CD14⁻PBMCs for activation upon subsequent stimulation, suggest that T cells of the adaptive immune system are responsible for the IFN-γ release. However, the finding that priming was mediated by iDCs, rather than mDCs, suggests that other lymphocytes such as NK cells or γδ or NKT cells may be activated. Alternatively, it is possible that the iDCs used were partially differentiated into mDCs as indicated by the expression of the maturation marker CD83 molecule in the presence of medium alone (section 3.2.5).

Based on the original hypothesis put forward by Bond and James, it was expected the mimic peptide but not the recogniser peptide would show immune stimulatory activity. The results however, show that both DSP and TMG were capable of stimulating CD14⁻ PBMCs to produce IFN-γ. These results were reproducible in four separate experiments using blood from 4 different healthy donors (data not shown).

3.2.7 Bioassay for the 'proof of concept' that DSP peptide is a mimic to peptide bound to Hsp70

To test the original hypothesis that a two-step reverse bio-panning process could generate peptide mimics of a protein used as a bait in the first round of bio-panning, it was necessary to prove a relationship between the mimic peptides such as the DSP peptide and the original bait material, Hsp70-PCs. To achieve this, a two-step CD14⁻ PBMC stimulation assay similar to that outlined in section 3.2.6 was used. The method used here builds on the strategy recently reported by Fleischer et al. *(Fleischer, Schmidt et al. 2004)* in which two rounds of CD14⁻ PBMCs stimulation were carried out using iDCs loaded with two different but related antigens. In the first round, CD14⁻PBMCs are incubated with iDCs loaded with a specific antigen (first stimuli). The second round of stimulation is carried out with iDCs loaded with a pool of antigens (second stimuli; cross priming). If the specific antigen used in the first stimulation resembles an

antigen(s) present in the pool of antigens used in the second round of stimulation, then CD14⁻ PBMCs will respond by secreting IFN-γ.

To test the validity of this approach, the ability of iDCs loaded with MDA-MB-231 tumour cell extracts (TC) to stimulate CD14⁻ PBMCs previously stimulated or primed with iDCs loaded with Hsp70-PC, Hsp70 or TCs was examined. Protein fractions enriched in Hsp70 or Hsp70-PC were obtained from MDA-MB-231 cells using ATP- agarose or ADP-agarose affinity chromatography, respectively, as described in the *Materials and Methods* section 2.1.8. This purification method is based on the ATP/ADP-binding properties of Hsp70: incubation with ATP strips the peptides associated with Hsp70 from the molecule. Western blot analysis using anti-Hsp70 antibodies and Coomassie Blue staining of the corresponding SDS gel show that the most prominent proteins eluted from the column with ATP or ADP are the constitutive (Hsp73) and inducible (Hsp72) forms of Hsp70 (Fig. 3.7.A and B, lane 3&5) confirming similar findings by others (Srivastava and Amato 2001). A small number of other proteins (less than 10%) are also eluted by this procedure. LPS was used as an adjuvant in the first but not in the second stimulation of CD14⁻ PBMCs, thereby avoiding any LPS effect on IFN-γ production following the second stimulation (Kambayashi, Assarsson et al. 2003). It was predicted that 24hr-incubation of iDCs with LPS plus peptides promotes their differentiation to mDCs as shown in the section 3.2.5. The level of IFN-γ production when cells were primed and challenged (first and second stimulations) with the same antigens [Fig 3.8.A, TC(1)+TC(2)] was set to 100%. IFN-γ levels of over 50% were obtained when the CD14⁻ PBMCs were initially primed by Hsp70 followed by challenge with total cell extract in the second stimulation [Fig 3.8.A&B, Hsp70(1)+TC(2)]. A significantly higher level of IFN-γ (over 90%) was secreted when primed and challenged with Hsp70-PC and tumour cell extract respectively [Fig 3.8.A&B, Hsp70-PC (1)+TC (2)]. Little or no IFN-γ was detected when CD14⁻ PBMCs received only a single exposure of tumour cell extracts [Fig 3.8.A, TC(1)], nor was IFN-γ produced if PBMCs were incubated with iDCs stimulated by LPS alone in the first stimulation and then challenged with iDCs loaded with TCs without LPS in the second stimulation (data not shown). These results support earlier observations (Udono and Srivastava 1994; Srivastava, Menoret et al. 1998; Srivastava 2002) that the ability of TCs to raise immune responses reside in the Hsp-PC fraction. This experiment was successfully repeated using PBMCs isolated from 4 individual

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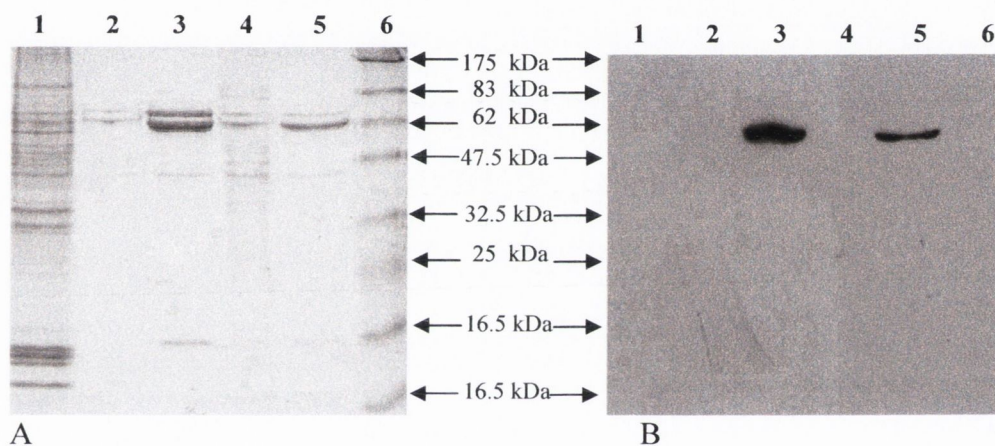
Fig 3.7 Purification of Hsp70 and Hsp70-PC from MDA-MB-231 Cells By Affinity Chromatography

Hsp70 and Hsp70- peptide complexes (Hsp70-PC) were isolated from whole cell extracts from MDA-MB-231 cells using ATP-Agarose and ADP-Agarose mini-columns, respectively. A. Coomassie-Blue stained 12% SDS-polyacrylamide gel and B: Western blot using anti-Hsp70 antibody. Lane 1: MDA-MB-231 cell membrane fraction. Lane 2: Flow-through from an ATP-agarose mini-column. Lane3: Proteins eluted from ATP-agarose column with 3mM ATP. Lane 4: Flow-through from an ADP-agarose column. Lane 5: Proteins eluted from ADP-agarose mini-column with 3mM ADP. Lane6: Molecular weight markers. Lane 3&5 were loaded with 2μg protein as determined by Bradford assay.
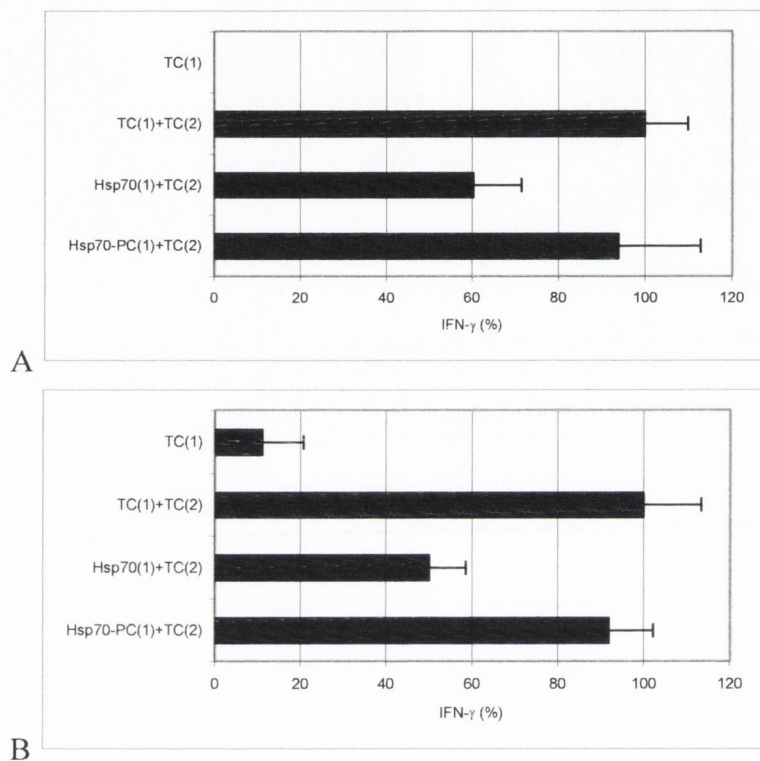
Fig 3.8 Secretion of IFNγ by CD14⁻ PBMCs primed with iDCs loaded with LPS and Hsp70, Hsp70-PC or TC upon secondary stimulation with iDCs loaded with TC

Hsp70 (5μg/ml), Hsp70-PC (5μg/ml) from MDA-MB-231 cells or TC (110μg/ml) were incubated with 10^4 iDCs and LPS (10μg/ml) for 24 hours. PBMCs from two healthy blood donors (A and B) were used. The loaded iDCs were washed and incubated with 10^5 CD14⁻ PBMCs for 48 hr. The cells were cultured with IL-2 (25ng/ml) for 10 days and then, incubated with 10^4 iDCs loaded with TC (110μg/ml) without LPS for 48hr [TC(2)]. Supernatants from the cells exposed to one round of stimulation [TC(1)] or two consecutive stimulations were tested for IFN-γ production by ELISA. Values were plotted as ratio to the IFN-γ level produced after using TC for both priming (1) and stimulation (2).

donors and the results of 2 such experiments shown in figure 3.8.A and B, clearly demonstrate their reproducibility. In some experiments, it was shown that if the numbers of iDCs were limiting (below 5,000 iDCs/100μl of medium) or if the iDC/CD14⁻PBMC ratio were less than 1:10, IFN-γ production by the PBMCs was not observed.

3.2.8 The mimic peptide DSP is an antigen with properties similar to antigens from tumour and normal breast cells

The ability of DSP and TMG peptides to prime CD14⁻ PBMCs against protein(s) of MDA-MB-231 cells was tested using the two step T-cell stimulation assay outlined above. CD14⁻ PBMCs were primed with iDCs loaded with LPS and either DSP, TMG or TC extract and stimulated 10 days later with iDCs loaded with the TC (Fig. 3.9; TC). Panels A and B in Figure 3.9 represent results from two individual blood donors. CD14⁻ PBMCs primed initially with DSP responded to a second stimulation with TC extract by secreting IFN-γ at 56% and 21% the levels obtained when the TC was used for both stimulations, respectively in donors A and B, [Fig.3.9.A, B; DSP(1)+TC(2)]. IFN-γ levels of 12% and 21% in donors A and B, respectively were produced by CD14⁻ PBMCs stimulated first with TMG and then with TC [Fig 3.9.A, B;TMG(1)+TC(2)]. No IFN-γ could be detected when CD14⁻ PBMCs received only a single exposure to TC [Fig3.9.A, B; TC(1)], nor was IFN-γ produced if PBMCs were incubated with iDCs stimulated by LPS alone in the first stimulation and then challenged with iDCs loaded with TCs without LPS in the second stimulation (data not shown).

The different levels of IFN-γ secretion by PBMCs from donors A and B when using DSP for the first stimulation and TC for the second stimulation suggest that the observed responses may have a certain degree of donor specificity.

To determine whether the T cell stimulation by DSP peptide was specific to the type of tumour cells, the experiment was repeated but this time the non-tumourigenic cell line extract (NTC) from MCF12A (Fig 3.9; NTC) was used. CD14⁻ PBMCs stimulated first with DSP and secondly with NTC, produced IFN-γ levels of 46 % and 32% of the levels obtained when the NTC extract was used for both stimulations in donors A and B, respectively. In contrast, when primed with TMG in the first stimulation and NTC in second stimulations, the relative IFN-γ levels were 3% and 28% in donors A and B respectively. There was no detectable IFN-γ response by cells
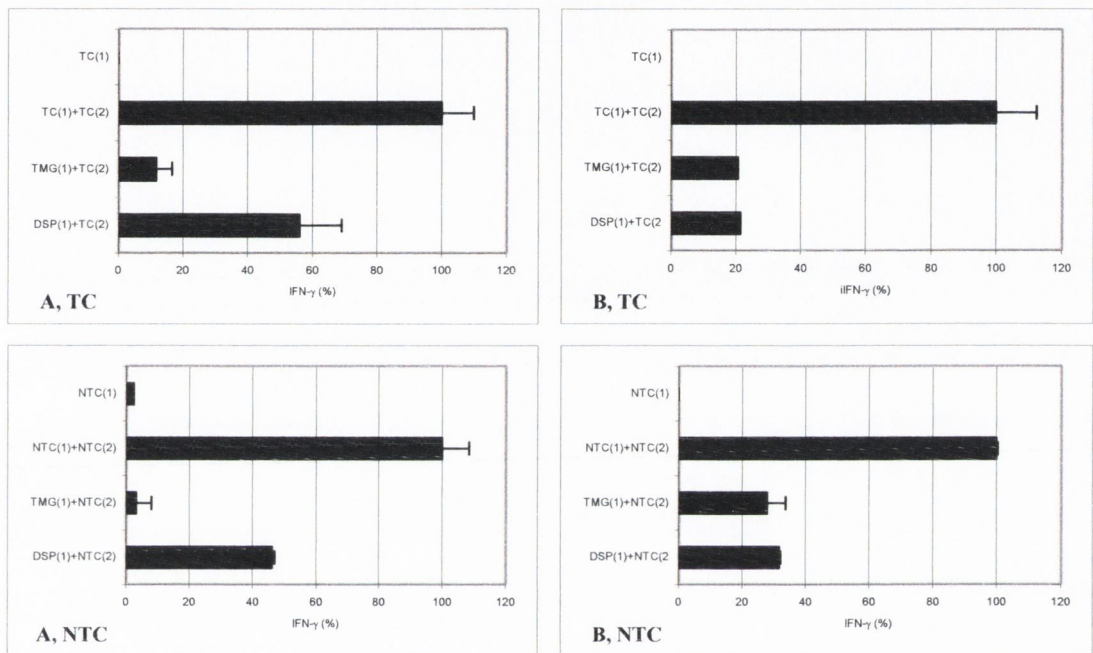
Fig 3.9 Priming of CD14⁻ PBMCs for IFN-γ production in response to tumour cell (TC) and non-tumour cell (NTC) extracts, with DSP and TMG peptides

DSP peptide (25µg/ml), TMG peptide (25µg/ml), cell extracts from MDA-MB-231 cells (TC, 110µg/ml) or cell extracts from MCF-12A cells (NTC, 110µg/ml), were incubated with 10^4 immature DCs and LPS (10µg/ml) for 24 hours. The loaded immature DCs were washed and incubated with 10^5 CD14⁻ PBMCs for 48 hr (1). The cells were cultured in the presence of with IL-2 (25ng/ml) for 10 days and then, incubated with 10^4 immature DCs loaded with 110µg/ml TC or NTC extracts without LPS for 48hr (2). Supernatants were tested for IFN-γ production by ELISA. Results with PBMCs from two healthy blood donors (A and B) were used. Values were plotted as ratio to the IFN-γ level produced after using TC or NTC for both priming (1) and stimulation (2).

stimulated by a single exposure to NTC [Fig. 3.9.A, B, NTC(1)], nor was IFN-γ produced if PBMCs were incubated with iDCs stimulated by LPS alone in the first stimulation and then challenged with iDCs loaded with NTCs without LPS in the second stimulation (data not shown).

Taken together, these data suggest that mimic peptide DSP resembles immunogenic peptides and/or protein components from both tumourigenic and non-tumourigenic breast cell lines and have the ability to stimulate human CD14⁻ PBMCs *in vitro*.

3.2.9 DSP is a mimic peptide with properties similar to antigens present in the antigen pool associated with Hsp70. 'Proof of concept' that DSP peptide is a mimic of Hsp70-PC

To determine if the DSP peptide resembles any Hsp70-associated MDA-MB-231 cellular peptide (Hsp70-PC) CD14⁻ PBMCs were first stimulated with LPS and Hsp70 or Hsp70-PC and then exposed to the mimic peptide DSP (second stimulation). As shown in figure 3.10.A, CD14⁻ PBMCs primed with LPS and Hsp70-PC respond very effectively to a second stimulation by DSP peptide with relative levels of IFN-γ over 155% in both donor A and B. IFN-γ levels are relative to that observed when the DSP peptide is used in both the first and second stimulation [DSP (1) +DSP (2)] (set at 100% to allow comparisons between individual experiments). When LPS and Hsp70 (that has been depleted of the associated peptides) was used for the first stimulation and DSP for the second stimulations, IFN-γ relative levels of 118% and 87% in donor A and B, respectively were detected. Thus the IFN-γ levels in this case were lower than those observed when the cells were primed with LPS and Hsp70-PC. As observed in Fig. 3.10.A, a single stimulation of CD14⁻ PBMCs with DSP peptide was not sufficient to elicit detectable IFN-γ production [Fig. 3.10.A&B; DSP (1)] nor was IFN-γ produced if PBMCs were incubated with iDCs stimulated by LPS alone in the first stimulation and then challenged with iDCs loaded with the DSP peptide without LPS in the second stimulation (data not shown). These data suggest that DSP peptide may represent an immunogen structurally resembling a cellular peptide within the pool of peptides complexed with Hsp70 and present in the Hsp70-PC enriched fraction of MDA-MB-231 cells.
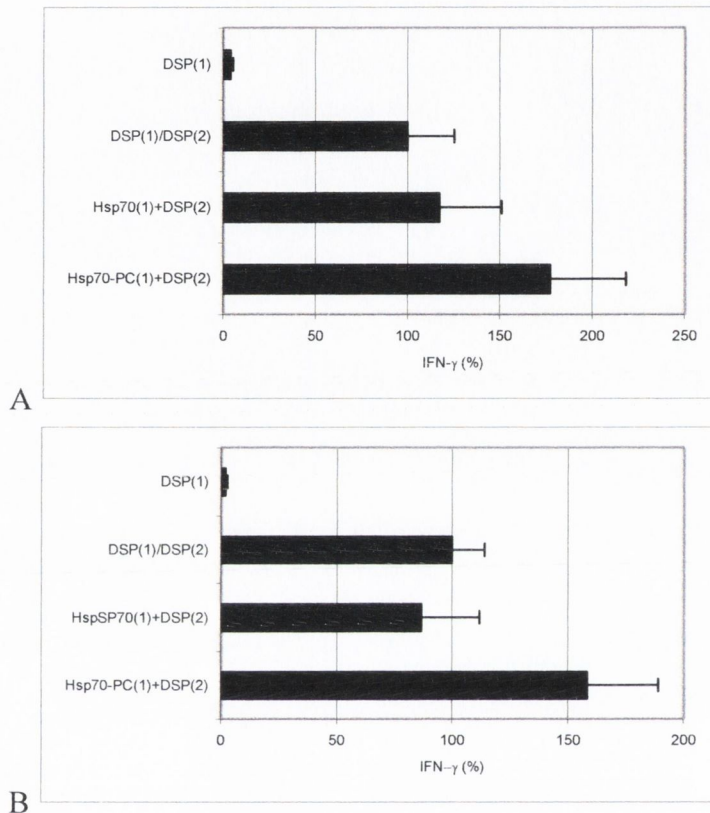
Fig 3.10 Secretion of IFN-γ by CD14⁻ PBMCs primed with iDCs loaded with LPS and Hsp70, Hsp70-PC from MDA-MB-231 cells or DSP peptides upon secondary stimulation with immature DCs loaded with DSP peptide

Hsp70 (5μg/ml), Hsp70-PC (5μg/ml) from MDA-MB-231 cells or DSP peptide (25μg/ml) were incubated with 10^4 iDCs and LPS (10μg/ml) for 24 hours. PBMCs from two healthy blood donors (A and B) were used. The loaded iDCs were washed and incubated with 10^5 CD14⁻ PBMCs for 48 hr (1). Supernatant from the wells containing LPS and DSP were tested for IFN-γ production by ELISA [DSP(1)]. The cells were cultured with IL-2 (25ng/ml) for 10 days and then, incubated with 10^4 iDCs loaded with DSP (25μg/ml) without LPS for 48hr (2). Supernatants were tested for IFN-γ production by ELISA. Values were plotted as ratio to the IFN-γ level produced after using DSP for both priming (1) and stimulation (2).

3.3 DISCUSSION

The primary aim of the work described in this chapter was to determine if the peptide DSP, isolated by the two-step reverse bio-panning procedure, is a functional mimic of peptides associated with Hsp-70 from MDA-MB-231 cells, in particular, if this peptide possesses immunogenic properties similar to those of the native Hsp70-PC. An *in vitro* assay was employed in which the ability of the synthetic peptides to activate CD14⁻ PBMCs to release IFN-γ, was tested.

Initially, the steps for the isolation, differentiation and maturation of PBMCs-derived iDCs were optimised. The plastic adherence method employed routinely by other groups to isolate CD14⁺ PBMCs yielded lower cell recovery than the CD14 retention method and the CD14 depletion method, both of which are based in the selection of magnetically-labelled specific cell populations. The CD14 depletion method yielded the best cell recovery but contamination with CD14⁻ cells was higher (low purity) than when using the CD14 retention method. Therefore the CD14 retention method was used in the subsequent experiments described in this chapter.

The differentiation of CD14⁺ PBMCs into iDCs and their subsequent maturation into mDCs was followed by flow cytometric analysis of cell surface marker expression. Incubation of CD14⁺ PBMCs for 6 days with 60ng/ml hrGM-CSF and 150ng/ml hrIL-4 led to their differentiation into iDCs as shown by the disappearance of CD14, the increased expression CD11c and the absence of expression of the maturation marker CD83. iDCs incubated with LPS for 24hr achieved a certain degree of maturation as was shown by upregulation of the expression of the maturation marker CD83. The inclusion of DSP, TMG and WHK did not augment LPS-induced DC maturation, as observed by examination of CD83 regulation. Surprisingly, DCs failed to express the co-stimulatory molecule CD86. This may be due to the incubation time being too short. It has previously been shown that while expression of the maturation marker CD83 can be induced following 4hr-incubation with LPS, CD86 surface induction required at least 24hr-incubation with LPS (Cao, Lee et al. 2005). The effect of the peptides should have been tested in the absence of LPS and therefore the results regarding the role of these peptides in DC maturation are inconclusive.

In vivo DC maturation can be influenced by a variety of factors, notably, inflammatory mediators such as IL-1, TNF, and microbial toxins such as LPS. However, it is unclear whether these factors are essential for DC maturation *in vitro* or whether

presented by some HLA class I molecules (data not shown). However, based on our model of recognisers and mimics described above, it is quite possible that peptides homologous to recogniser peptides may also be present in the pool of Hsp70-PC. For example, it is possible that antigenic peptides representing a recogniser/mimic pair such as the EGF Receptor/EGF ligand could be present in the PC pool associated with Hsp70 in the cell. EGF receptor is activated by autocrine or paracrine growth factor loops and is over-expressed in at least 50% of all epithelial malignancies (Earp, Calvo et al. 2003). Therefore in this scenario, the possibility may exist where both recogniser and mimic could be part of the Hsp70-PC pool. Supporting this view, the present study found that both TMG and DSP peptides, but not WHK, specifically bound to MDA-MB-231 cells indicating that either peptide can interact with cellular components within these cells.

Having established an assay to measure T-cell stimulation in response to peptides, it was then possible to investigate whether the DSP peptide represents a mimic of true tumour antigens present in tumour cells and in particular tumour antigens in the Hsp70-PC pool. To do this, T-cells were subjected to two consecutive stimulations by iDCs, loaded with either the same or different antigen pools. T-cells were found to be activated only if the iDCs were pulsed with the same or similar antigen(s) in the two stimulations. The method used here builds on the strategy in a recently reported *in vitro* test system for recognition of a particular antigen (first stimuli) in a pool of unknown antigens (second stimuli) (Fleischer, Schmidt et al. 2004).

Not surprisingly, MDA-MB-231 tumour cell extracts were found to contain certain antigens in common with those present in the Hsp70-PC fraction as they successfully stimulated T-cells previously primed with Hsp70-PC from the same tumour cells. Additionally, Hsp70 alone, in the absence of any associated peptides, was found to prime T-cells to respond to MDA-MB-231 tumour cell extracts. Thus Hsp70 in addition to chaperoning peptides into the antigen processing pathway of iDC, may also trigger IFN-γ production in a similar way to that of LPS (Blanchard, Djeu et al. 1986; Tough, Sun et al. 1997; Vabulas, Ahmad-Nejad et al. 2002; Wallin, Lundqvist et al. 2002). These findings are in agreement with previous data showing that Hsp70s can enhance the ability of APCs for antigen uptake (Todryk, Melcher et al. 1999; Noessner, Gastpar et al. 2002) and can activate T cells *in vitro* and *in vivo* (Breloer, Fleischer et al. 1999; MacAry, Javid et al. 2004). Therefore, one could envisage a pool of antigens (peptides) being chaperoned by adjuvant molecules such as Hsp70 which can also facilitate their uptake by the APCs through Hsp-specific receptors (e.g., CD91) in the case of tumour

tissue damage or necrosis. These peptides may be then re-presented to T-cells, through the MHC class I antigen processing pathway (cross-priming). Thus, the reconstitution of peptides with heat shock proteins such as Hsp70, might be an important strategy to ensure an enhancement of the T-cell response to peptides (Blachere, Li et al. 1997; Suzue, Zhou et al. 1997).

Using the same technique, it was also possible to demonstrate that DSP peptide resembles antigens present in total cell extracts from either tumourigenic (MDA-MB-231) or non-tumourigenic (MCF-12A) the breast carcinoma cell lines. Thus, DSP peptide may be mimicking a common antigen in both cell lines. Furthermore, it was found that the degree of T-cell stimulation by DSP peptide appeared to be dependant on the donor source of the PBMCs. This may be due to differences in the immune system of the two donors. In general, the number of iDCs used to stimulate T-cells was found to critical for the production of detectable levels of IFN-γ using the ELISA technique. The use of at least 10^4 iDCs and a T-cell:iDC ratio of at least 10:1 were key conditions to obtain detectable levels of the cytokines IL-12 and IFN-γ. The TMG peptide showed lower levels of T-cell stimulation following a second exposure to either extracts from MDA-MB231 or MCF-12A cell lines. This suggests that DSP may indeed represent a true mimic peptide with properties similar to cellular antigens of breast cell lines irrespective of their tumourigenic status. Furthermore, T-cells incubated initially with iDCs loaded with Hsp70-PC could be re-stimulated with iDCs loaded the DSP peptide, again suggesting that this peptide resembles antigenic peptides associated with the Hsp70 in these cell lines.

In conclusion, this study has shown that peptides isolated by a two-step reverse bio-panning approach can stimulate T-cells to secrete IFN-γ. However, since only one mimic peptide with T-cell stimulatory activity has been identified, it will be necessary to characterise additional mimic peptides before firm conclusions can be drawn on the general applicability of this approach. Adjustments to the bio-panning protocol such as (1) a pre-incubation of the phage library with non-tumour cell extracts prior to specific biopanning with the Hsp70-PC as bait, (2) the use of other model tumour cell lines, (3) the use of highly purified Hsp70-PC and at a higher concentration and (4) the use of mass spectroscopy to identify the peptides associated with Hsp70-PC followed by the use of their synthetic equivalents for biopanning may improve our chances of obtaining tumour specific mimics. Additionally it may be possible to conduct the initial bio-

panning *in situ* on tumour cell lines and/or solid tumours to identify 'recognisers' specific to the cell surface molecules of the tumour.

INTERACTION OF DSP AND TMG PEPTIDES WITH HUMAN CELL LINES AND BREAST TISSUE SECTIONS

4.1 INTRODUCTION

The results obtained in the previous chapter, using the two step T-cell stimulation assay, support a hypothesis that the DSP and TMG peptides resemble proteins present in the MDA-MB-231 (tumour) and MCF-12A (normal) breast cell lysates. Proteins in the MDA-MB-231 and MCF-12A cell lysates were able to interact with the same T cell clones as the DSP and TMG peptides. Thus, a certain degree of homology must exist between the DSP and TMG peptides and T-cell epitopes on proteins present in the cell extracts as the same T-cell clone can only be stimulated by different peptide sequences with equal or conserved amino acids in certain positions (active amino acids).

Since the peptide TMG was isolated as a recogniser phage that interacts with HSP70-PCs, in theory, this recogniser peptide should bind to Hsp70-PCs in the breast tumour cell line or to the original proteins from which the processed peptides associated with Hsp70 are derived. As expected by their definition of a mimic and recogniser pair, the DSP and TMG peptides were shown by phage ELISA (see chapter 3) to interact with each other. Therefore, it can be speculated their cellular homologous proteins could interact not only with each other but also cross-react with the peptides. Thus, DSP could interact with the TMG-homologous protein(s) and vice versa, TMG could interact with the DSP-homologous protein(s). To examine this hypothesis, biotinylated peptides were incubated with the breast cell lines MDA-MB-231, MCF-7 and MCF-12A grown to different confluency stages. The results presented in this chapter show that the DSP and TMG interact with proteins present in breast tumour cell lines in a highly reproducible manner and that this interaction decreases with increasing confluency of the cells. This pattern was also observed in a number of other cell lines such as normal (BPH-1) and tumour (PC-3) prostate cell lines, non-small cell lung carcinoma (NCI-H358) cell lines and non-transformed keratinocytes. MTT-based proliferation assays indicated that the non-small cell lung carcinoma NCI-H358 and normal breast cells MCF-12A showed the slowest growth rate . Interestingly, the levels of DSP and TMG binding molecule(s) in these two cell lines is much lower than in the rest of the cell lines tested.

In conclusion, the results indicate that the peptides DSP and TMG interact with proteins present in many cell types and that the expression of these binding partners appears to be down-regulated by cell-cell contact. This conclusion is supported by the staining patterns observed in breast tissue sections with the peptides.

4.2 RESULTS

4.2.1 Interaction of DSP and TMG peptides with MDA-MB-231, MCF-7 and MCF-12A cells is confluence-dependent

The results in Chapter 3 indicate that the DSP and TMG peptides could resemble protein motifs present in breast cells and are capable of inducing immune responses. To investigate the possibility that these peptides have binding partners in breast cells, the biotinylated DSP and TMG peptides were incubated with the cell lines MDA-MB-231, MCF-7 and MCF-12A. The mimic peptide WHK which was previously shown to lack immunomodulatory activity and which represented only 8% of the peptides displayed on phage isolated by the **NNYDDISLRARP** recogniser peptide (see Table 1 *Preface to this thesis* section) was used as a negative control.

MDA-MB-231, MCF-7 and MCF-12A cells were seeded at three different concentrations into a flat bottom 96-multiwell plate and incubated for 2 days at $37^{\circ}C$ in a humidified atmosphere of $5\%CO_2$. The confluency stage reached by the cells was 10-25%, 50-65% and 85-100%, respectively. The cell monolayers were fixed with paraformaldehyde, blocked and incubated with biotinylated DSP, TMG and WHK peptides as described in *Materials and Methods* section 2.2.3. To visualise peptide binding, the cells were subsequently incubated with ExtrAvidin-HRP conjugate and the HRP substrate 3,3'-diaminobenzidine (DAB) as described in *Materials and Methods* section 2.2.3.1. Bound peptide is detected by the formation of a brown precipitate as a result of oxidation of the substrate.

The results in Figure 4.1 show that the DSP and TMG peptides bind to MDA-MB-231 (Fig 4.1.DSPa and TMGa) and MCF-7 cells (Fig 4.1.DSPb and TMGb) in cells grown to 10-25% and 50-80% confluency. The intensity of the staining indicates that the quantity of peptide binding to cells is confluency-dependent with a higher staining intensity observed in cells grown to 10-25% confluency compared to the staining in cells grown to 50-65% confluency. Little or no staining is observed as cells reach 85-100% confluency. No binding to any of the cells tested was observed with the WHK peptide. This result suggests that there is a higher availability of the binding partner(s) when cell-cell contact is at a minimum and a lower or lack of availability as cell-cell contact increases. In MCF12-A cells (Fig 4.1.DSPc and TMGc), DSP and TMG peptide binding is only detected in cells grown to 10-25% confluency, indicating a different

Fig 4.1 Detection of DSP and TMG peptides binding to breast cell lines by cytochemistry
a: MDA-MB-231 cells, b: MCF-7 cells, c: MCF-12A cells. Cells at 10-25%, 50-65% and 85-100% confluency, were incubated with 65μM biotinylated DSP, TMG and WHK. Biotinylated peptides were detected with ExtrAvidin-HRP conjugate as described in *Materials and Methods* section 2.2.3 and 2.2.3.1 Magnification 200 x. The areas (1), (2), (3) and (4) were magnified to reveal more detail of the staining patterns.

DSP
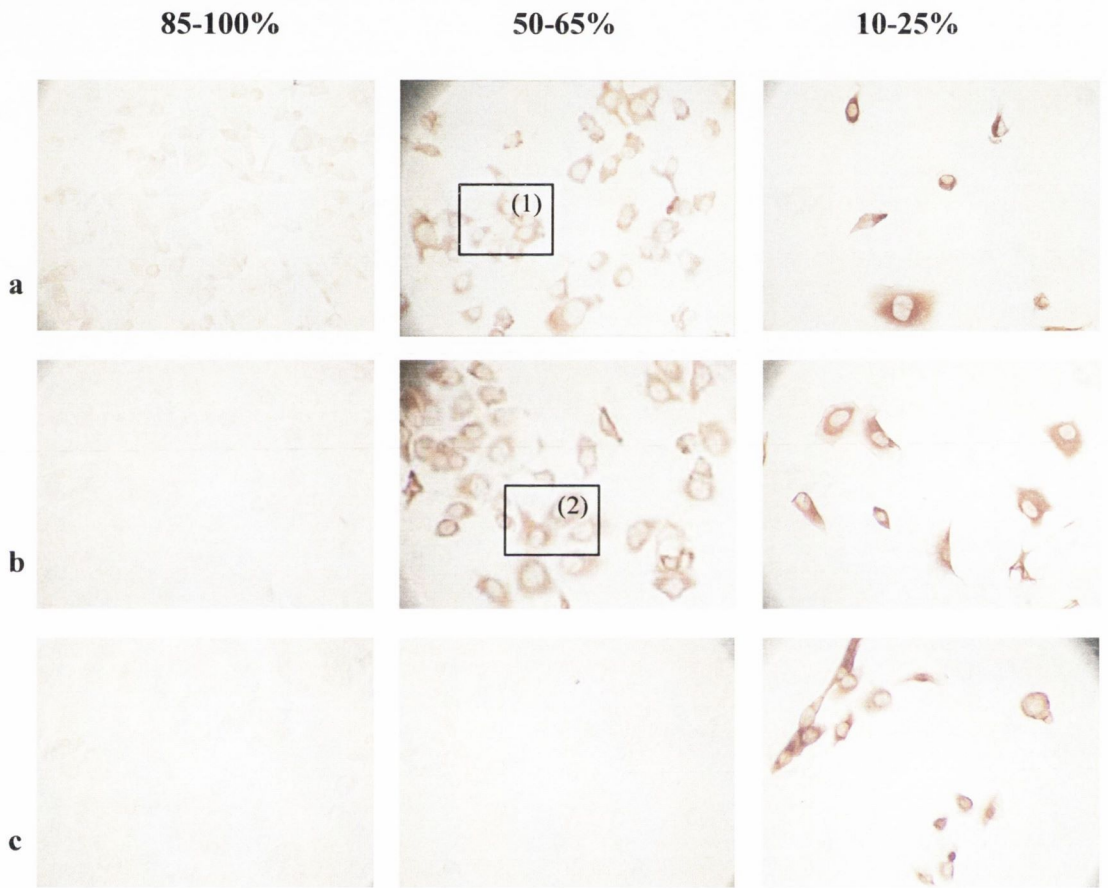
TMG
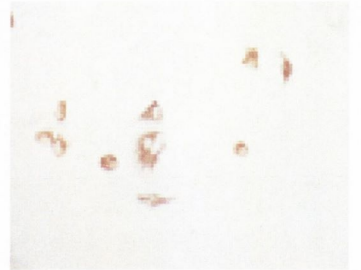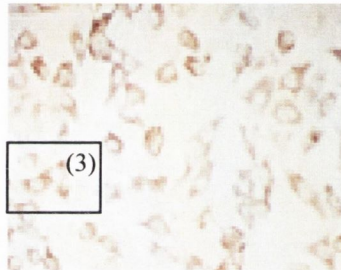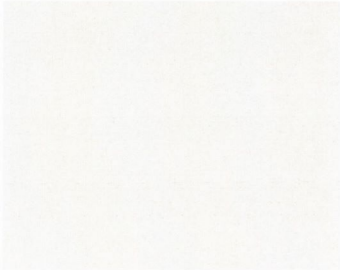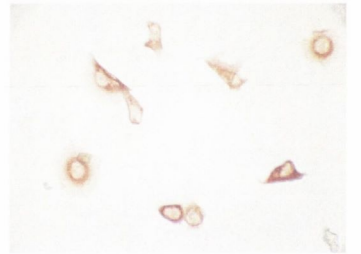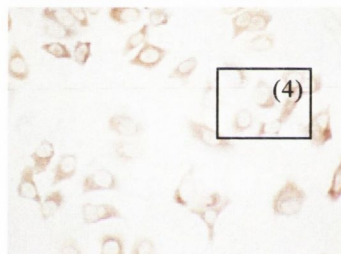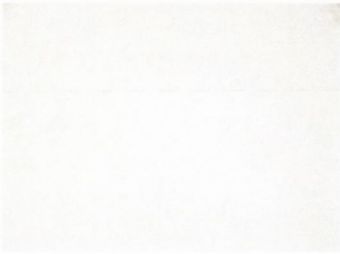
	85-100%	50-65%	10-25%
a		(3)	
b		(4)	
c			
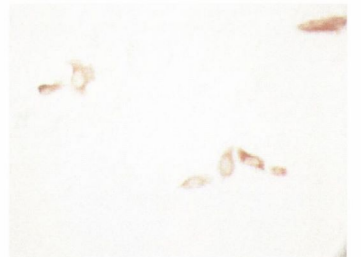
WHK

	85-100%	50-65%	10-25%

(1) **a**

(2) **b**

(3) **a**

(4) **b**
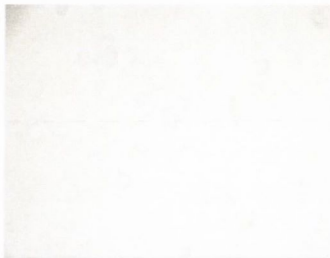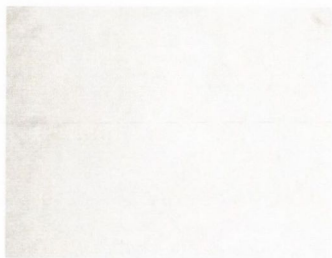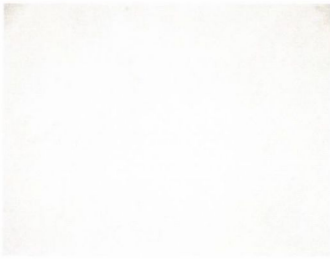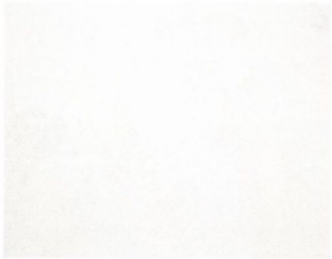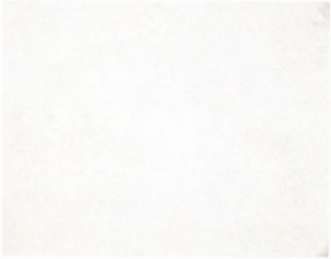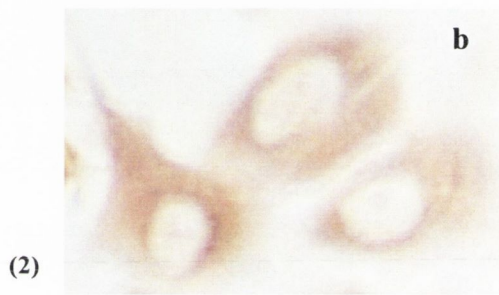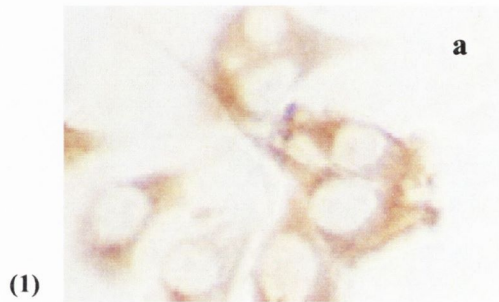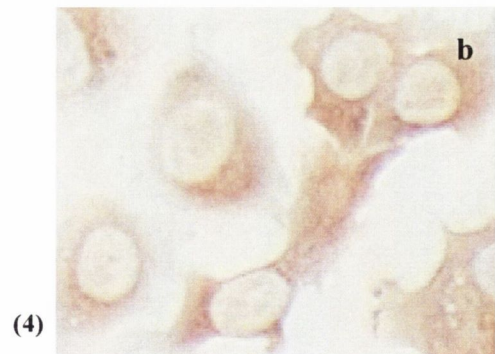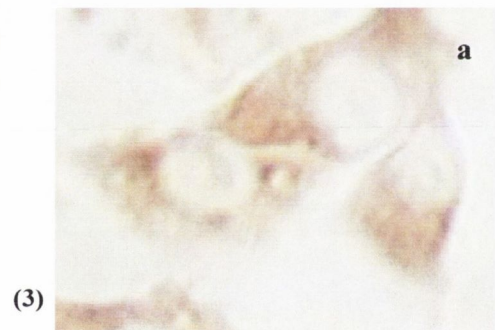
pattern of binding partner availability in this cell line. MCF-12A cells grow in domes once they reach 50-65% confluency (Fig 4.7), therefore cell-cell contact tends to be maximised. These cells, while not forming a fully confluent cell monolayer on dishes, behave as being fully confluent cells in terms of cell-cell contact. This increased cell-cell contact appears to correlate with lack of binding to DSP and TMG peptides. Therefore, the availability of the binding partner(s) appears to correlate with differential levels of expression at different cell-cell contact stages of the cells.

Re-sized pictures [Fig 4.1.(1), (2), (3) and (4)] of DSP and TMG peptides bound to MDA-MB-231 and MCF-7 cells grown to 50-80% confluency indicates that the binding is exo-nuclear. The diffuse nature of the staining indicates a cytoplasmic localisation.

The above experiment, with cells grown to 50-65% confluency, was repeated using chamber slides and peptide binding was detected with Streptavidin-Cy3 as described in the *Materials and Methods* section 2.2.3.2. Cells emitting red fluorescence following irradiation at 510-560 nm indicate binding of the biotinylated peptide. The cell nuclei were stained with the fluorochrome 4′,6-diamidino-2-phenylindole (DAPI) which was visualised using a 340-380 nm excitement filter. The images obtained with both filters were superimposed (*) using Adobe Photoshop software. Figure 4.2 shows binding of DSP (Fig 4.2.DSP.A and DSP.B) and TMG (Fig 4.2.TMG.A and TMG.B) peptides to MDA-MB-231 and MCF-7 but not MCF-12A cells (Fig 4.2 DSP.C and TMG.C). No staining was observed if cells were incubated in the absence of peptide (Fig4.2 NP.A, NP.B and NP.C). No binding to any of the three cell lines was observed with the WHK peptide (negative control) (Fig 4.2 WHK.A, WHK.B and WHK.C). The results shown in this section are representative of six independent experiments.

4.2.2 Characterisation of the interaction of DSP and TMG peptides with MDA-MB-231 cells

The cell-peptide interaction experiment described in section 4.2.1 was further validated using MDA-MB-231 cells, since this was the cell line from which the Hsp70-PC protein preparation, used as bait for the bio panning experiment that led to the isolation of the DSP/TMG peptides, was isolated.

Fig 4.2 Immunofluorescence detection of DSP and TMG peptides binding to breast cell lines

A: MDA-MB-231 cells, B: MCF-7 cells, C MCF-12A cells. Cells at 50-65% confluency were incubated with 65µM biotinylated DSP, TMG and WHK and labelled with Streptavidin-Cy3 as described in *Materialsl and Methods* section 2.2.3 and 2.2.3.2. Bound peptide is detected by Cy3 (red) fluorescence using a 510-560 nm excitation filter. DAPI (blue) staining of DNA is detected using a 340-380 nm excitation filter. NP: Cells in which no peptide was added (negative control). Magnification 200 x. Pictures taken with both filters were superimposed (*) using Adobe Photoshop software.

DSP

(*)

A

B

C

TMG

(*)

A

B

C

WHK

(*)

A

B

C

NP

(*)

A

B

C
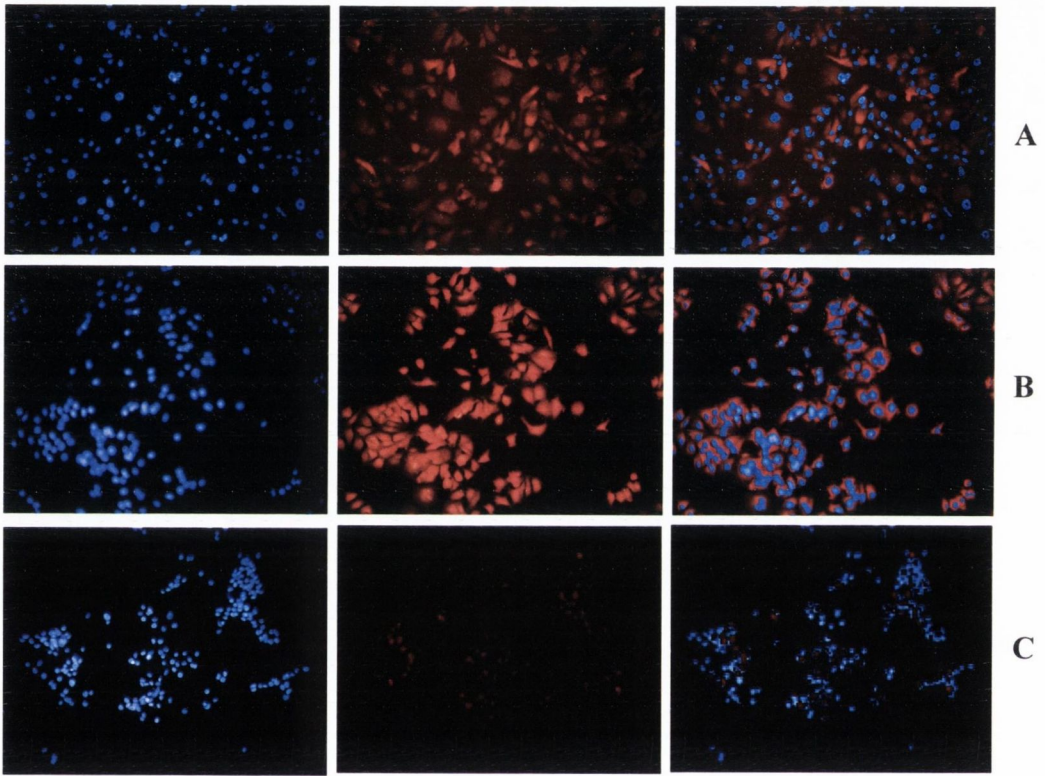
4.2.2.1 The interaction of DSP and TMG peptides with MDA-MB-231 cells is concentration-dependent

Approximately 5×10^3 MDA-MB-231 cells were seeded into a flat bottom 96-multiwell plate and grown until 50-65% confluent. The cells were then incubated in triplicate with biotinylated DSP and TMG peptides as described in *Materials and Methods* section 2.2.4 and 2.2.4.1. Duplicate samples were additionally permeabilised by incubation with 0.05% NP-40 PBS for 10 min.
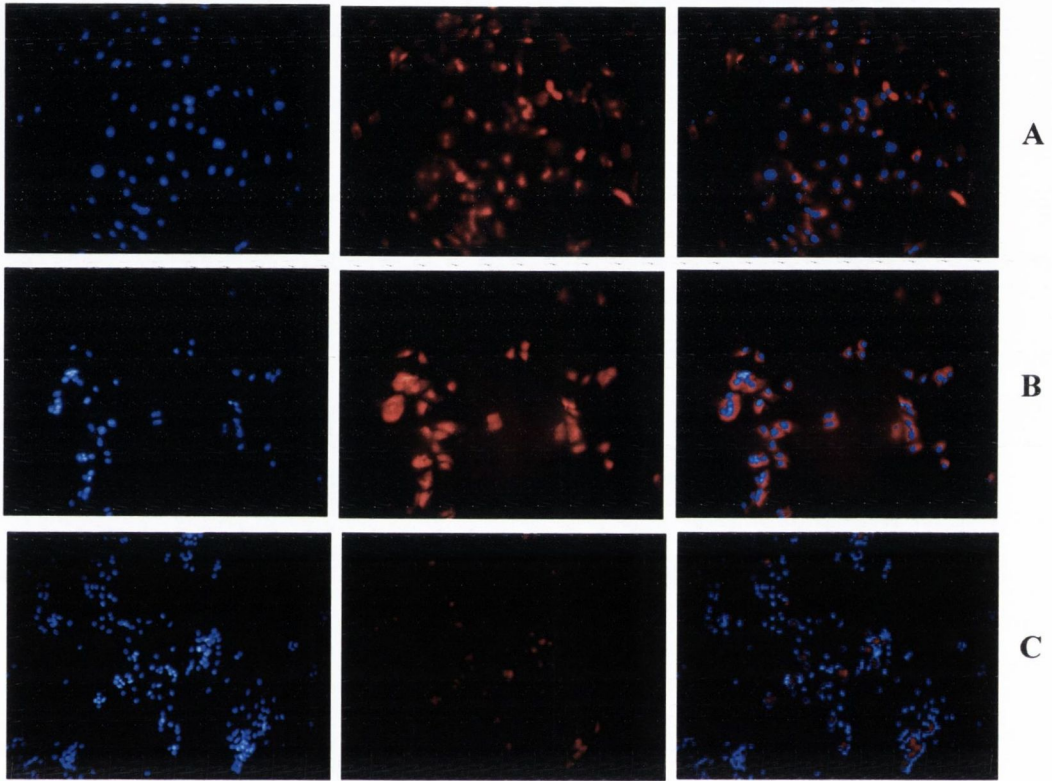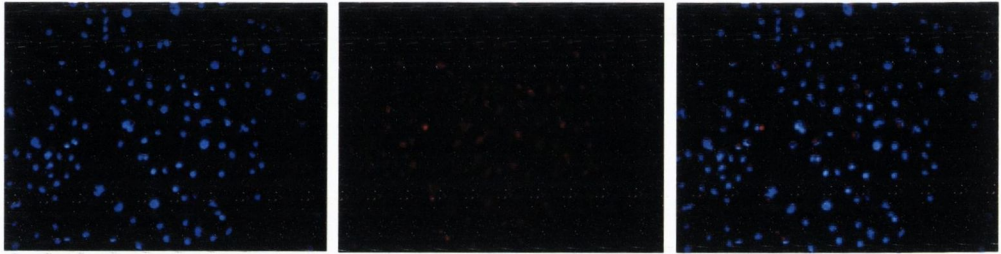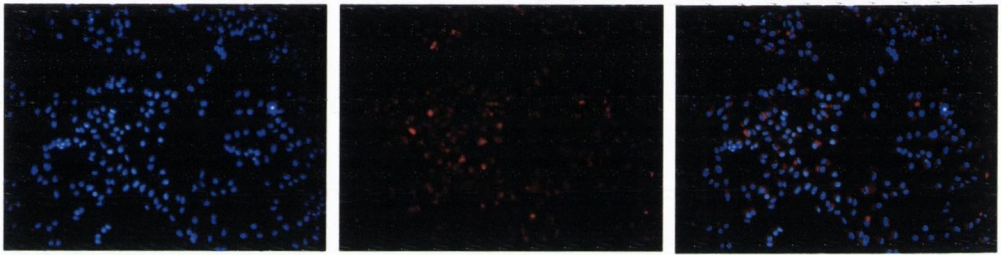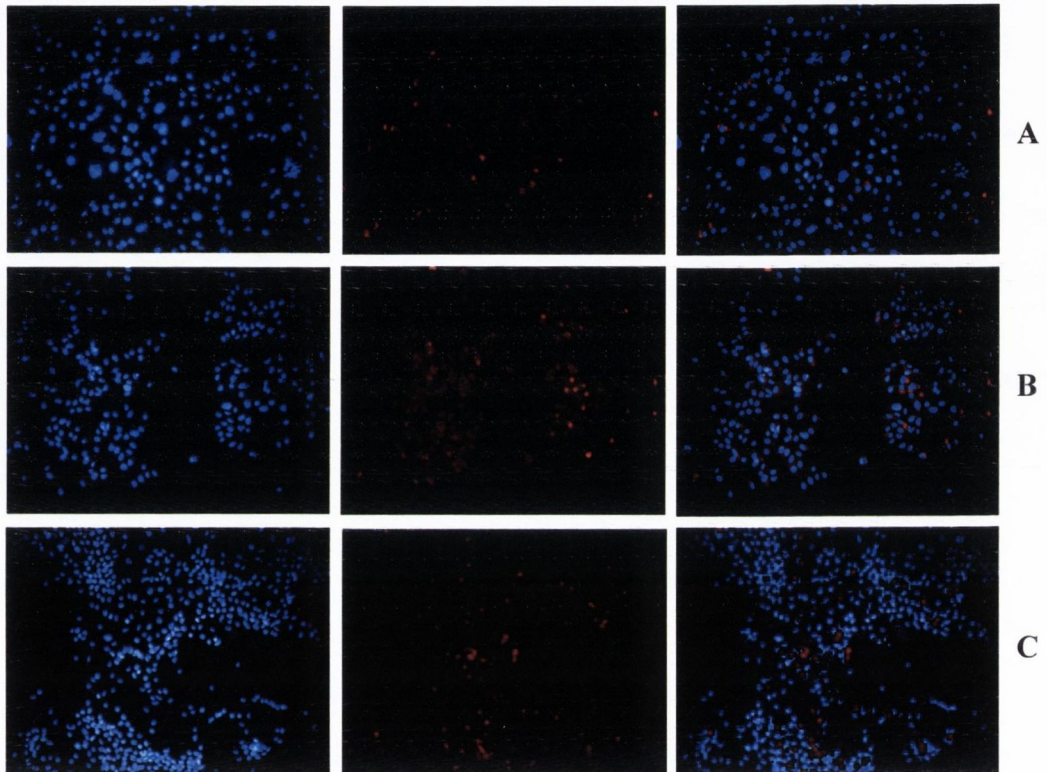
Figure 4.3 shows that the amount of DSP and TMG peptides binding to MDA-MB-231 cells increases with increasing concentration of peptide. The sensitivity limit for detection of DSP and TMG binding is 40μM (Fig 4.3.DSP column) (Fig 4.3.TMG column), however, after permeabilisation of the cell membranes with NP-40 binding is observed at a concentration of 20μM (Fig 4.3.DSP+NP-40 column) (Fig 4.3.TMG+NP-40 column). Treatment of the cells with NP-40 augmented the detection of the DSP and TMG peptide interactions with the cells. The intensity of the staining after incubation of permeabilised MDA-MB-231 cells with the DSP peptide is higher (Fig 4.3.DSP+NP-40 column) than that observed with the TMG peptide (Fig 4.3.TMG+NP-40 column) suggesting a higher expression of DSP-binding partner(s) than TMG-binding partner(s). The results shown in this section are representative of three independent experiments.

4.2.2.2 The interaction of DSP and TMG peptides with MDA-MB-231 cells is not biotin-dependent

The results outlined in section 4.2.1 indicate that DSP and TMG peptides bind to MDA-MB-231 cells grown to 50-65% confluency. The peptides employed are biotinylated, thus there is a possibility that the biotin moiety may be contributing to the binding observed. To test this hypothesis MDA-MB-231 cells at 50-65% confluency were fixed, permeabilised, blocked and incubated in triplicate with D-biotin prior to incubation with the biotinylated peptides as described in the *Materials and Methods* section 2.2.4.and 2.2.4.2. Samples treated without D-biotin were used as positive controls and without peptides as negative controls.

The results in Figure 4.4 show that the intensity of the staining due to binding of the peptides to the cells (Fig4.4 DSP-BIOTIN and TMG-BIOTIN) was not diminished by incubation with D-biotin (Fig4.4 DSP+BIOTIN and TMG+BIOTIN).

Fig 4.3 DSP and TMG peptides binding to MDA-MB-231 cells is concentration-dependent

Cells were fixed at 50-65% confluence stage, incubated with 130µM, 90 µM, 65 µM, 40 µM, 20 µM and 1 µM biotinylated DSP and TMG and detected with ExtrAvidin HRP conjugate as described in *Material and Methods* section 1.2.3 and 1.2.3.1. DSP+NP-40 and TMG+NP-40: Duplicate samples were additionally permeabilised by incubation with 0.05% NP-40 PBS. Magnification 200 x.

DSP   DSP+NP-40

1 μM

20 μM

40 μM
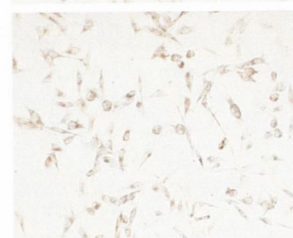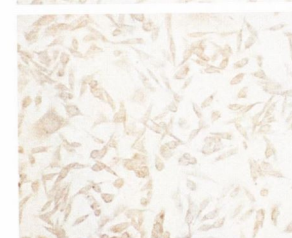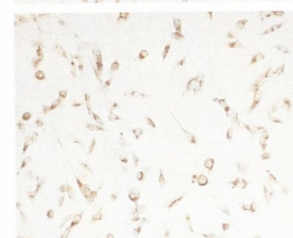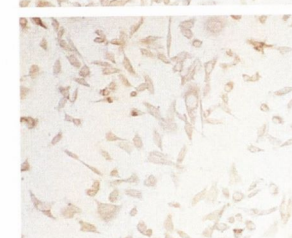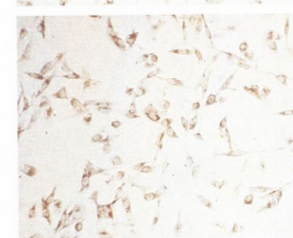
65 μM

90 μM

130 μM

	TMG	TMG+NP-40	

1 μM

20 μM

40 μM

65 μM

90 μM

130 μM

+BIOTIN　　　　　**−BIOTIN**

DSP

TMG

NP
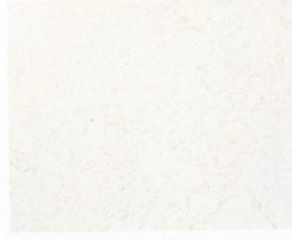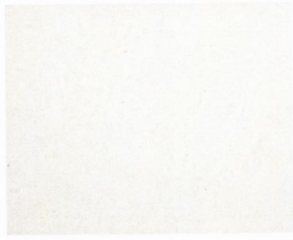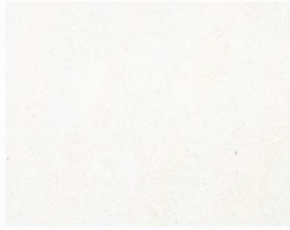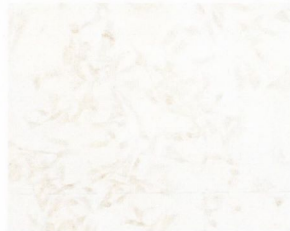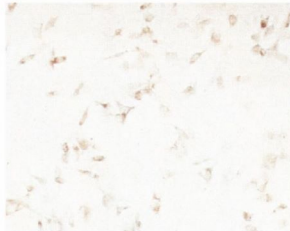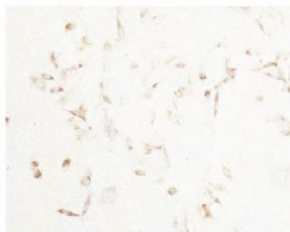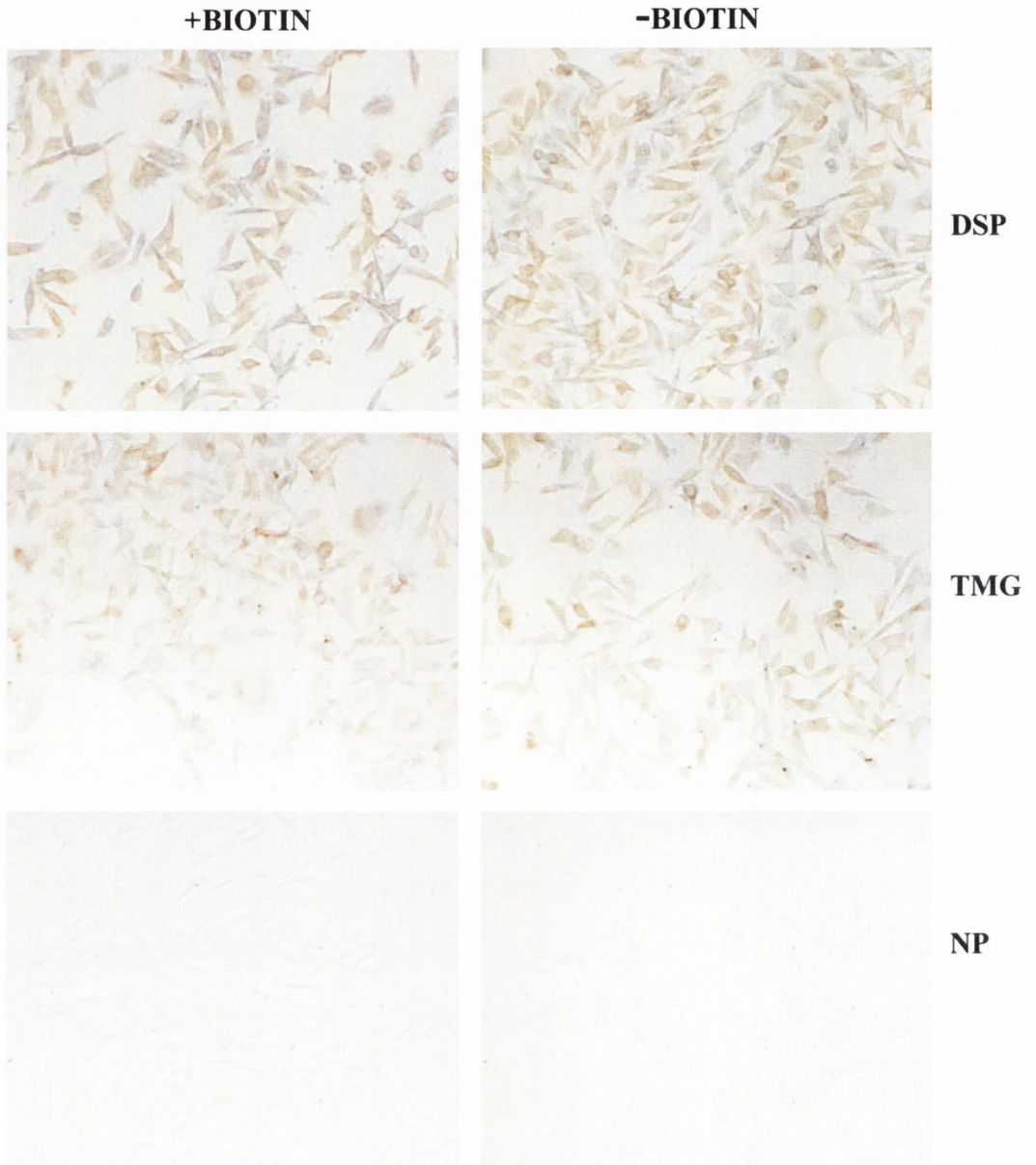
Fig.4.4 DSP and TMG peptides binding to MDA-MB-231 cells is not biotin-dependent

Cells were fixed at 50-65% confluency stage, incubated with 130µM biotinylated DSP and TMG or no peptide (NP) in the presence (+BIOTIN) or absence of 130µM D-biotin (-BIOTIN) and labelled with ExtrAvidin HRP conjugate as described in *Materials and Methods* section 2.2.4 and 2.2.4.2 Magnification 200 x.

Cells incubated with no peptide in the presence or absence of D-biotin (Fig4.4 NP+BIOTIN and NP-BIOTIN) did not show staining. In conclusion, D-biotin does not interfere with the binding of DSP and TMG peptides to MDA-MB-231 cells. This result, in addition to the failure of WHK to bind cells, shows that the interaction of DSP and TMG peptides with MDA-MB-231 cells is specific.

4.2.2.3 The interaction of DSP and TMG with MDA-MB-231 cells can be competed with non-biotinylated peptide

To examine the specificity of DSP and TMG interaction with MDA-MB-231 cells, cells at 50-65% confluency were fixed, permeabilised, and incubated in triplicate with 400 μM non-biotinylated DSP, TMG or no peptides, followed by incubation with 40 μM biotinylated DSP and TMG peptides as described in *Materials and Methods* section 2.2.4 and 2.2.4.3. As shown in Figure 4.5, incubation of MDA-MB-231 cells with non-biotinylated DSP or TMG peptides blocks the binding of biotinylated DSP (Fig 4.5.bDSP+DSP) or TMG (Fig 4.5.bTMG+TMG) peptides, respectively. In the absence of non-biotinylated peptide, normal staining with biotinylated DSP (Fig 4.5.bDSP-DSP) and TMG peptides (Fig 4.5.bTMG-TMG) is observed (positive control). This result adds to the findings in sections 4.1, 4.2.2 and 4.2.3 and corroborates the validity of the methodology used to test peptide-cell interactions. In conclusion, the interaction between DSP and TMG peptides and MDA-MB-231 cells is specific.

4.2.3 Interaction of DSP and TMG peptides with non-breast cells

To determine if the interaction of the TMG and DSP peptides with cellular components was specific to breast cells, the experiment described in section 4.2.1 was repeated using the following non-breast cell lines: human prostate normal cell line BPH-1, human tumour prostate cell line PC-3, human non-small cell lung carcinoma cell line NCI-H358 and human keratinocytes .

The results shown in Figure 4.6 indicate that both the DSP and TMG peptides show binding to BPH-1, PC-3 and human keratinocytes grown to 10-25% and 50-80% confluency while the WHK peptide does not bind to any of the cells tested. As previously observed, the intensity of the staining indicates that peptide binding to cells is confluency-dependent with a higher staining intensity in cells grown to 10-25%

bDSP−DSP

bDSP+DSP
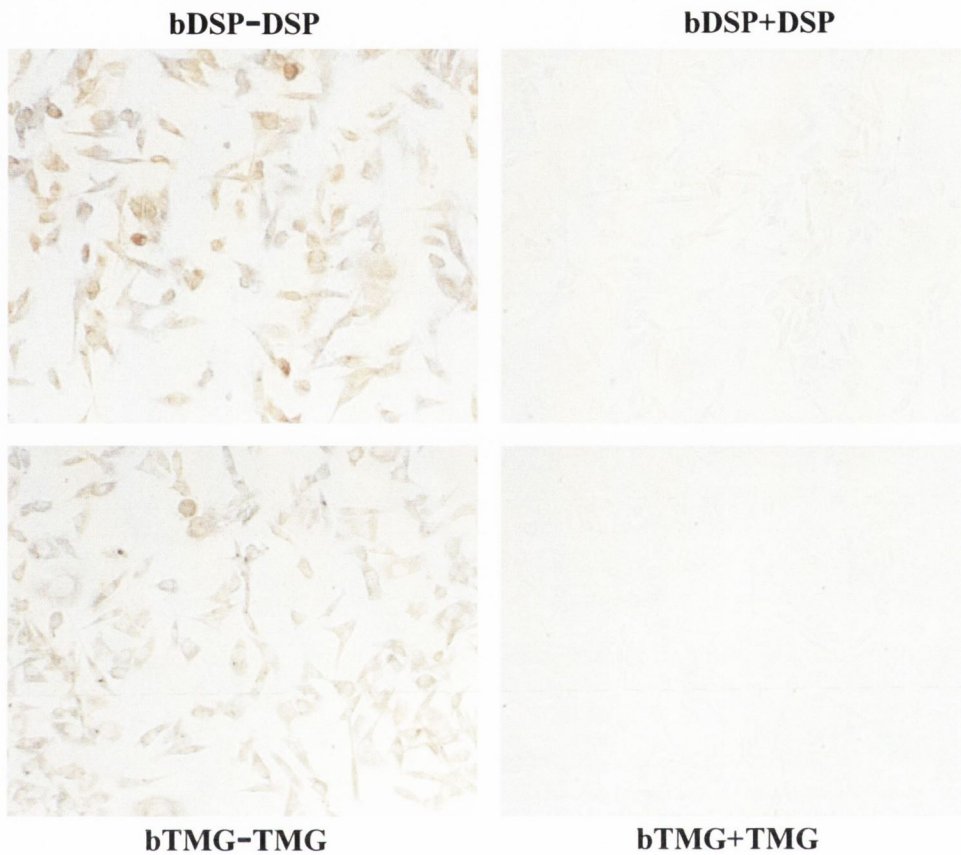
bTMG−TMG

bTMG+TMG

Fig.4.5 DSP and TMG peptides binding to MDA-MB-231 cells is specific

Cells were fixed at 50-65% confluency stage, incubated with 400µM non-biotinylated DSP (+DSP), TMG (+TMG) or no peptide (-TMG, -DSP) firstly, and then with 40µM biotinylated DSP (bDSP) and TMG (bTMG). Bound peptide was detected with ExtrAvidin HRP conjugate as described in *Materials and Methods* section 2.2.4 and 2.2.4.3 Magnification 200 x.

Fig 4.6 DSP and TMG peptides binding to non-breast cell lines

BPH-1: normal prostate cell line, PC-3: tumour prostate cell line, NCI-H358: non-small cell lung carcinoma. Cells were fixed at 10-25% and 50-65% confluency stage, and incubated with 65µM biotinylated DSP, TMG and detected with ExtrAvidin HRP conjugate as described in *Materials and Methods* section 2.2.3 and 2.2.3.1. Magnification 200x

BPH-1

50-65%C	10-25%C	

DSP

TMG

WHK
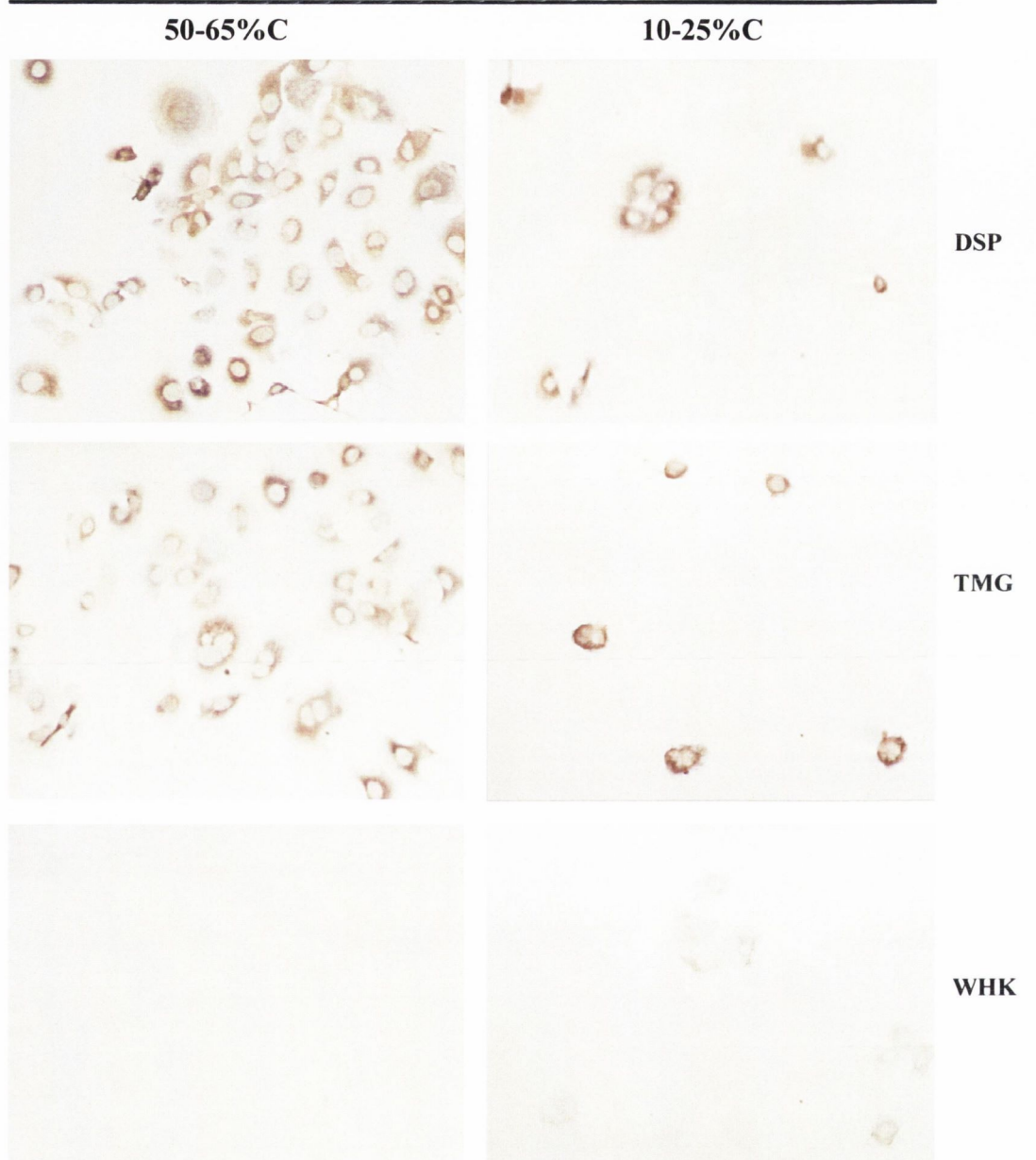
PC-3
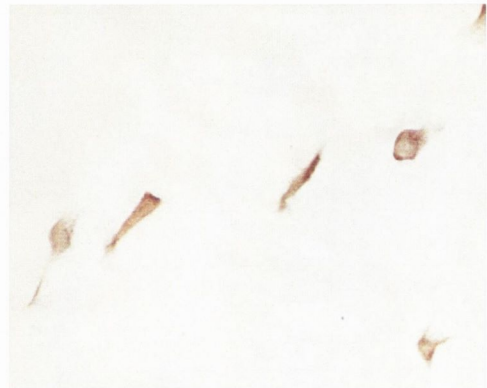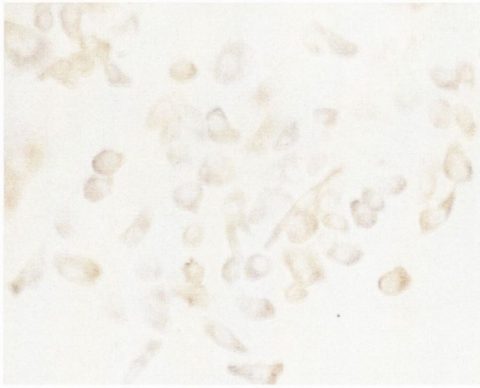
50-65%C	10-25%C	

DSP
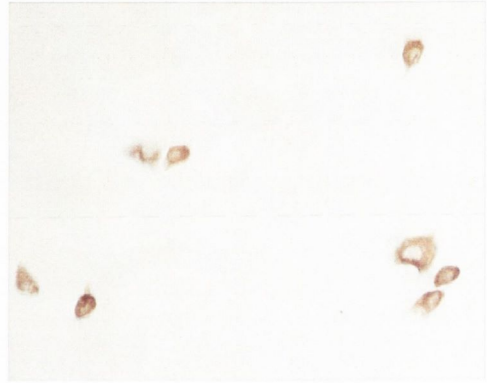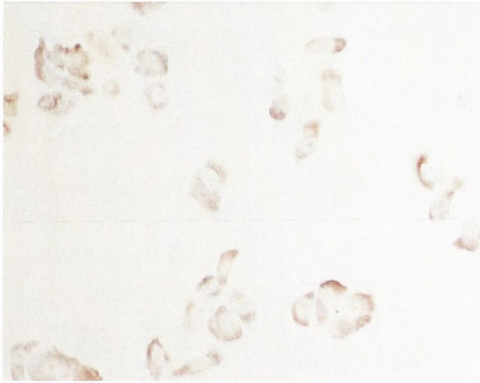
TMG

WHK

KERATINOCYTES
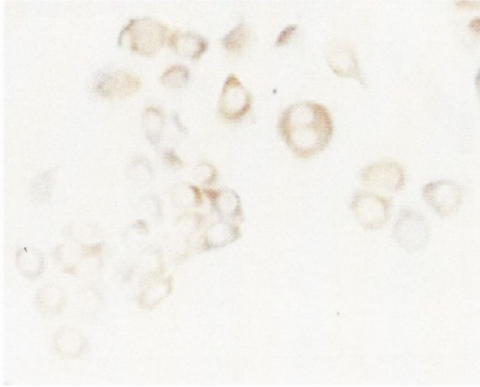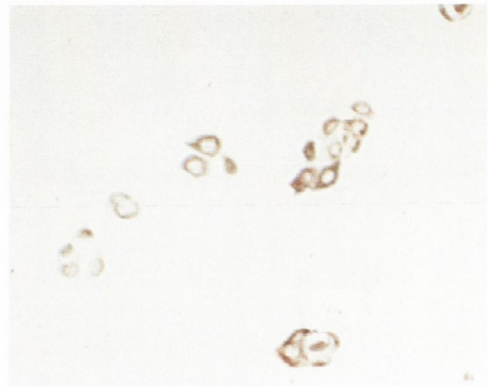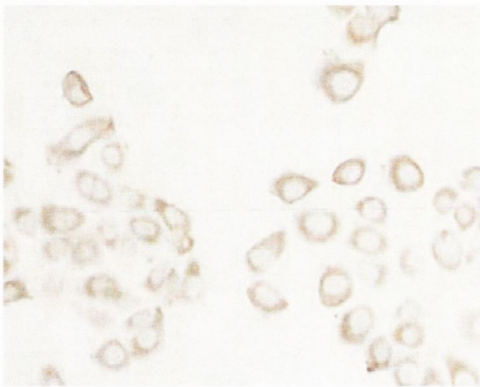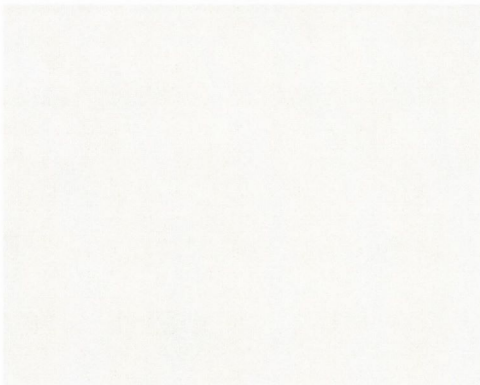
50-65%C	10-25%C	
		DSP
		TMG
		WHK

NCI- H358
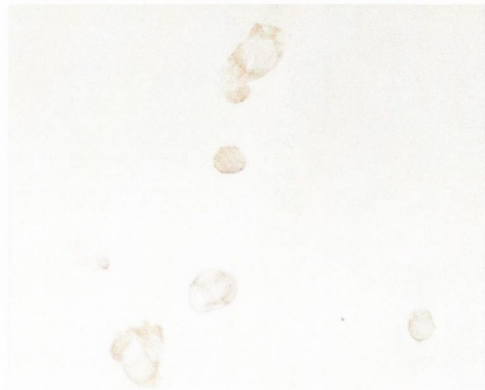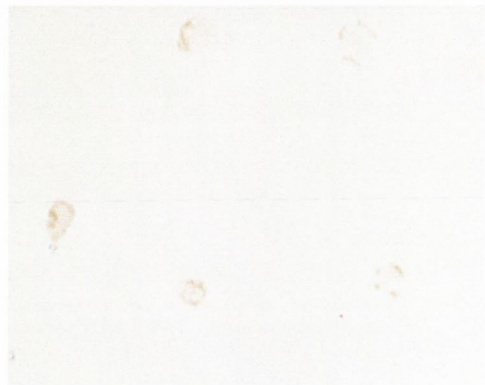
	50-65%C	10-25%C	
			DSP
			TMG
			WHK

confluency compared to the intensity observed in cells grown to 50-80% confluency. Very weak staining is observed in cells 85-100% confluent (data not shown). This result could indicate a higher expression of the binding partner/s at stages with diminished cell-cell contact and a lower or lack of expression in the case of greater cell-cell contact. In NCI-H358 cells, DSP and TMG binding is only observed in cells grown to 10-25% confluency, indicating a different pattern of binding partner expression, when compare to the rest of the cell lines tested. This staining pattern is similar to that observed with MCF-12A cells. As shown in Figure 4.7 MCF-12A and H358 grow in domes, therefore cell-cell contact tends to be maximized even at low cell density. These cells although not confluent, behave as fully confluent cells in terms of binding to DSP and TMG peptides. These new results support the hypothesis formulated in section 4.1 that the expression of TMG and DSP binding partners correlates with the degree of cell-cell contact between the cells.

4.2.4 Cell growth curves: MDA-MB-231, MCF-7, MCF-12A, Keratinocytes, NCI-H358 and BPH-1 cells.

To determine if the binding of the DSP and TMG peptides is dependent on the metabolic activity of the cells, the metabolic activity in each of the cell cultures was examined. This assay employs the MTS tetrazolium compound (Owen´s reagent) that is bioreduced by the cells into a coloured formazan product that is soluble in tissue culture medium. This conversion requires Dihydronicotinamide Adenine Dinucleotide phosphate (NADPH) or, Dihydronicotinamide Adenine Dinucleotide (NADH) produced by Dehydrogenase enzymes in metabolically active cell. Fig 4.8.A shows the metabolic activity of several cell lines for 3.5hr at time points 12, 24, 36 and 48 hr. The slope of the curves plotted for each cell line was calculated using GraphPad Prism program (Fig. 4.8B). The lowest rates of metabolic activity is observed (in bold) in the two cell lines MCF-12A and H358 as indicated by the slopes of the curves. This result indicates that cells such as MCF-12A and NCI-H358 that grow with a higher capacity for cell-cell contact (domes, Fig 4.7) have a lower growth rate than those cell lines which tend to form monolayers. These characteristics are correlated with lack of binding of the TMG and DSP peptides. Interestingly, under the conditions where peptide binding is observed with these cell lines (10-25% confluency) the cells have not formed domes and are attached as a single layer to the dishes.
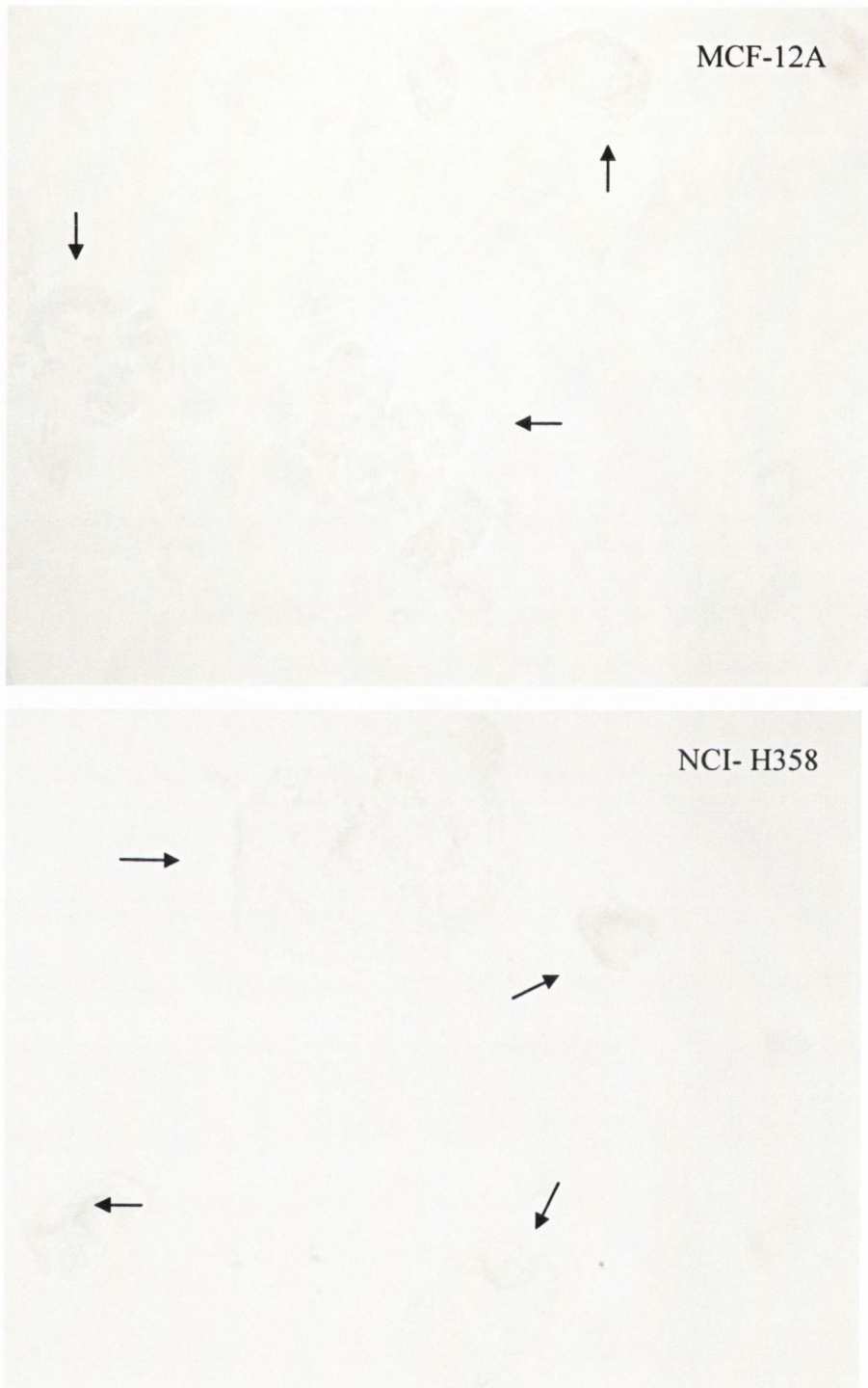
Fig 4.7 Demonstration of dome formation by MCF-12A and NCI-H358 cells
Re-sizing of pictures (200x) of MCF-12A and H358 cells at 50-65% confluency
in flat bottom 96-multiwell plate. Arrows indicate the localization of the domes.

A
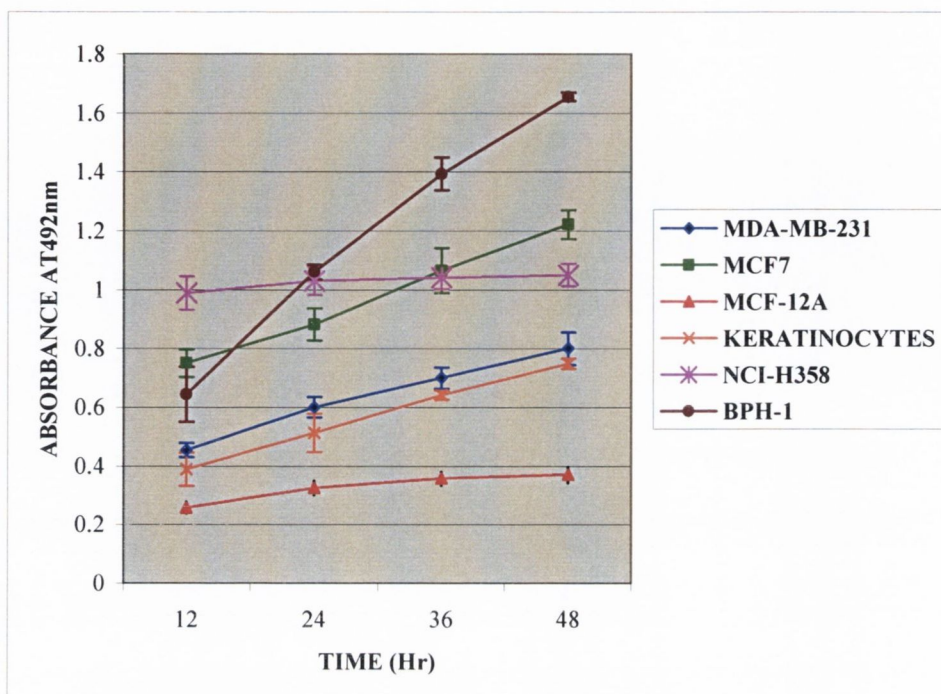
B

CELL LINES	SLOPE	ERROR
MDA-MB-231	0.009483	± 0.0006640
MCF-7	0.01330	± 0.0005672
MCF12A	**0.003085**	**± 0.0007216**
KERATINOCYTES	0.01005	± 0.0002804
NCI-H358	**0.001617**	**± 0.0004643**
BPH-1	0.02805	± 0.002027

Fig 4.8 Growth of MDA-MB-231, MCF-7, MCF-12A, Keratinocytes, NCI-H358 and BPH-1 cell lines for 48h

Aproximately 10^4 cells were seeded in flat bottom 96-multiwell plates and cultured for 48 hr. At time points 12hr, 24hr, 36hr and 48hr, absorbance readings at 492 nm were taken to measure transformation of the reagent CellTiter Aquous by the mitochondrial respiration chain as described in the *Materials and Methods* section 2.2.5.1.A: Plot of the absorbance reading data at 492nm at 12hr, 24hr, 36hr and 48hr. B: Table containing the slopes and slope errors of the curves plotted in A. Slopes were calculated with the first three time point corresponding absorbance values.

4.2.5 Cytotoxicity test of DSP and TMG on MDA-MB-231, MCF-7, Keratinocytes and BPH-1 cells

Guided by the growth curve plotted for each cell line in Figure 4.8, each cell line was seeded at different cell densities in order to approximately synchronise their exponential growth phases (see Materials and Method section 2.2.5.1). The metabolic activity of the cells was measured for a period of 3.5 hrs at specific intervals following seeding.

Figure 4.9 shows the metabolic activity at time points 60hr, 84hr, 108hr and 132 hr. It can be observed that all the cell lines are in exponential phase, apart from MCF-7 that appears to be reaching stationary phase at time point 132hr. Therefore time points 84 and 108 hr were chosen to test the effect of different concentrations of DSP and TMG peptides on the growth of MDA-MB-231, MCF-7, Keratinocytes and BPH-1 cells lines. The cell lines examined had previously shown an interaction with DSP and TMG peptides at 50-65% confluency.

Non-biotinylated peptides DSP, TMG and WHK, at increasing concentrations, were added in quadruplet wells 84hr after seeding and absorbance in these wells was read 24hr later (see Materials and Method section 2.2.5.2). The results in Figure 4.10 show that incubation of cells with the DSP peptide led to slight stimulatory effects in MDA-MB231 and MCF-7 cells at the highest concentration of the peptides while no effect was observed with the BPH1 cells. A slight inhibitory effect was observed in keratinocytes incubated with the DSP peptide at high concentrations.

Incubation of the cells with the TMG peptide had no effect on MDA-MB 231 cells or keratinocytes but a slight stimulatory effect was observed in MCF-7 and BPH-1 cells. Finally, incubation of the cells with the WHK peptide resulted in a slight stimulatory effect in MDA-MB 231 and MCF-7 cells but a slight inhibitory effect in keratinocytes and BPH-1 cells. Interestingly, more cell debris are observed in WHK treated cells (data not shown) when compared to DSP or TMG treated cells at higher peptide concentrations. Thus, differential effects by the peptides on the metabolic activity are seen in different cells. Due to the differential effects of the peptides on the metabolic activity of the various cell lines, it is hard to draw any firm conclusions regarding the effects of these peptides on these cells.
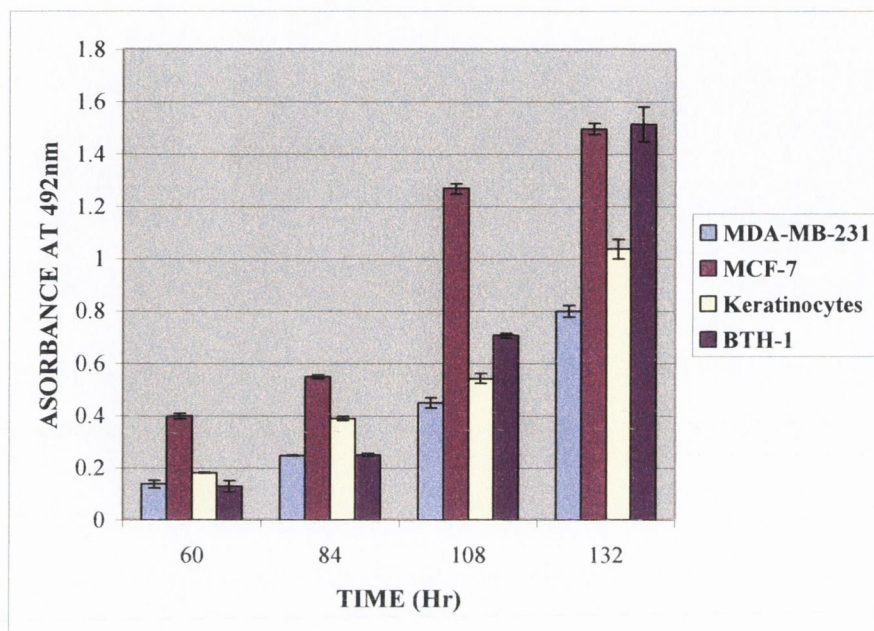
Fig 4.9 Growth of MDA-MB-231, MCF-7, MCF-12A, Keratinocytes, and BPH-1 cell lines for 132hr
Approximately the following cell numbers were seeded in flat bottom 96-multiwell plates and cultured for
132 hr: 10^3 MCF-7, MCF-12A and keratinocytes, 2×10^3 MDA-MB-231 and 4×10^2 BPH-1 cells. At time
points 60hr, 84hr, 108hr and 132hr, absorbance readings at 492 nm were taken to measure transformation
of the reagent CellTiter Aquous by the mitochondrial respiration chain as described in the Materials and
Methods section 2.2.5.1.**A:** Plot of the absorbance reading data at 492nm at 12, 24, 36 and 48hr.

Fig 4.10 Cytotoxicity of DSP and TMG peptides on MDA-MB-231, MCF-7, Keratinocytes, and BPH-1 cell lines

Approximately the following cell numbers were seeded in flat bottom 96-multiwell plates and cultured for 132 hr: 10^3 MCF-7 and keratinocytes, $2x10^3$ MDA-MB-231 and $4x10^2$ BPH-1 cells. Non-biotinylated peptides DSP, TMG and WHK at concentration of 0, 650fM, 650pM, 650nM and 65μM were added in quadruplet wells 84hr since seeding, and absorbance at 492nm was read 24hr later to measure transformation of the reagent CellTiter Aquous by the mitochondrial respiration chain as described in the Materials and Methods section 2.2.5.2.
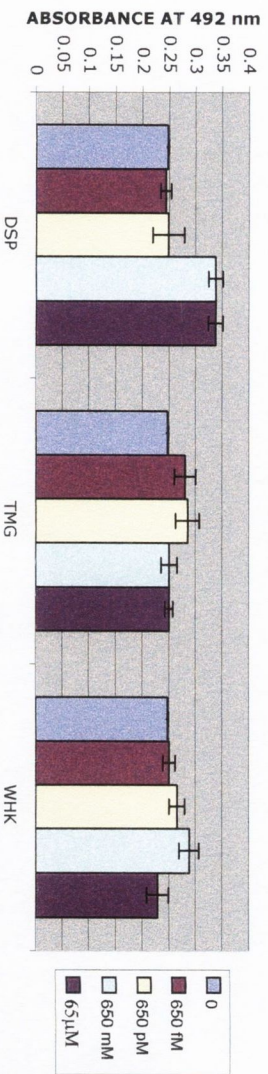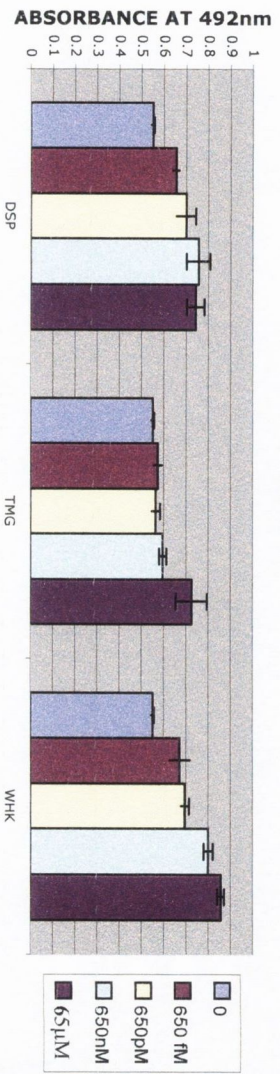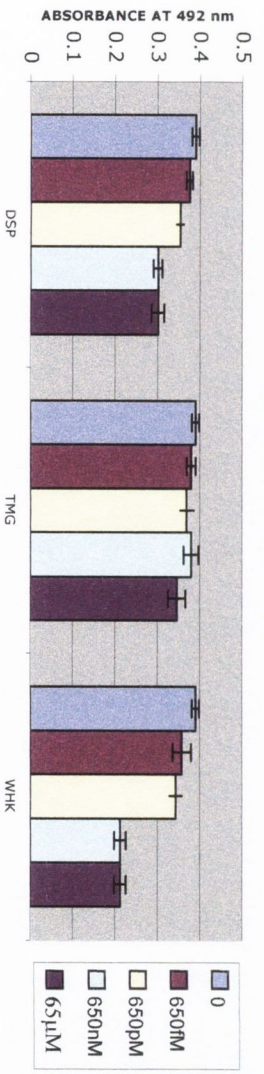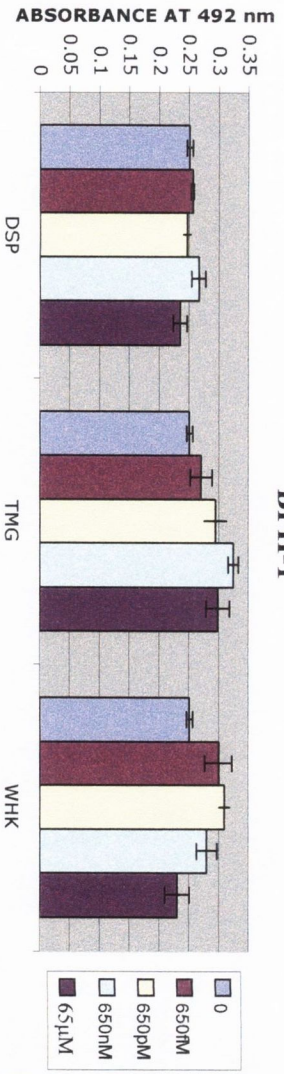
4.2.6 Histology

MDA-MB-231 is a cell line originated from ductal mammary carcinoma and it was employed for the extraction of the Hsp70-PC fraction used for the generation of the mimic/recogniser pair DSP/TMG. Since the TMG and DSP peptides both bound to breast tumour and non-tumour cell lines, it was of interest to determine if these peptides could also bind to breast tumour and non-tumour tissue. Normal breast and ductal tumour tissue were obtained as paraffin-embedded tissue sections. The tissue samples were stained with hematoxilin and eosin (H & E) to reveal the morphology of the samples as outlined in the Methods and Material section 2.3.1.

4.2.6.1 Main anatomical part of the breast. Nomenclature

A brief anatomical description of the breast will help to understand the morphology of the breast sections. Nine to ten major or large ducts open to the nipple by a separate orifice. They include a dilated part with sinuous contours termed lactiferous or milk sinus. Then they divide into medium and small ducts ending in the terminal ductal-lobular unit (TDLU) which contains the extra and intralobular terminal ducts ending in the smallest structure within the lobule termed terminal ductile or acinus which are mainly only functional during pregnancy and lactation.

4.2.6.2 Morphology of normal mammary tissue and mammary carcinoma

4.2.6.2.1 Normal mammary tissue from adult human

H and E staining reveals ducts lined by two cell layers: a well-differentiated columnar epithelial layer and myoepithelial outer layer. Columnar epithelia show nuclear polarity and apical snouts. Dead ductal cells can be found in the lumen of the ducts. Connective tissue surrounds the ducts (Fig 4.11).

4.2.6.2.2 Mammary ductal carcinoma from adult human

Within the same blocks tumour cells are also observed. These are defined by poorly differentiated ductal cells invading the surrounding connective tissue (*) and the
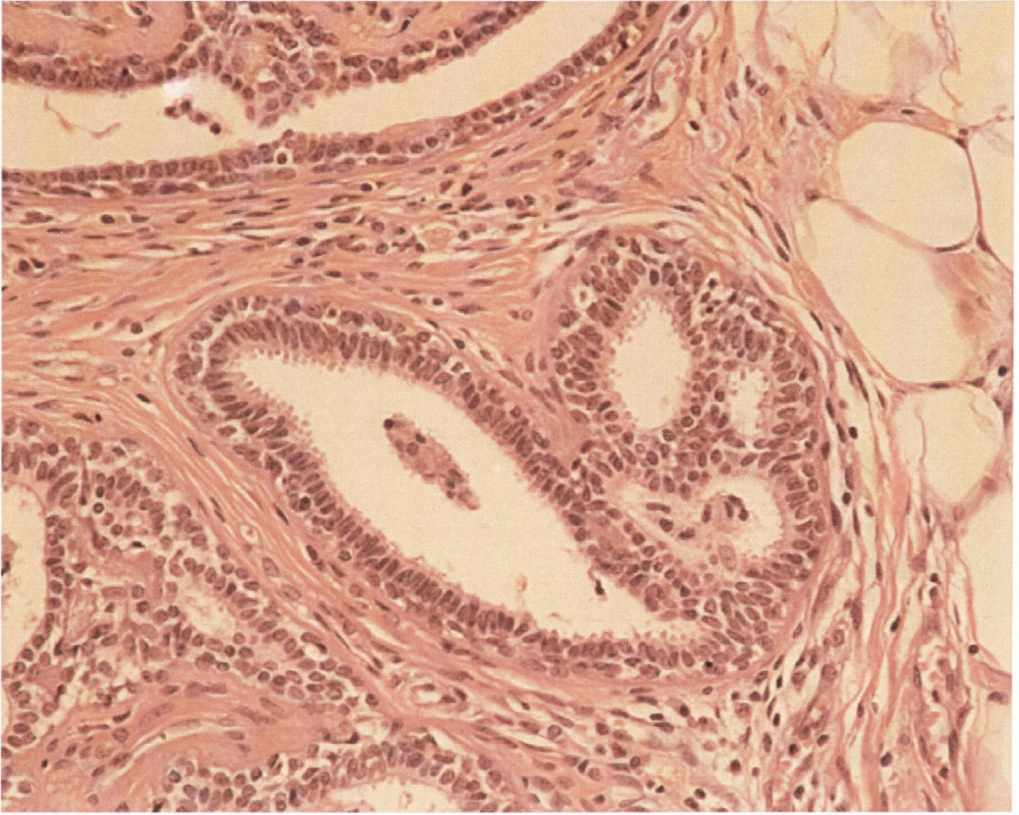
Fig 4.11 Normal mammary tissue from adult human. H&E

Magnification 200 x
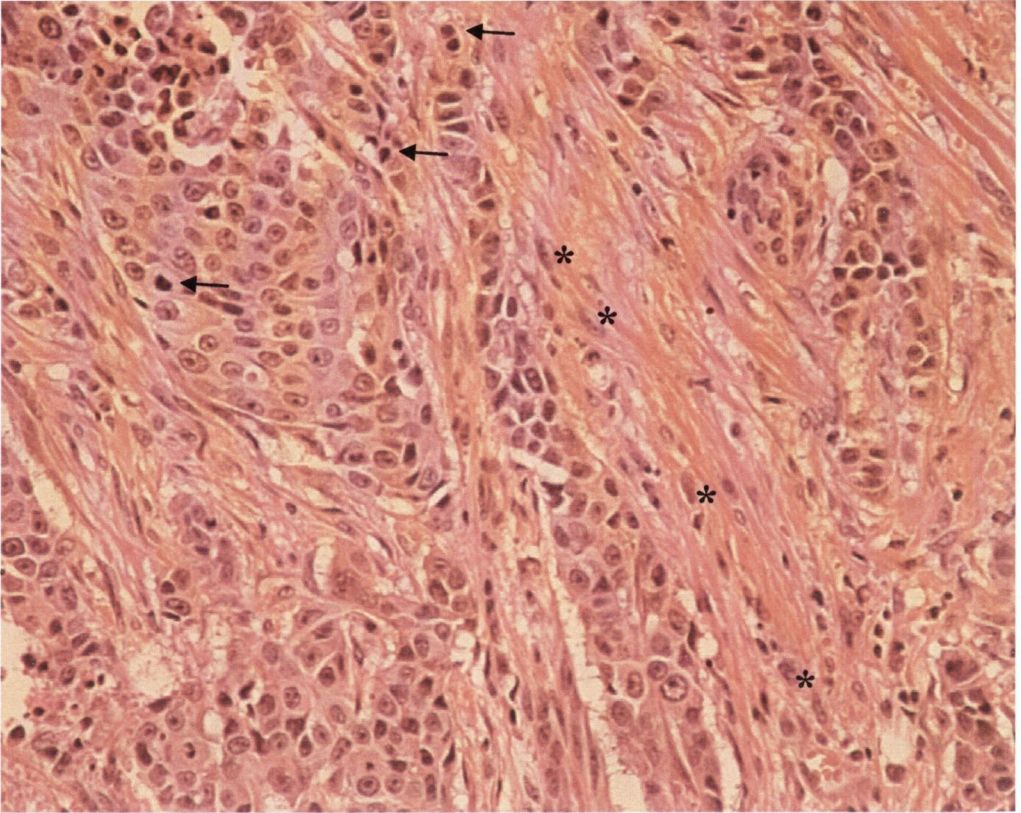
Fig 4.12 Ductal carcinoma from adult human. H&E

Magnification 200 x. **Arrows**: mitotic figures

(*): Invading single ductal carcinoma cells

lumen of the ducts loosing the two cell layer structure observed in normal mammary tissue (Fig 4.12). Mitotic forms (arrows), nuclear pleomorphism and hyperchromasia (sign of heteroploidy) can be observed.

4.2.6.3 Interaction of DSP and TMG peptides with human breast tissue sections

Paraffin embedded mammary tissue sections (normal and tumour) were dewaxed, rehydrated, blocked and incubated with DPS, TMG and WHK biotinylated peptides or no peptide (NP) and fixed with paraformaldehyde followed by incubation with the fluorescence-quencher sodium borohydride (0.1%). Once the tissue autofluorescence and the fluorescence due to paraformaldehyde was quenched (Clancy and Cauller 1998), cell-bound biotinylated peptide was detected with the red fluorescence tag (Cy3) conjugated to Streptavidin. Actively dividing cells were identified by incubation of the slides with a rabbit anti-human ki67 antibody tagged with the green fluorescence tag FITC and nuclei were stained with DAPI in blue. (See *Materials and Methods* section 2.3.3). Ki67 is a protein expressed in all the active phases of the cell cycle (G_1, S, G_2 and M), and absent from resting cells (G_0). During interphase, the antigen can be exclusively detected within the nucleoplasm, whereas during mitosis most of the protein is associated with chromatin. It is used as a proliferation marker to measure the growth fraction of a given cell population. In particular, it is often used in the context of prostate and breast carcinomas (Scholzen and Gerdes 2000).

Figure 4.13 reveals that both DSP and TMG peptides interact with ductal cells predominantly and at a lesser extent with fibroblasts (red) whereas the WHK peptide showed no staining to the tissue (red). The nature of the staining is faint and diffuse, and probably cytoplasmic. Areas with cells with high proliferation events (green) correlates with ductal carcinoma cells (tumour). Ductal carcinoma cells show a stronger red staining intensity indicating a preference of the DSP and TMG peptides for ductal carcinoma cells rather than normal ductal cells or fibroblasts. This observation is supported by the differential intensity of the red staining when comparing ductal cells in tumour and normal mammary tissue incubated with DSP or TMG peptides [Fig 4.13(*) and (**)]. The nature of the red staining is more intense on ductal cells, both normal and tumour, compared to staining of connective tissue (Fig 4.13). Occasionally, it was possible to detect single cells in the tissue sections. Detection of single cells was rare in

Fig 4.13 DSP and TMG peptides bind to human breast tissue

Paraffin embedded mammary tissue sections (normal and tumour) were dewaxed, rehydrated, blocked and incubated with 40 µM DPS and TMG biotinylated peptides. Bound peptide was detected by Cy3 (red) fluorescence using a 510-560 nm excitation filter. Ki67 was detected with FITC-conjugated secondary antibody (green). DAPI (blue) staining of DNA is detected using a 340-380 nm excitation filter. Biotinylated WHK and no peptide (NP) were used as negative controls. Magnification 100 x. (*): cy3 staining is peptide-specific.   (**): Composite image of the three-colour pictures using AdobePhotoshop program.
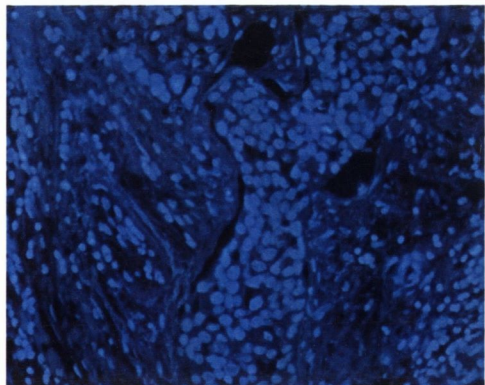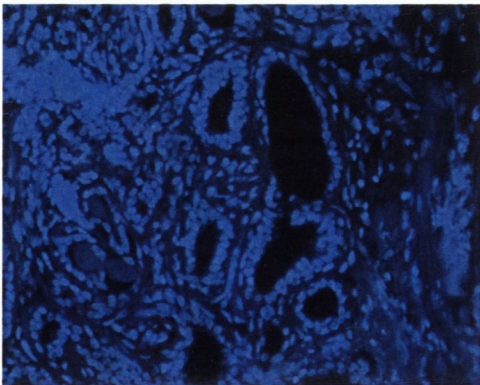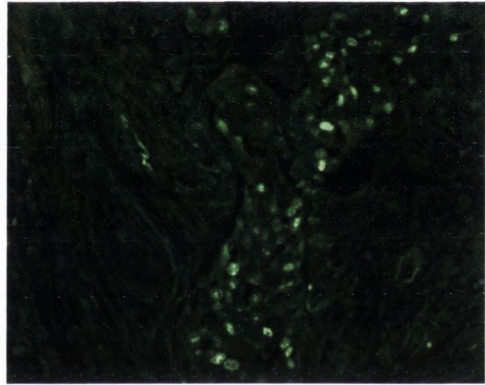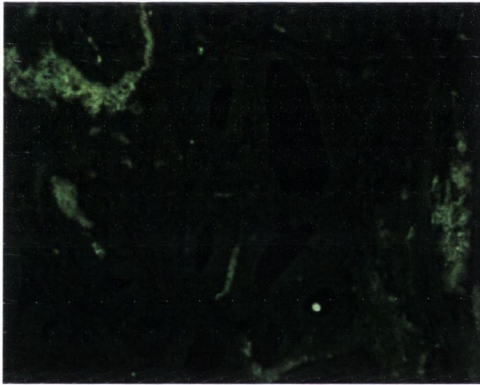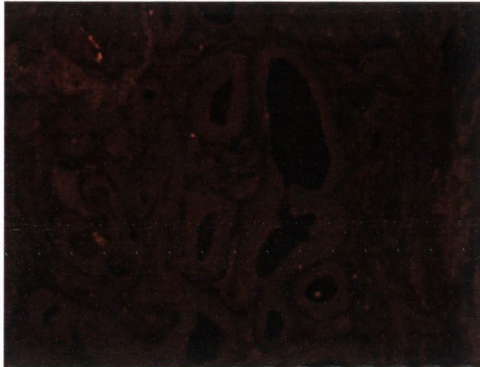
NORMAL	TUMOUR
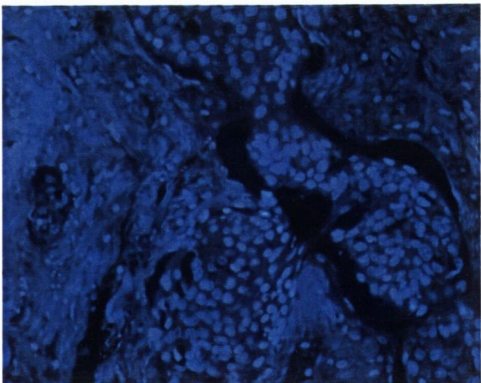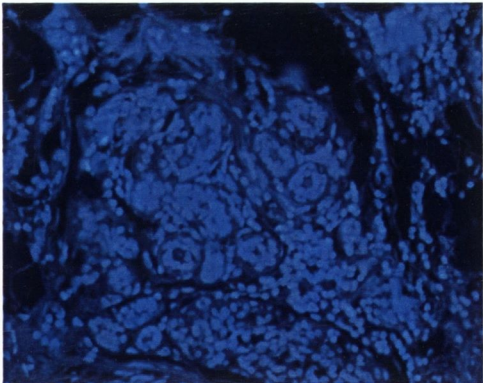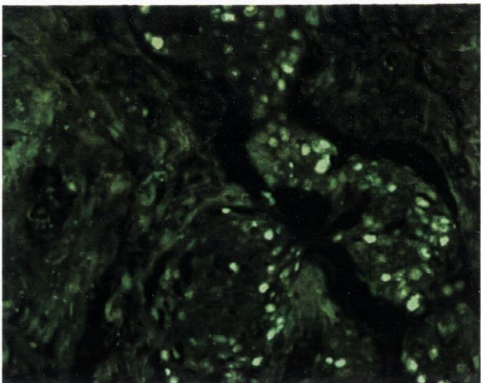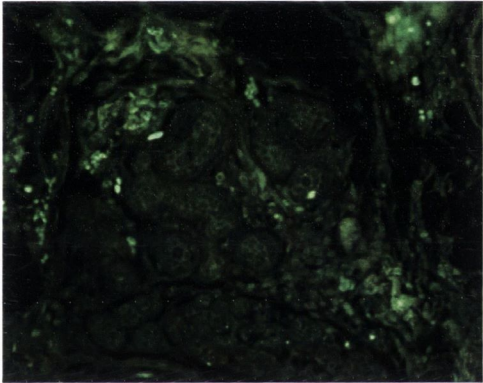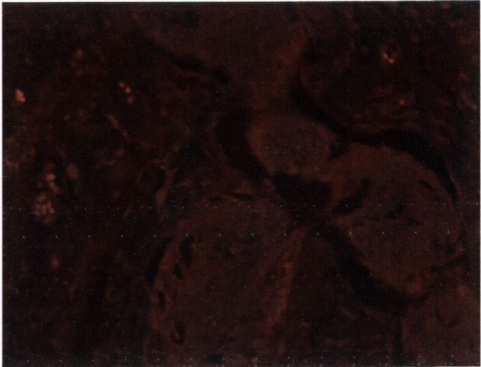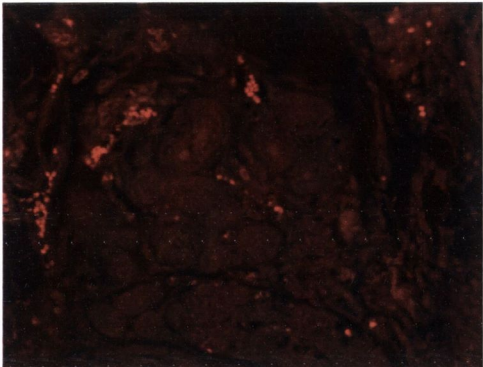
NORMAL   TUMOUR
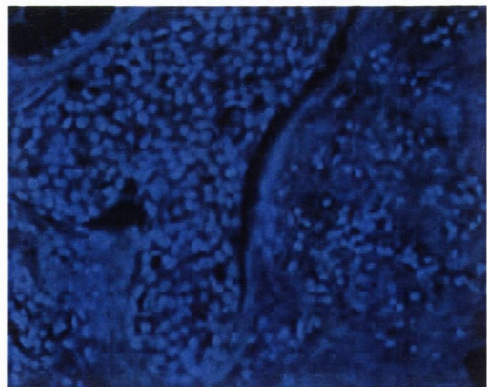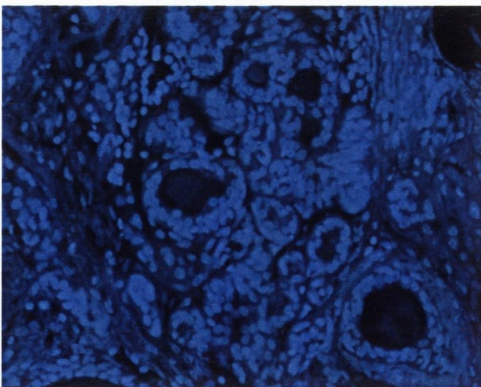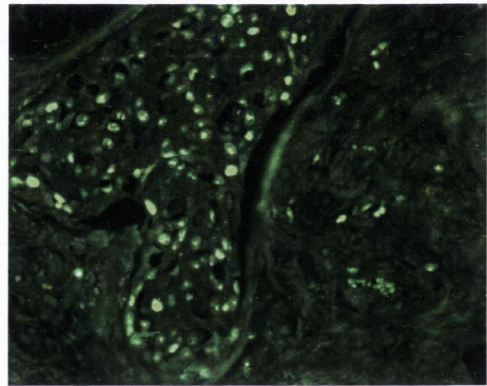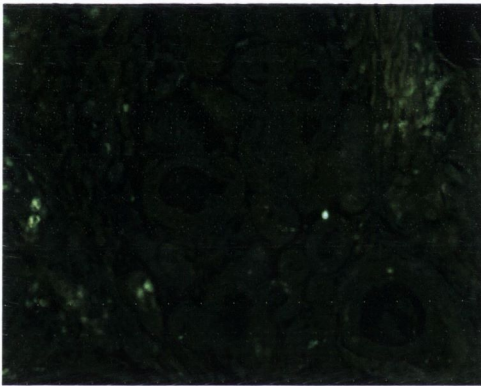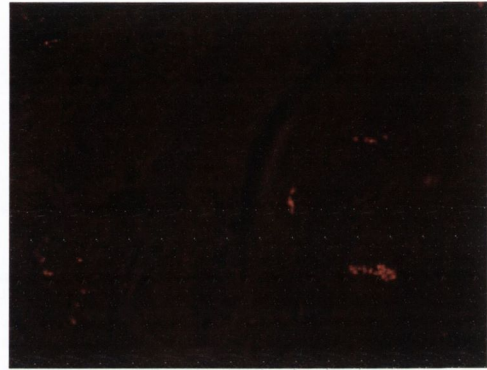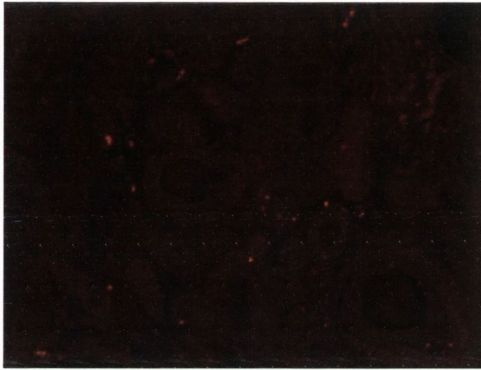
NORMAL	TUMOUR

NORMAL	TUMOUR

(*)
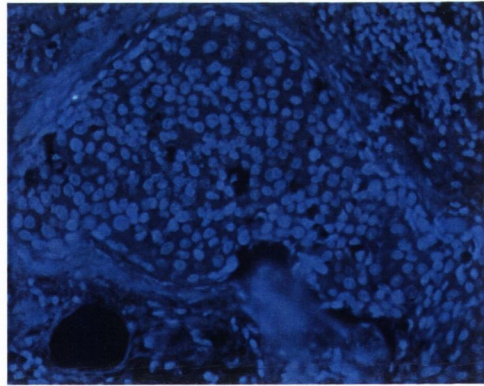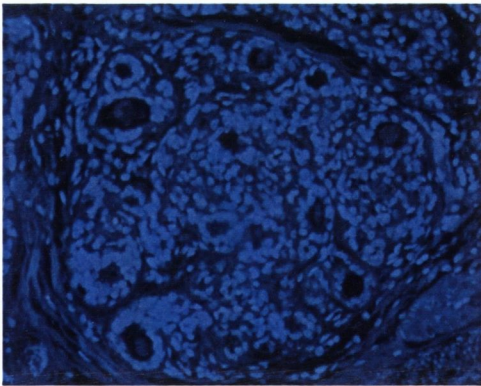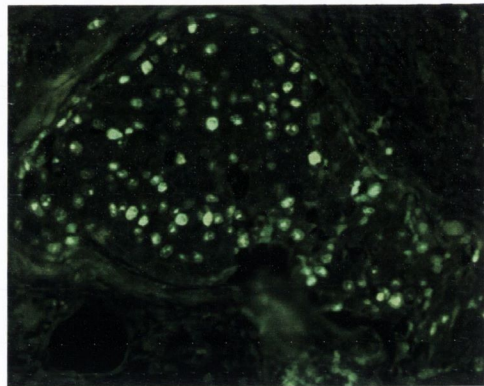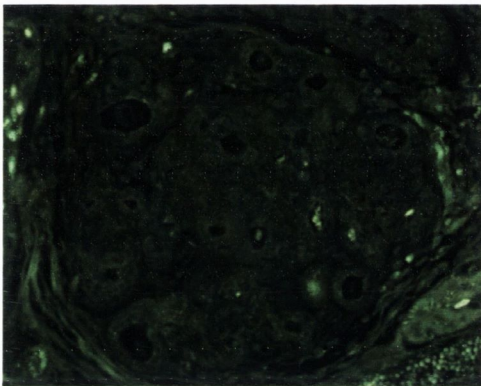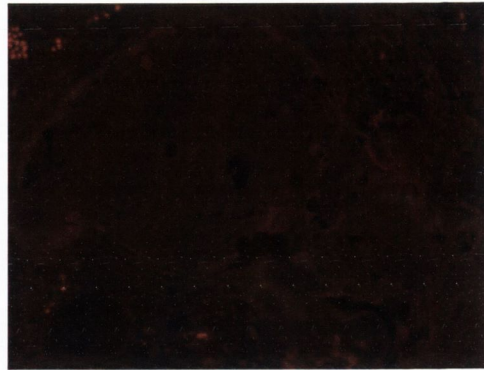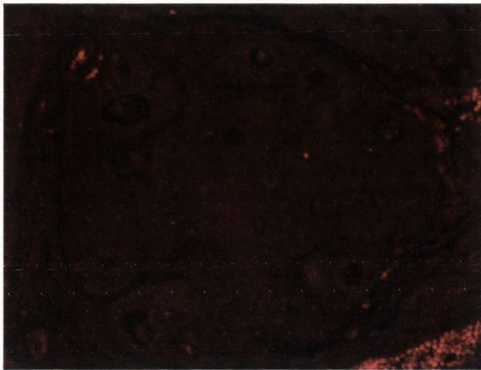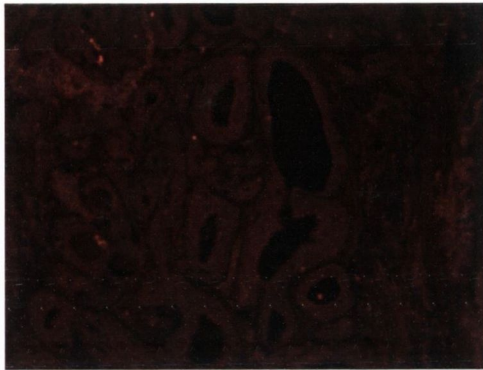
NORMAL   TUMOUR

DSP
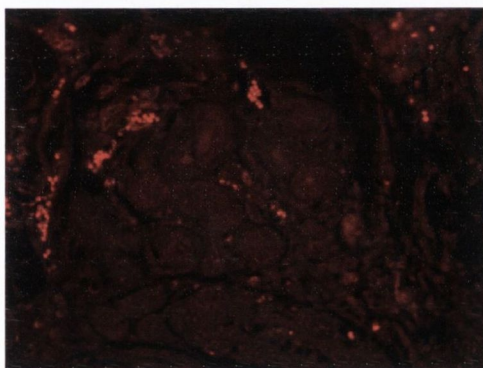
TMG
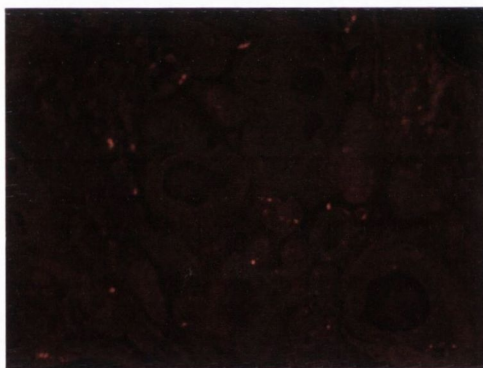
WHK
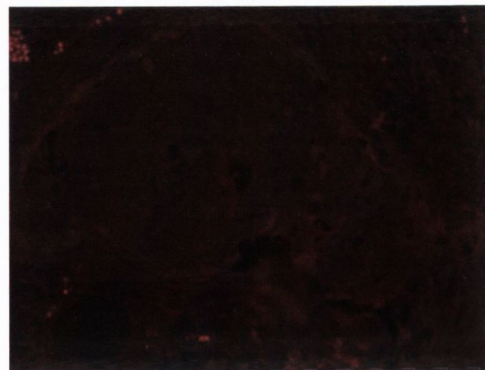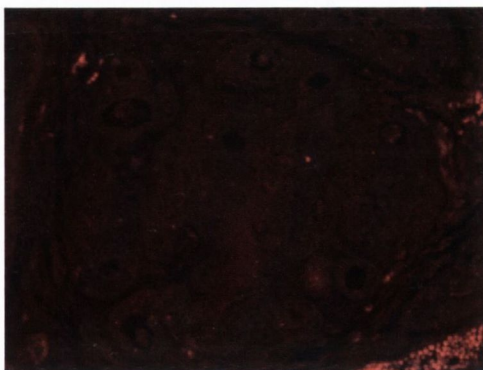
NP

DSP (**)
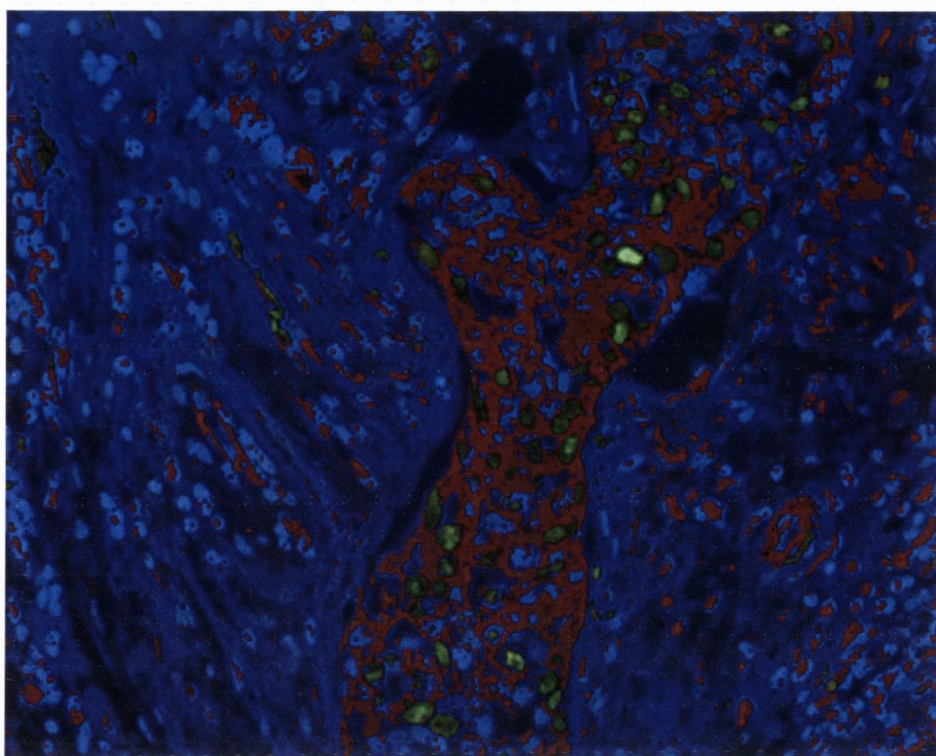
TUMOUR
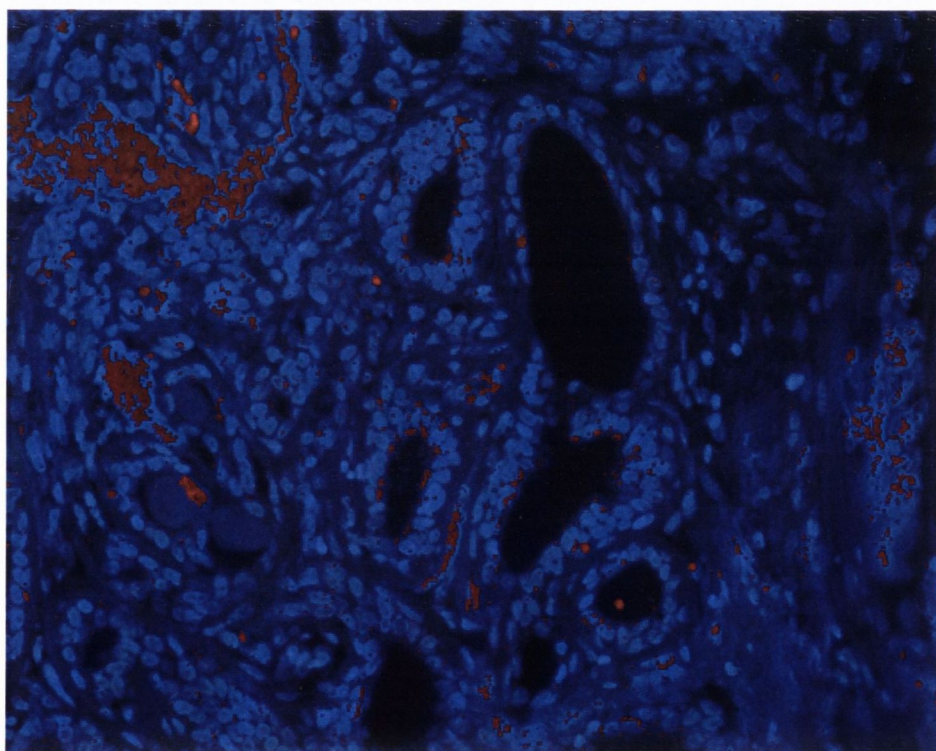
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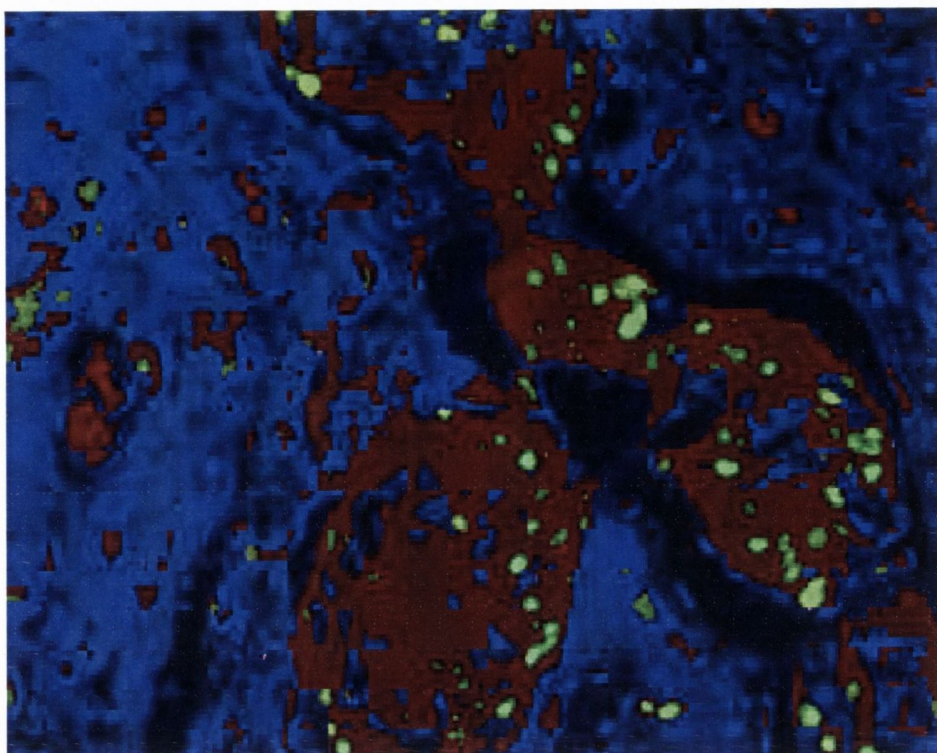
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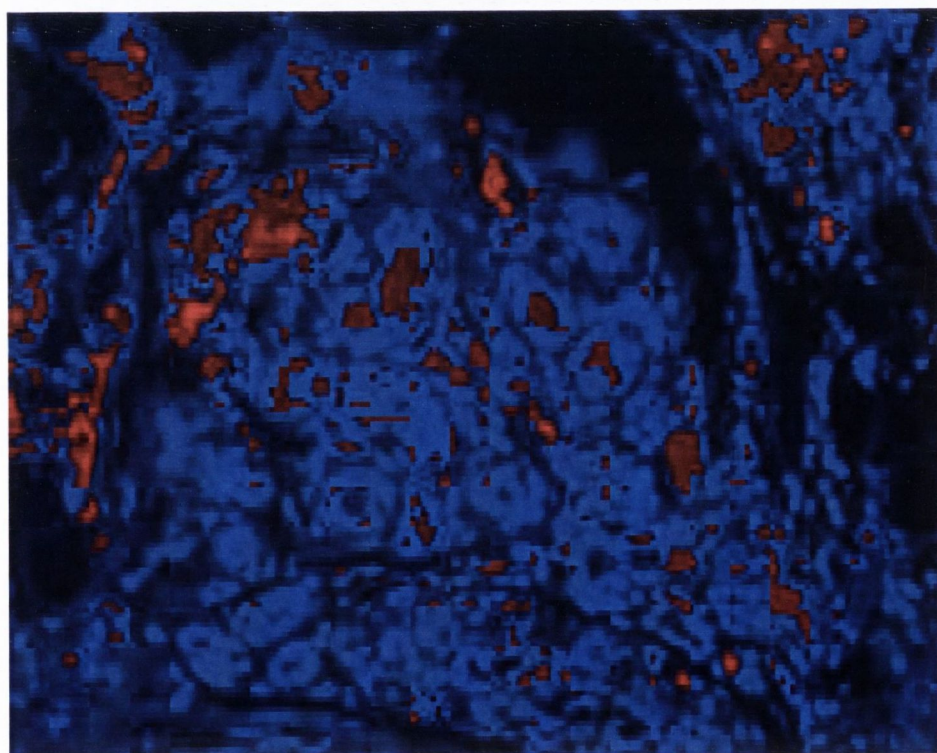
NORMAL

the tissue sections employed and none were found in the tissue incubated with DSP peptide. The staining with the TMG peptide was more intense in these single cells compared to the staining intensity observed with cells within a tissue mass (Fig. 4.14). This result supports the previous observations with the cell lines (described in section 4.2.1 and 4.2.3) that the availability of TMG peptide binding partner(s) increases with diminished cell-cell contact. Incubation of ductal carcinoma cells with increasing concentrations of TMG peptide as described in *Materials and Methods* section 2.3.3 showed the interaction to be concentration-dependent (Fig 4.15). Binding of the peptides to MDA-MB-231 cells was also saturatable when higher concentrations of peptides are used. To obtain a semi-quantitation of the binding, DSP and TMG binding was detected using Cy-3-conjugated Streptavidin. The fluorescence staining was quantified by measuring the red intensity of the staining using Adobe Photoshop. As shown in Fig 4.16, binding of the TMG peptide saturated at approximately 110 μM. Similar results were obtained for the binding kinetics of the DSP peptide (data not shown).

4.2.2.3 DISCUSSION

The purpose of the experiments outlined in this chapter was to further characterise the "recogniser" peptide TMG and the putative "mimic" peptide DSP. These peptides were isolated in a phage display screening of TAs associated with the heat shock protein Hsp70 (Arnaiz et al, submitted). The Hsp70-peptide complexes were isolated from the breast cancer cell line MDA-MB-231. In theory, the recogniser peptide should bind to TAs associated with Hsp70 in the cell or even with the original protein from which the TA peptide originated from. These TAs or the proteins from which they were derived are expected to be present in breast cells. Binding of the peptide TMG to the three breast cell lines and mammary tissue sections was observed. However the interaction of TMG with its binding partner was not restricted to breast cells and tissues as binding was also observed in keratinocytes, prostate PC-3 (tumour) and BPH-1 (normal) and non-small cell lung carcinoma NCI-H358 cell lines. The mimic DSP peptide also shows an interaction with the same cells. This is not surprising because if DSP and TMG constitute a mimic/recogniser pair, then it is possible there exists in the cells protein pair/s with binding motifs homologous to DSP and TMG peptide sequences. The peptide DSP is a putative mimic of a breast antigen. Many antigens are

Fig 4.14 Isolated tumour cells with intense Cy3 staining

Paraffin embedded mammary tissue sections (normal and tumour) were dewaxed, rehydrated, blocked and incubated with 40 μM TMG biotinylated peptides. Bound peptide was detected by Cy3 (red) fluorescence using a 510-560 nm excitation filter. DAPI (blue) staining of DNA is detected using a 340-380 nm excitation filter. The areas (1) and (2) were magnified to reveal more detail of the staining patterns.

TUMOUR+TMG
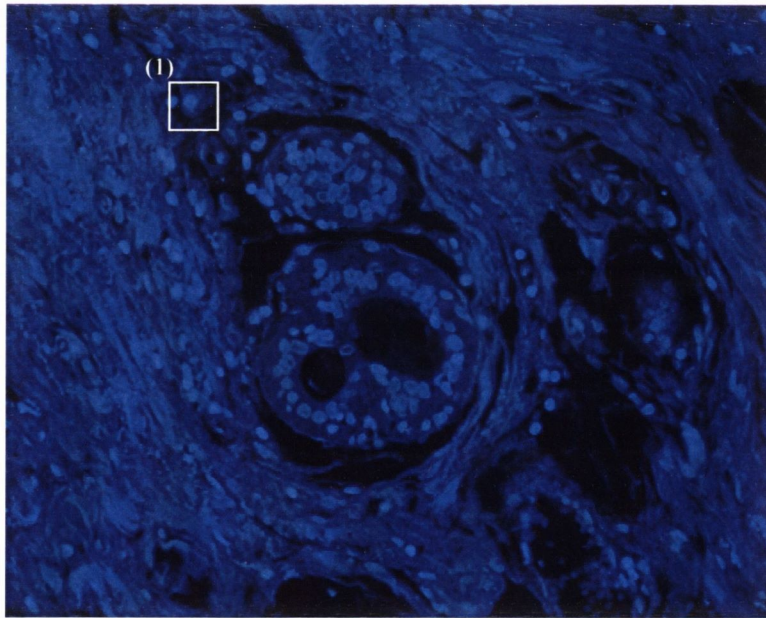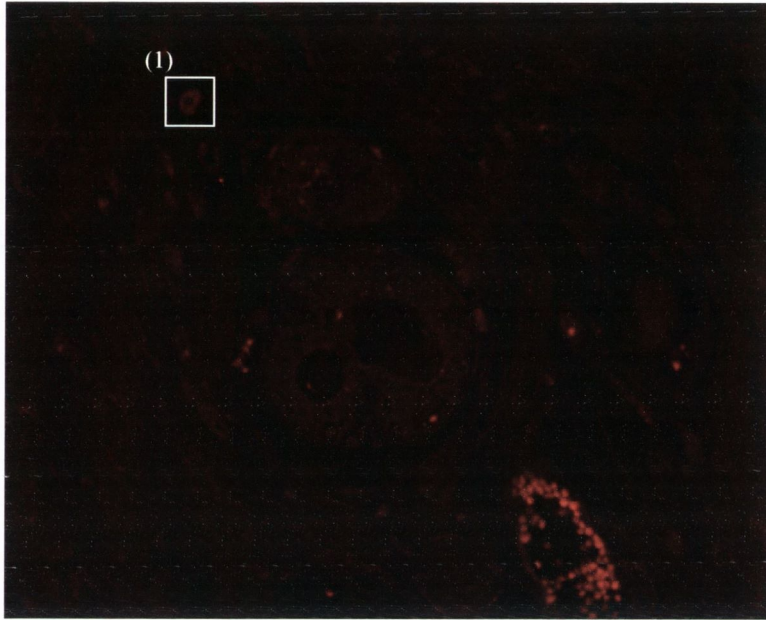
NORMAL+TMG
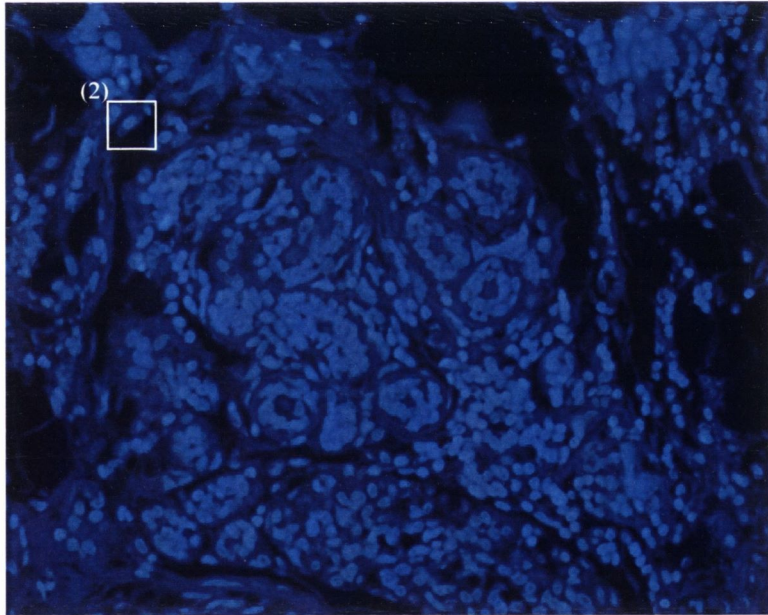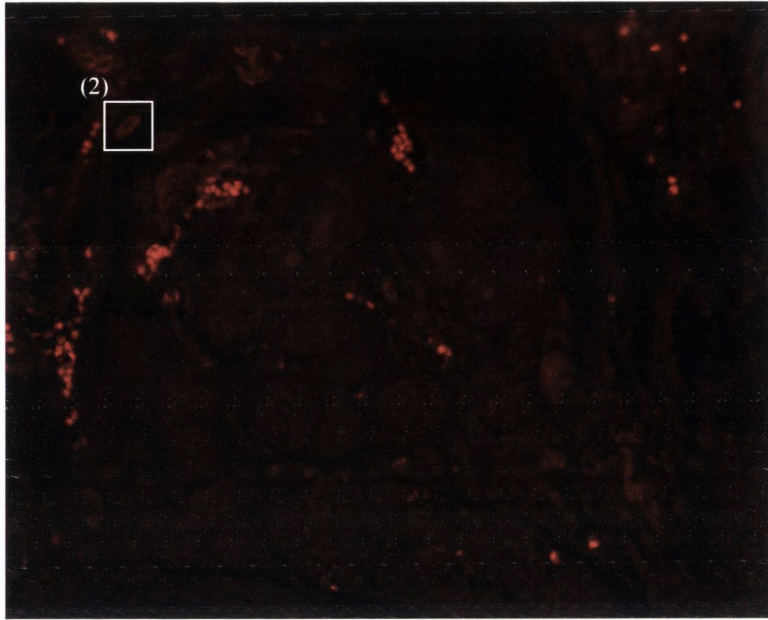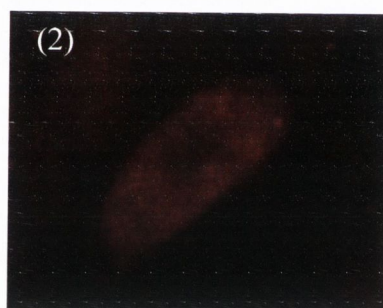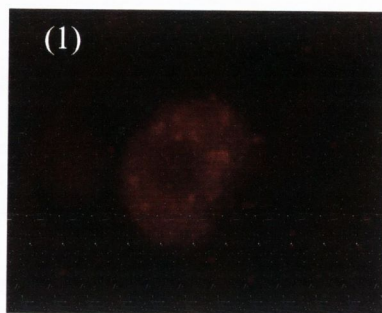
Fig 4.15. Demonstration of TMG peptide interaction with ductal mammary carcinoma cells is concentration-dependent

Paraffin embedded ductal mammary carcinoma sections were dewaxed, rehydrated, blocked and incubated with 0, 40, 65, 90, 110 or 130 μM biotinylated TMG peptide described in *Material and Methods* section 2.2.2. Red: Peptide detection with streptavidin-conjugated cy3. Magnification 100 x.
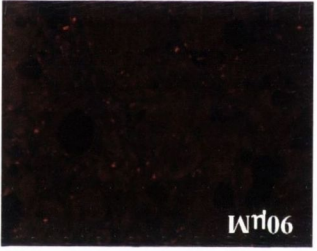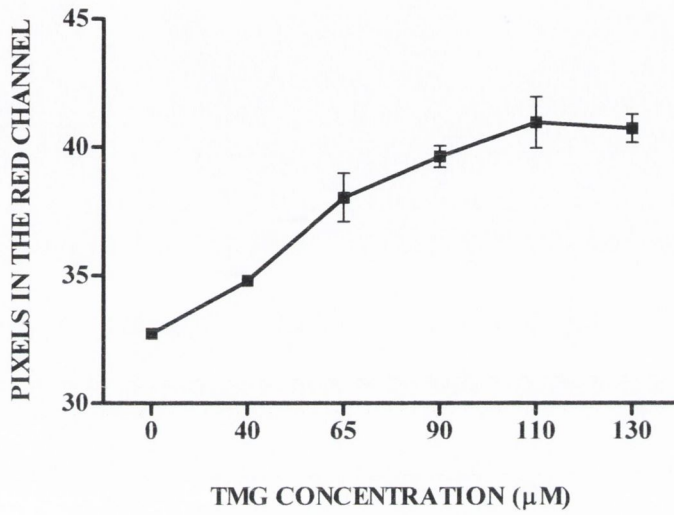
Fig 4.16. Saturation curve of TMG peptide interaction with ductal mammary carcinoma sections Equal areas on each of the pictures in figure 4.15 were selected, and the mean and standard deviation of the intensity of the red pixels was calculated by the red channel option in the Histogram tool with the program Adobe Photoshop version 6.0. The different concentrations of biotinylated TMG peptide used in the incubation with ductal mammary carcinoma sections were plotted versus the mean of the read intensity given by the fluorochrome streptavidin-conjugated cy3.

derived from proteins that show intracellular interactions with other proteins, for example the oncogene c-Ras interacts with a variety of proteins in signal cascades in the cells while the EGF interacts in an autocrine manner with the EGF receptor. Moreover, the T-cell stimulation assay described in chapter 3 showed protein(s) in MDA-MB-231 and MCF-12A cell lysates could stimulate the same T-cell clones previously stimulated by DSP and TMG peptides to produce IFN-γ. T-cell clones can be stimulated only by peptides/antigens with sequences having a very particular homology in the active amino acids (Linnemann, Tumenjargal et al. 2001) and the canonical MHC anchor residues according to SYFPEITHI database (http://www.uni-tuebingen.de/uni/kxi/). Therefore, the results in Chapter 3 also support the existence of proteins with motifs homologous to DSP and TMG peptides.

DSP and TMG peptides bind to molecule(s) present at higher levels in cells plated at 10-25% confluency compared to cells plated at 50-65% or 85-100% confluency. Generally cells plated at low concentrations lack cell-cell contact and grow as individual cells in a monolayer. However, certain cell lines, in particular NCI-H358 and MCF-12A, displayed unusual cell growth phenotypes. These cells grow as a monolayer at low cell densities, however as cell concentrations increased, the cells formed domes or balls of cells once they reached a confluency of 50-65%. Interestingly, DSP and TMG peptides only bind to these cells at 10-25% confluency. Thus, it could be speculated these two cell lines have more capacity for cell contact growth inhibition than the other cell lines tested and that this might also be the reason why they show a lower proliferation rate. A similar observation has previously reported (Orford et al. 1999) when studying the effect of β-catening-expressing kidney cell mutants on colony formation in soft agar plates. β-catening plays an important role in cell-cell adhesion as it links E-cadherin, a transmembrane glycoprotein, to the actin cytoskeleton through the protein α-catenin. E-cadherin expression is frequently lost or significantly reduced in malignantly transformed cells with an invasive phenotype that show a low degree of cell contact growth inhibition. Down regulation of E-cadherin allows EGFR activation by its ligand (Takahashi and Suzuki 1996) and liberates β-catenin, bound to its intracellular domain, into the cytoplasm where its accumulation drives its interaction with members of LEF/TCF family of nuclear transcription factor eventually resulting in altered gene expression. β-catenin- overexpressing cell mutants dramatically increased the rate of colony growth and generated a more scattered, mesenchymal phenotype, suggesting that enhance motility by avoiding the contact

inhibitory effect of tight cell-cell adhesion, may contribute to an increase in colony cell growth rate (Orford et al. 1999). Therefore, it is possible that the peptides TMG and DSP bind to cellular components present in greater abundance in cells plated at low cell confluency and that binding is inhibited by cell-cell contact. This result was confirmed by MTT-based cell proliferation assays which showed that the growth rate of MCF-12A and NCI-H358 is lower than the rest of the cell lines tested, thus, a correlation between cells with high proliferation rates and expression of DSP and TMG binding partner/s may be established.

To determine if the binding observed in breast cell lines was replicated in breast tissue, the staining patterns of the peptides were examined in a number of mammary tissue sections. The results indicate that both peptides show a preferential staining of tumour ductal cells within the mammary tissue. Supporting this idea is the fact that ki67 tissue localisation seems to correlate with a highest expression of DSP and TMG binding partner(s) in tumour ductal cells when compared to its ki67 negative counterparts on normal ductal cells as shown by the composite image of peptides, ki67 and DAPI. Occasionally, some single ductal cells could be observed in the sections. Two individually isolated ductal cells were identified and they showed higher levels of TMG staining compared to the staining observed in dense tissue cell masses, confirming the earlier findings that peptide binding partners are more abundant in cells with low cell-cell contact. Ideally, isolated ductal breast cells should have been localised on slides treated with H&E, then, localised in the contiguous tissue section treated with fluorescently-visualised DSP and TMG peptides. Unfortunately, optimisation of the assay consumed most of the sections.

The experiments performed in this chapter employing cell lines has accumulated enough evidence as to encourage further investigations employing human tissue of different types. DSP and TMG binding partners could be part of a cell growth pathway up regulated in cells whose proliferation is uncontrolled. This is the case of cell lines both normal and tumour with decreased cell-cell contact growth inhibition and tumour tissue. In the breast tissue sections tested there is a differential level of interaction with DSP and TMG between normal and tumour ductal cells. TAAs are often up regulated in tumour cells when compared to their counterparts in normal cells and are molecules often involved in cell proliferation pathways. Evidence accumulated in this thesis support the idea of that the DSP/TMG (mimic/recogniser) pair could be homologous to a TAA/TAA-binding partner pair in the cells as initially hypothesised for

its generation through the bio panning approach. While the TMG/DSP peptide pair do not show specificity for breast tumour cells only, modifications of the bio panning approach by the introduction of a subtracting step using Hsp70-PC from normal breast cells could eliminate those Hsp70-PC chaperoning peptides produced by all human cells and allow the isolation of tumour specific antigens (TSA).

Regarding the cytotoxicity of DSP and TMG peptides, DSP appeared to have a negative effect in the growth of the two normal cell lines tested, BPH-1 and keratinocytes, at 65µM concentration. Surprisingly, the peptide WHK, although having been shown not to bind to the cells tested, appeared to decrease cell growth in all the cell lines tested at 65µM concentration except MCF-7. The presence of abundant debri in the wells of the affected cells could be dead cells or precipitation of proteins present in the medium. To confirm if some of the mild effects on DSP and TMG are meaningful the assay could be repeated using only two peptide concentrations 0 and 65µM and extending the incubation period of the cells and peptides, for instance, from 24 to 48hr. In its present format, it is difficult to draw any firm conclusions on the effects of the peptides on cell growth.

In conclusion, there seems to be a correlation between the level of expression of DSP and TMG binding partners, the cell growth rate of the cells and the cell-cell contact growth inhibition of cells. Both features of high cell growth rate and cell-cell contact independence are characteristic of tumour cells, therefore DSP and TMG peptides constitute an interesting finding for its potential therapeutic value.

5.1 INTRODUCTION

Evidence accumulated in chapters 3 and 4 indicate that the mimic/ recogniser pair DSP/TMG has interacting partners in MDA-MB-231 cells. Identification of TMG interacting molecules could give us information about the antigen(s) associated with Hsp70 in MDA-MB-321 cells that DSP mimics. If achieved, it would mean that bio panning of M13 phage display libraries using Hsp70-PC from tumour cells could lead not only to the isolation of antigen mimics with potential beneficial use in vaccination strategies but, also to the identification of new antigens to be exploited in the development of drug targeting and tumour therapies. In this chapter, detection, isolation and identification of DSP and TMG-binding molecules by biochemical approaches was intended. After repeated attempts at detecting DSP and TMG binding partners by Far-Western without success, the approach of maintaining the intracellular interaction conditions by cross linking of the biotinylated peptides to the cells before homogenisation was employed. This approach led to the detection by Far-Western of DSP-binding molecule(s). Digestion with endoproteinase Glu-C and trypsin showed the proteinaceous nature of the DSP-binding molecule(s). Biotinlylated- TMG and DSP peptides, immobilised on Streptavidin-coated magnetic beads, were employed to isolate their specific binding partner(s) from MDA-MB-231 extracts. This led to the identification of a protein of approximately 70 kDa in size. The identification of this protein by mass spectrometric fingerprinting and data mining failed to yield reliable results due to low amount of sample, however, among the proteins with higher percentage of homology was the HSP70 protein in the case of TMG-binding protein and the ATP-dependent RNA helicase DEAD-box protein DDX18 in the case of DSP-binding protein.

Another approach taken for the identification of DSP and TMG binding proteins was the screening of a breast ductal tumour cDNA expression library using the DSP and TMG peptides immobilised on streptavidin-coated magnetic beads. None of the selected polypeptide sequences showed 100% homology to proteins in NCBI databases but the proteins with higher homology selected had a role in cell proliferation or survival, supporting the evidence described in chapter 4, regarding DSP and TMG-binding partners being molecules involved in the regulation of cell growth. The experiments generating these results are outlined in this chapter.

DETECTION, ISOLATION AND IDENTIFICATION OF DSP AND TMG-BINDING PROTEINS FROM MDA-MB-231 CELL EXTRACTS

5.2 RESULTS

5.2.1 Detection of DSP and TMG-binding partners in MDA-MB-231 cells by Far-Western

In Chapter 4, it was observed that the DSP and TMG peptides interacted with cellular components of MDA-MB-231 cells. Maximum binding was detected in cells grown at low confluency while low binding was detected in fully confluent cells. Intermediate levels of binding were detected in cells grown to 50-60% confluency. Therefore, in order to identify and isolate these peptide binding proteins, cells grown to 50-60% confuency were chosen to maximise cell numbers and the amount of the binding partners.

5.2.1.1 Far-Western analysis of protein extracts from MDA-MB-231 cells using DSP and TMG peptides as probes

MDA-MB-231 cells were harvested at 50-65% confluency and cell extracts prepared as described in the *Materials and Methods* section 2.4.1 and 2.4.2. 50 μg of protein extracts from MDA-MB-231 cells were separated on 12% SDS-PAGE and the proteins were transferred to a nitrocellulose membrane. The membrane was divided in eight pieces, each corresponding to one lane of the gel. Half of the membranes were blocked with 5% casein PBS with biotin (Fig 5.1. lane 2, 4, 6 & 8) and the other half were incubated with 5% casein PBS without biotin (Fig 5.1. lane 1, 3, 5 & 7) as described in *Materials and Methods* section 2.4.2. The membranes were incubated with either the biotinylated peptides DSP, TMG, WHK or PBS alone for 2 hr, washed and subsequently incubated with streptavidin-HRP conjugate.

Results in figure 5.1 indicates the protein in lanes 1, 3, 5 & 7 of 83 kDa molecular weight binds non-specifically to streptavidin-HRP as it is also present when no peptide was used for the incubation of the membrane (Fig 5.1. lane 7). When the membrane pieces were incubated with biotin-containing blocking buffer, the band is not observed (Fig 5.1. lane 2, 4, 6, 8). This result indicates the 83 kDa protein corresponds to a streptavidin-conjugated HRP-binding protein, as it is not observed when biotin is used prior to incubation of the membrane with the biotinylated peptides. It could be concluded the conditions used in the Far-Western technique to allow detection of
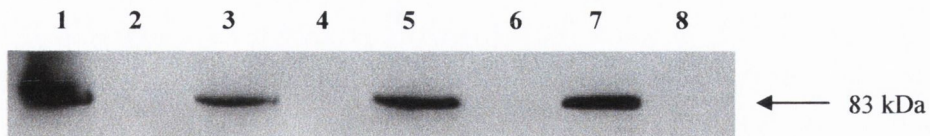
Fig 5.1 Detection of DSP and TMG *in vitro* binding proteins by Far-western analysis
MDA-MB-231 cells were lysed and separated by 12% SDS-PAGE. The proteins were transferred to a nitrocellulose membrane and blocked with 5% casein PBS with biotin (lane 2, 4, 6 &8) or without biotin (lane 1, 3, 5&7) and probed with 65μM biotinylated DSP (lane 1&2), TMG (lane 3&4),WHK (lane 5&6) peptides and no peptide (lane 7&8). The peptides were labelled with ExtrAvidin HRP conjugate and SuperSignal Westpico Chemiluminescent Substrate. The peptide WHK was used as negative control.
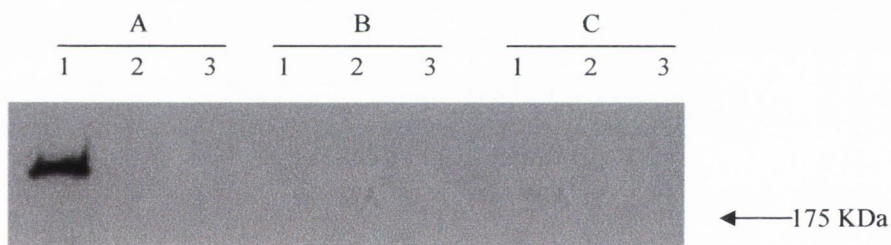
Fig 5.2 Detection of DSP, TMG *ex-vivo* binding proteins by Far-western analysis
MDA-MB-231 cells were crosslinked to biotinylated DSP, TMG, and WHK peptide. The cells were lysed and the membrane and cytosolic proteins were extracted and pooled. Equal amounts of each protein sample, proteins crosslinked to biotinylated DSP (lane 1), TMG (lane 2) and WHK peptide (lane 3) were either not treated (A), digested with trypsin (B) or with endoproteinase Glu-C (C). The samples were separated by a 7.5% SDS-PAGE, transferred to a nitrocellulose membrane, the peptides were labelled with ExtrAvidin HRP conjugate and SuperSignal Westpico Chemiluminescent Substrate as described in *Materials and Methods* section 2.4.3.

interactions between the peptides and their binding partners, denatured protein/s on the nitrocellulose membrane, were not adequate. Re-naturing of the proteins on the nitrocellulose membrane also failed to show specific bands (data not shown).

5.2.1.2 Far-Western analysis of protein extracts from MDA-MB-231 cross-linked to DSP peptide

To improve the liklihood of detecting interactions between the peptides and cellular proteins, MDA-MB-231 cells were fixed with 3.7% paraformadehyde when they were 50-65% confluent in 175 cm^2- TC flasks (approximately 9x10^7 cells). The cells were permeabilised with 0.5% NP-40 PBS and blocked with 5% casein PBS containing biotin. Then they were divided in three equal parts and incubated with 65 μM of either biotinylated DSP, TMG or WHK peptides for 2hr, washed and fixed again with 3.7% paraformaldehyde. The monolayers were washed and the cells scrapped off the flasks. Cell extracts were prepared as described in the methods section 2.4.1 and 2.4.2. Each total protein extract was then further divided into three equal parts each, and incubated for 14 hr with either endoproteinase Glu-C (Roche,UK), 1xTrypsin solution (Sigma,UK) or PBS only. The protein samples were separated on 7.5% SDS-PAGE, after five minutes boiling and then transferred to a nitrocellulose membrane. The membrane was blocked with 5% casein PBS containing biotin and incubated with streptavidin-HRP conjugate (see *Materials and Methods* section 2.4.3).

Figure 5.2A, lane 1, reveals a unique band present in the stacking gel for the protein sample cross linked to biotinylated DSP which is absent in samples incubated with trypsin (4.2 B, lane 1) or endoproteinase Glu-C (C, lane 1). The size of the band, at greater than 175kDa, most likely due to cross-linking, prevented the sample from running into the separating gel. The fact that the band is absent in extracts treated with trypsin (B1) and endoproteinase Glu-C (C1), indicates that DPS-binding partner/s is proteinaceous in nature. No binding of biotinylated peptide was detected in extracts from cells cross linked to the TMG (5.2 A, lane 2) or the WHK biotinylated peptides (5.2 A, lane 3). These results indicate that under the conditions used here, it was not possible to identify proteins cross-linking to these two peptides. From the results obtained in chapter 4, we did not expect WHK to have any binding-partner in MDA-MB-231 cells as no interaction was detected by cytochemistry or histochemistry techniques. It was also observed that the interaction between the peptide TMG and its binding partner in MDA-

MB-231 cells at 50-65% confluency, was less intense that the one between the DSP peptide and its binding partner/s as the staining of these cells with the TMG peptide was fainter than with the DSP peptide. Both results support the idea that TMG- binding partner/s is expressed at lower levels or interacts weakly in MDA-MB-231 cells compared to the DSP- binding partner/s.

5.2.2 Isolation of DSP and TMG-binding partners in MDA-MB-231 cells using streptavidin-coated magnetic beads

Having identified putative binding partner/s for the DSP peptide, and shown it to be proteinaceous in nature, the isolation of these protein/s by employing streptavidin coated magnetic beads to retain the protein-peptide complexes was attempted. As a starting material, either protein extracts from MDA-MB-231 cells incubated *in vitro* with the biotinylated peptides as described in section 5.2.2.1 or extracts from cells *ex-vivo* cross linked with the peptides as described in section 5.2.2.2 were used. In order to improve detection of DSP-binding partner/s by silver staining of acrylamide gels, the starting number of MDA-MB-231 cells was $9x10^8$. Prior to their use, Streptavidin-coated magnetic beads were treated with high salt buffers as described in *Materials and Methods* section 2.4.4 to remove BSA, which is a component of the buffer the beads are stored in.

5.2.2.1 Isolation of DSP *in vitro* binding proteins on streptavidin-coated magnetic beads

MDA-MB-231 cells were harvested and cell extracts prepared as described in the Methods section. Approximately 2mg protein extract (in duplicate) was incubated with 20 µg biotinylated DSP peptide for 2hr, and then mixed with pre-washed Streptavidin coated magnetic beads for 30 min. The unbound fractions were collected and the beads were washed and each wash was collected. The bound fraction was eluted by incubation of the beads with 200µg DSP and TMG peptides as described in *Materials and Methods* section 2.4.4 The washes were precipitated with TCA and resuspended in SDS sample buffer. The eluted and the unbound fraction were mixed with 2x SDS sample buffer, boiled for 5 min and separated by 12% PAGE. The gel was silver stained as described in the section 2.4.7.

The results in figure 5.3 shows three bands on the fractions eluted with DSP peptide (A: lane 2) at approximately 83, 70 and 55kDa. The same bands are present in the fractions eluted with TMG peptides (B: lane 2) although in this case the gel is slightly over stained, thus some bands had turned translucent loosing contrast in the scanned gel. The three bands were specifically eluted with both peptides as they are not present in the washes (A & B; lanes 6-10). The 83kDa protein band was fully eluted from the beads (A & B; lane 3). Since none of the proteins eluted with the peptides appear to be specific, this approach was not pursued.

5.2.2.2 Isolation of DSP and TMG *ex-vivo* binding proteins on streptavidin-coated magnetic beads

MDA-MB-231 cells were fixed with 3.7% paraformaldehyde when they were 50-65% confluent in 175 cm^2- TC flasks. The cell membranes were permeabilised with NP-40 and the cell monolayers were blocked with 5% casein PBS containing biotin. Then they were divided into two equal parts and incubated with either biotinylated DSP or TMG peptides for 2hr, washed and fixed again with 3.7% paraformaldehyde. This protocol is an modified version of the one used to detect binding of the peptides to cell lines described in the *Materials and Methods* section 2.2.3. The monolayers were washed and the cells scraped off the flasks. Cell extracts were prepared and then incubated with streptavidin-coated magnetic beads as described in the *Materials and Methods* section 2.4.1 and 2.4.5. The unbound fractions were collected. The beads were washed with high salt-containing buffer and each wash was collected and precipitated with trichloro-acetic acid as described in the *Materials and Methods* section 2.4.6. The crosslinked protein-peptide and protein-protein complexes, were eluted from the beads by boiling the beads for 15 min in 2x SDS Sample Buffer (Layh-Schmitt, Podtelejnikov et al. 2000) prior to separation by SDS-PAGE. The first, seventh and tenth washes, one eight of the unbound fraction and the bound fraction were separated on 10% SDS-PAGE and the gel was silver stained using the European Molecular Biology Laboratory (EMBL) silver staining protocol (Shevchenko, Wilm et al. 1996) which allows subsequent protein analysis by mass spectrometry.

The results in fig 5.4 shows that two major bands with similar molecular weights are present in the fractions eluted with either the DSP or the TMG peptide. (Fig 5.4. A&B lane 5). The molecular weights of the two major protein bands are
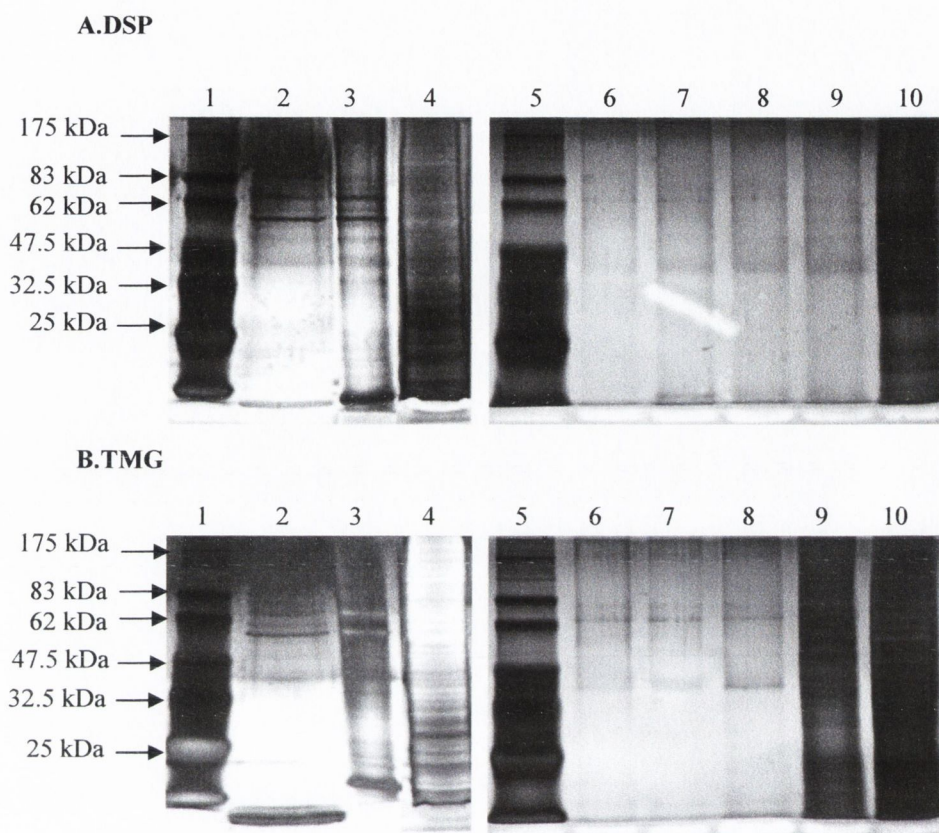
Fig 5.3 Isolation of DSP *in vitro* binding proteins on streptavidin-coated magnetic beads
Biotinylated DSP peptide incubated with MDA-MB231 cell extracts for 2hr and was immobilised on streptavidin-coated magnetic beads as described in *Materials and Methods* section 2.4.4. The bound fraction was eluted with non-biotinylated peptides. The samples were separated by 12% SDS-PAGE. The silver stained gels show A.DSP: samples obtained using DSP peptide in the elution and B.TMG: samples obtained using TMG peptide in the elution; lane 1 & 5: molecular weight markers, lane 2: eluted fraction, lane 3: beads after elution with non-biotinylated peptide, lane 4: unbound fraction, lane 6: tenth wash, lane 7: ninth wash, lane 8: fifth wash, lane 9: third wash, lane 10: first wash.

A.DSP
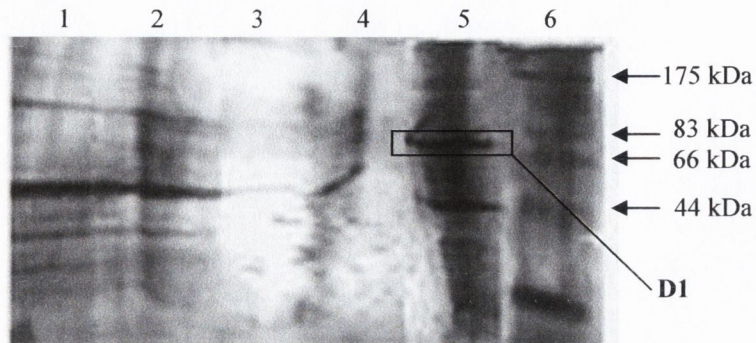
B.TMG
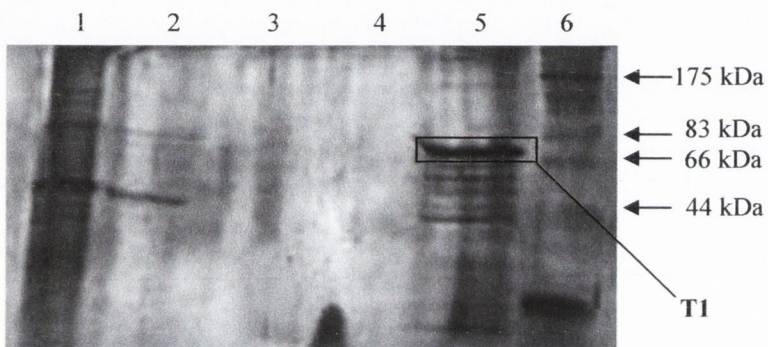
Fig 5.4 Isolation of DSP and TMG *ex-vivo* binding proteins on streptavidin-coated magnetic beads

Biotinylated DSP and TMG peptides were crosslinked with MDA-MB231 cells, the cells were lysed, incubated with streptavidin-coated magnetic beads for 30 min and washed thoroughly as described in *Materials and Methods* section 2.4.5. The retained fraction was eluted disrupting the crosslinks by boiling for 15 min in 2x sample buffer and the sample separated by 10% SDS-PAGE. The silver stained gels show A.DSP: samples obtained from the immobilised DSP peptide and B.TMG: samples obtained from the immobilised TMG peptide; lane 1: Unbound fraction, lane 2: fist wash, lane 3: seventh wash, lane 4: tenth wash, lane 5: eluted fraction; lane 6: molecular weight markers. D1 and T1: bands excised for identification by MALDI-TOF.

approximately 40 kDa and 70 kDa. The intensity of the 70kDa band is similar in both eluates containing DSP and TMG-interacting partners, but the intensity of the 40kDa band is less intense in the eluate containing TMG-binding partner/s. This result suggests that the DSP-and TMG-binding proteins could be one and the same but the affinity of some of those proteins for the peptides (ie 40kDa protein) could differ. It is also possible that the 40kDa protein is a breakdown product of the 70kDa protein. From the three major bands visualised in the experiment described in the section 5.2.2.1, only the 70kDa band is present in the experiment described in this section. Taken together, the results suggest that both TMG and DSP interact with similar proteins in the cell, one of which has a molecular weight of 70 kDa.

5.2.3 Identification of DSP and TMG-binding partners in MDA-MB-231 cells

5.2.3.1 Identification of DSP binding proteins by mass spectrometry

The protein bands of approximately 70kDa in size present in the fractions eluted with the DSP (D1) and TMG (T1) (shown in figure 5.4) were excised under sterile conditions and sent to be analysed by MALDI-ToF Mass Spectrometry in the Moredun Research Institute (Pentlands Science Park, Bush Penicuik EH26, Scotland, UK). Matrix Assisted Laser Desorption Ionisation Time of Flight Mass Spectrometry (MALDI-ToF) is a highly sensitive technique (femtomole level) that is used primarily for peptide mass profiling and subsequent protein identification via database mining.

The results of the Mass Spectrometry analysis are shown in Table 5.1. Unfortunately, none of the top hits for T1 band are significant which means that there was not enough protein in the gel piece to allow identification with any confidence. The fact that these proteins are listed in the top twenty hits is encouraging but the only way to obtain correct identification with any significance is to increase the amount of the protein sample analysed. Interestingly, one of the proteins identified as a TMG binder is Hsp70 from newt.

Mass spectrometry analysis of the D1 band again did not identify significant hits, however, a number of putative proteins were identified, in particular, the human ATP-dependent RNA helicase DDX18. A homology search in protein databases for short nearly exact matches of the TMG peptide, which binds to the DSP peptide, showed it to have 85% homology to the DEAD-box protein P68 that belongs to the

Table 5.1 Mascot top score search results within the mass range 66-83000dal

Analysis of T1 band

Name	Mass	Score*	Organism
Serum albumin precursor (Allergen Bos d 6)	71244	54	Bovine
Keratin, type II cytoskeletal 1 (Cytokeratin 1)	66018	50	Human
Heat shock 70 kDa protein (HSP70)	71218	41	Newt
UvrABC system protein B (UvrB protein) (Excinuclease ABC subunit B)	76177	40	Clostridium

Analysis of D1 band

Name	Mass	Score*	Organism
Serum albumin precursor (Allergen Bos d 6)	71244	101	Bovine
ATP-dependent RNA helicase DDX18 (Myc-regulated DEAD-box protein)	75702	46	Human

*Protein scores greater than 65 are significant ($p < 0.05$)

DDX5/DDX17 subfamily (Table 5.5), suggesting that DDX proteins could be a DSP-binding partner with high homology to the TMG peptide. The biochemical characteristics and biological functions of the majority of human RNA helicases (HRH) are still not fully characterised but there are examples of dysregulation of HRH expression in various types of cancer. (Abdelhaleem 2004; Abdelhaleem 2004). Other proteins identified included Keratin a common hair or skin contaminant due to sample-handling and contaminating bovine serum albumin precursor (BSA). Contaminating proteins can saturate the detector and impede the detection of certain peptides, and create an inaccurate trace that will influence the significance of the matches in the databases.

5.2.3.2 Identification of DSP and TMG binding proteins by bio panning of a breast tumour cDNA T7 phage display library

Another approach was taken to isolate DSP and TMG-binding partners. This involved the screening of a cDNA T7 phage display library, by the bio panning method, for those polypeptides with affinity for DSP or TMG peptides. A cDNA library derived from breast tumour tissue was amplified using random primers and cloned in the T7Select10-3 vector (Novagen,UK). Each T7 phage displays between 10-15 copies of a recombinant minor coat protein fused to up to 1200 amino acid polypeptides encoded by the breast tumour cDNA. Firstly the library was amplified in *E.coli* BTL5615, extracted by phage precipitation with polyethylene glycol-8,000, purified through a cesium chloride gradient by ultracentrifugation and titred. Approximately 10^8 p.f.u. T7 phage were incubated with streptavidin-coated magnetic beads, pre-blocked with 0.5%BSA, 0.1mM biotin PBS. The unbound fraction of the library was then transferred to streptavidin-coated magnetic beads with 200 pmole immobilised DSP, TMG or WHK biotinylated peptides. After 1 hour incubation at ambient temperature, the beads were washed twenty times with PBS containing TWEEN-20 (0.05%). The bound fraction was eluted by incubation with 200 pmole DSP, TMG and WHK non-biotinylated peptides for one hour, respectively. The eluted fraction was titred and amplified. The bio-panning was repeated four times with approximately 10^8 p.f.u T7 phage amplified from each preceding round.

Figure 5.5 shows the titre of the eluted phage corresponding to each of the four rounds of bio panning against WHK, DSP and TMG peptides respectively. The titre of
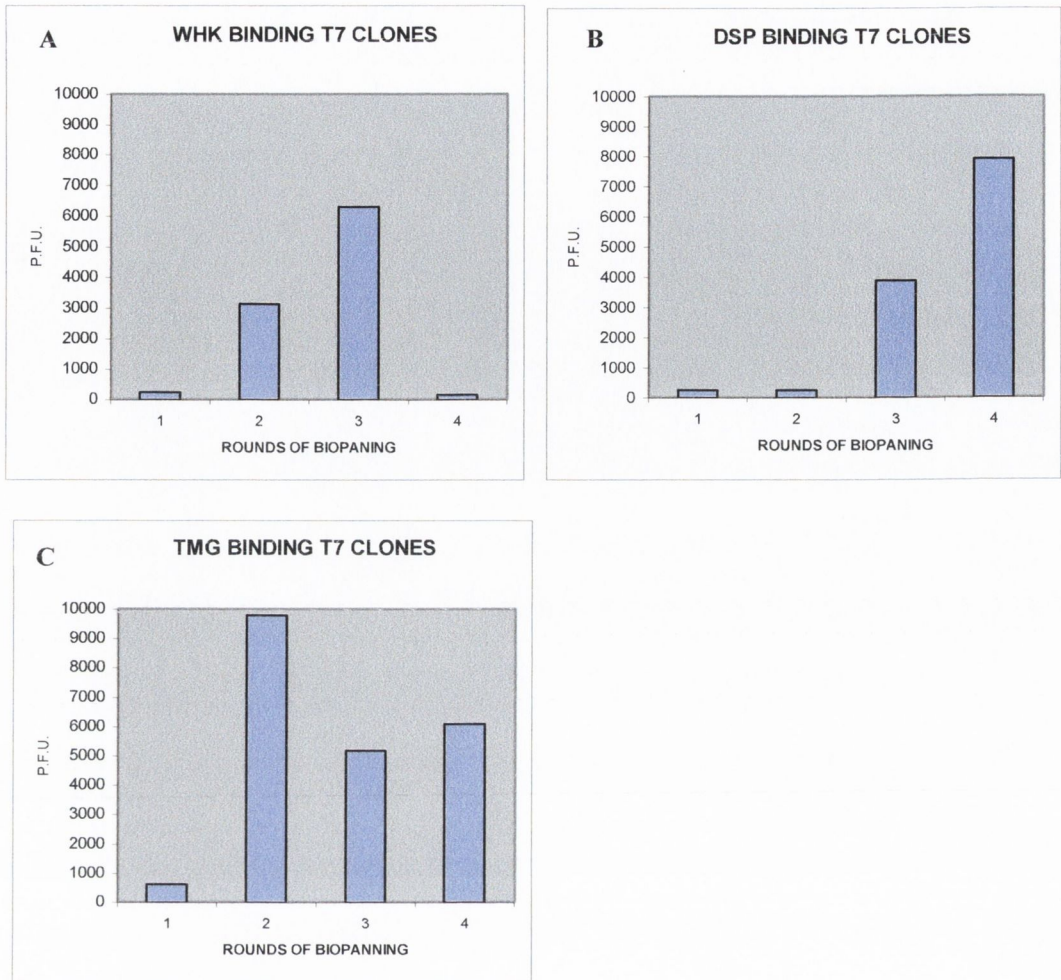
Fig 5.5   Titering of the eluate resulting from bio panning of the breast tumour cDNA T7Select10-3 library against WHK (A), DSP (B) and TMG(C) peptides

Round 1: 10^8 p.f.u. of the library was incubated against 200 pmole biotinylated peptides immobilised on streptavidin coated magnetic beads and the eluate obtained titered. (1). Round 2; 10^8 p.f.u. of the amplified eluate obtained from round 1 of bio panning was incubated against 200 pmole biotinylated peptides as before (2). Round 3; 10^8 p.f.u. of the amplified eluate obtained from round 2 of bio panning was incubated against 200 pmole biotinylated peptides as before (3). Round 4; 10^8 p.f.u. of the amplified eluate obtained from round 2 of bio panning was incubated against 200 pmole biotinylated peptides as before (4).

the recovered phage eluate increases in the three first rounds of bio panning against WHK (Fig 5.5.A) and DSP (Fig 5.5.B), but only up to the second round for TMG (Fig 5.5.C), indicating that the pool of phage becomes enriched with clones with high affinity for the target peptides after the successive rounds of bio panning. The increasing levels of Tween-20 in the washing buffer, allows selection of the strongest binders thus reducing the titre of WHK (Fig 5.5.A) and TMG (Fig 5.5.C) binders in the fourth (0.7 %Tween-20, PBS) and third (0.5% Tween-20, PBS) rounds to 175 and 5,200 p.f.u., respectively. After four rounds of bio panning using increasing stringency conditions in the washings, from a starting titer of 10^8 p.f.u., the recovery of high affinity binders to WHK, DSP and TMG are 175, 7925 and 6100 p.f.u., respectively.

To determine whether the phage identified in the fourth round of biopanning were indeed specific to the DSP (Fig 5.6.B) and TMG (Fig 5.6.C) peptides, the two fourth round eluates were plated onto two agar plates and then lifted onto nitrocellulose membranes in triplicate. The membranes lifted from each plate were incubated with either 100μg/ml biotinylated DSP, TMG or WHK peptides (Fig 5.6. A&B; DSP, TMG or WHK) and then incubated with ExtrAvidin HRP. Phage interacting with the biotinylated peptides were detected by autoradiography. The lack of signal after incubation of the filters with TMG, DSP or WHK peptide indicates that fourth round eluted phage clones obtained from biopanning against DSP and TMG are specific only for the relevant peptide. Each dot corresponds to a plaque on the agar plates, and each plaque to a single phage clone.

5.2.3.2.1 DNA sequencing, sequence analysis, and data mining

From each of the two agar plates containing the fourth round eluates from the TMG and DSP bio-panning, twenty plaques (clones) were chosen and amplified in 3 ml *E.coli BLT5615* cultures for DNA amplification by polymerase chain reaction (PCR) and sequence identification. The primers T7SelectUp anneals to the vector 43 base pairs upstream of the beginning of the cDNA insert while the primer T7SelectDown anneals to the vector 65 base pairs downstream the end of the cDNA insert (Fig 5.7). These primers were used for PCR amplification of the insert. PCR products were analysed on an agarose mini-gel and DNA detected by ethidium bromide staining. The size of the products was estimated by comparing their gel-migration to a 50bp DNA ladder (Roches, UK) run on the same gel. The PCR products were sent for sequencing to

Fig 5.6 Screening of the fourth round eluate obtained from bio panning against DSP and TMG peptides by plaque lift

The agar plates containing phage isolated in the fourth round of bio panning against DSP (A) and TMG (B) were lawned with *E. coli* infected with the eluate, transferred to three nitrocellulose membranes by plaque lift, incubated with the biotinylated peptides 100μg/ml DSP or TMG, and detected by autoradiography as described in *Materials and Methods* section 2.4.8.3. As negative controls biotinylated 100μg/ml WHK, TMG or DSP peptide was used for blotting the membranes in both A and B experiments.

WHK DSP

A

TMG

WHK TMG

B

DSP
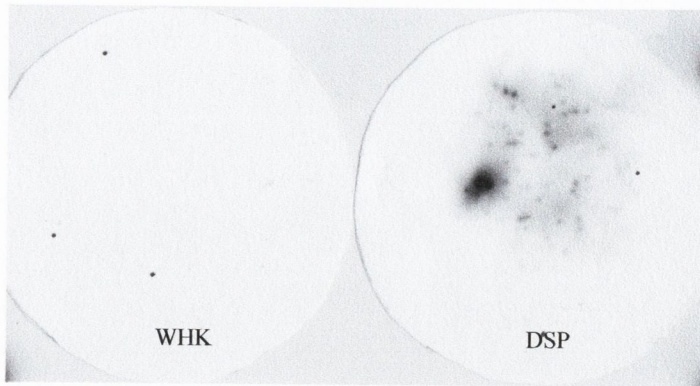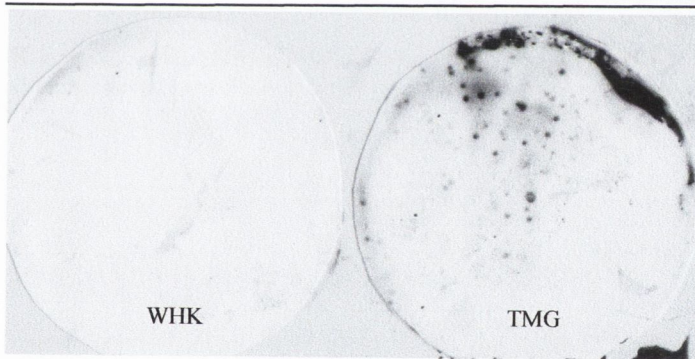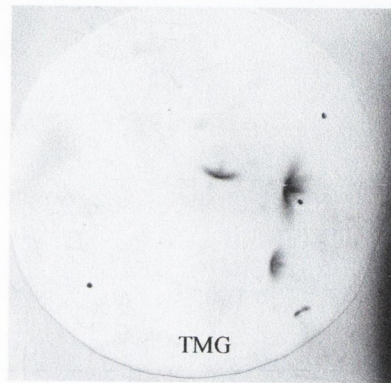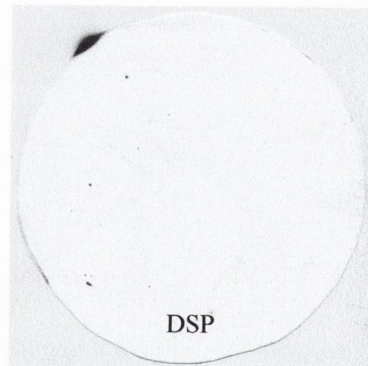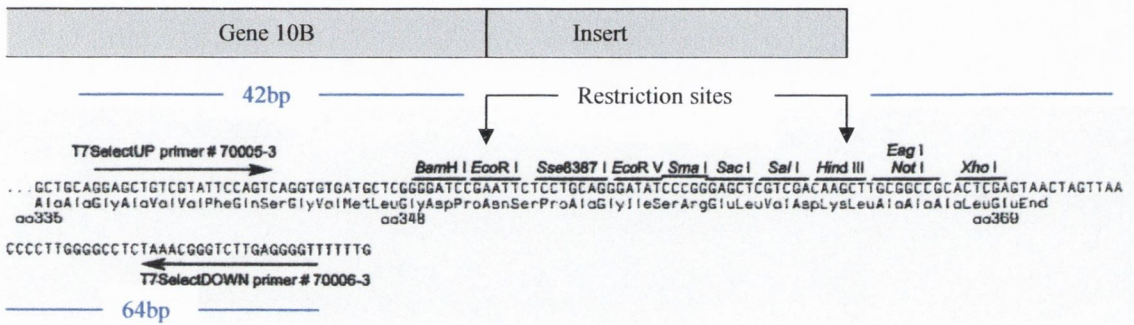
Fig 5.7 Map of the restriction sites employed for the cloning of the breast tumour cDNA library on the T7 Select10-3 vector and the annealing sites for the primers T7 SelectUP and T7 SelectDown (Novagen website; http://www.emdbiosciences.com/g.asp?f=NVG/T7Selecttable.html)

The distance between the T7SelectUP and T7 SelectDown primers and EcoRI and HindIII restriction sites are 42bp and 64bp respectively. The insert is translated as a C' terminal fusion protein of gene 10B minor coat protein.

MWGAG Biotech (Ebersberg, Germany) as described in the *Materials and Methods* section 2.4.8.4.

The gel in Fig 5.8 shows three different sizes of the PCR products; D4 and D6 with longest sequences over 500bp, for T2, T3, T4 T5, T7, T9, T10, T12, T13 with intermediate length sequences over 500bp and D1, D2, D3, D5, D7, D8, D9, D10, D11, D12, D13, D16, D18, D19, D20, T6, T8, T11, T15, T17, T18 with the shortest sequence below 500bp. The clones T1, T14, T16, T19 and T20 did not show any PCR product (data not shown), indicating the lack of a cDNA insert. Sequencing of the T1 clone and the search for homology with the T7Select10-3 vector sequence using the Blast program, corroborated the lack of insert. Clones representing each PCR product size range were also sequenced (Fig 5.8 D1, D3, D4, D5, D6, T1, T5, T6, T8, T11, T12, T13, T15, T17 and T18) and aligned using the multiple sequence alignment tool of the EMBL website. Table 5.2 is a summary of the alignment of the sequences using clustal W1.81 and indicates 100% sequence homology between the clones T5, T12 and T13, T17 and T18 and D1, D3 and D5. The clones with identical DNA sequences correspond with clones that show the same molecular weight on the agarose gels. Thus, it was assumed the clones with similar PCR product sizes had identical sequences. Based on this assumption, table 5.2 shows the frequency of the distinct sequences found among the total number of clones containing inserts in each of the two eluates. The sequences corresponding to the clones T5, T12 and T13 were the most abundant among the TMG-binders, followed by the ones corresponding to the clones T17 and T18. Among the DSP-binders, the sequence corresponding to the clones D1, D3 and D5 were the most abundant.

One clone for each of the distinct sequences present in each of the two eluates was further analysed. Using the nebcutter v.2 program, the location of the restriction sites for EcoRI and HindIII (Fig 5.9), the two restriction enzymes used for cloning the ductal breast tumour cDNA library into the T7Select10-3 vector, were identified within the DNA sequences analysed. Figure 5.9 shows the clones T6, T8, T11, T15, T18 and D5 have two identical inserts in opposite orientation, while T12 and D4 (incomplete sequence) contained only one. This was verified by inversion of the sequence of one of the two inserts and alignment of both inserts. The restriction sites suggest that the inserts are the same size. Thus, sequence and restriction enzyme digestion analysis suggests that some of the clones contained greater than one insert. This may arise from drawbacks inherent in the technique used for the construction of the library by the company

D1 D2 **D3** **D4** **D5** **D6** D7 D8 D9 D10 D11 D12 D13 D16 D18 D19 D20

← 2642 bp

← 500 bp

T2 T3 T4 **T5** **T6** T7 **T8** T9 T10 **T11** **T12** **T13** **T15** **T17** **T18** D4

← 2642 bp

← 500 bp
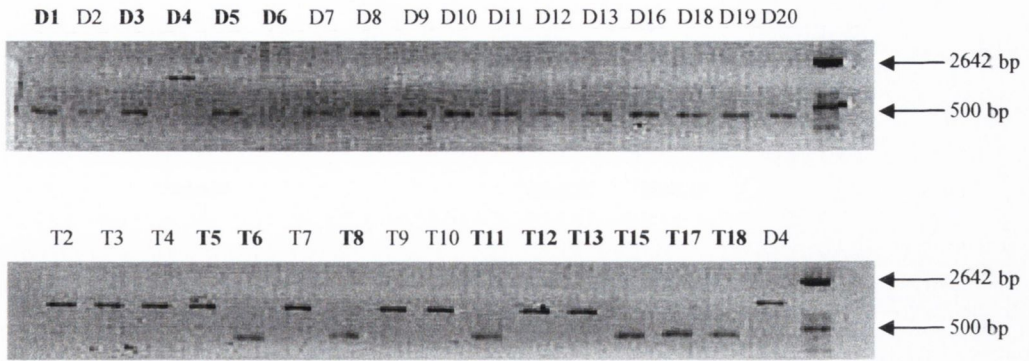
Fig 5.8 PCR products of the cDNA inserts from the clones selected from the T7 phage display library

D1-D20; phage clones obtained by bio panning against DSP peptide. T1-T18; phage clones obtained by bio panning against TMG peptide. T7SelectUp and T7Select Down primers were used for the PCR reaction, as described in Materials and Methods section 2.4.8.4, obtaining PCR products of roughly three different sizes for each set of clones. 1.8% agarose gel electrophoresis was performed to visualise the PCR products. Samples with their label in bold were sequenced.

A

CLONE	FREQUENCY (%)
T5,T12&T13	60
T17&T18	13.3
T6	6.6
T8	6.6
T11	6.6
T15	6.6

B

CLONE	FREQUENCY (%)
D1,D3&D5	88.2
D4	11.7
D6	11.7

Table 5.2 Frequency of clones with the same DNA sequence and same sized PCR products in the eluate obtained from bio panning against TMG peptide or DSP peptide

The calculations were done considering 20 clones as a representative sample of 6,100 phage present in the eluate obtained from bio panning against TMG peptide (A) and 7,925 clones present in the eluate obtained from bio panning against DSP peptide (B).
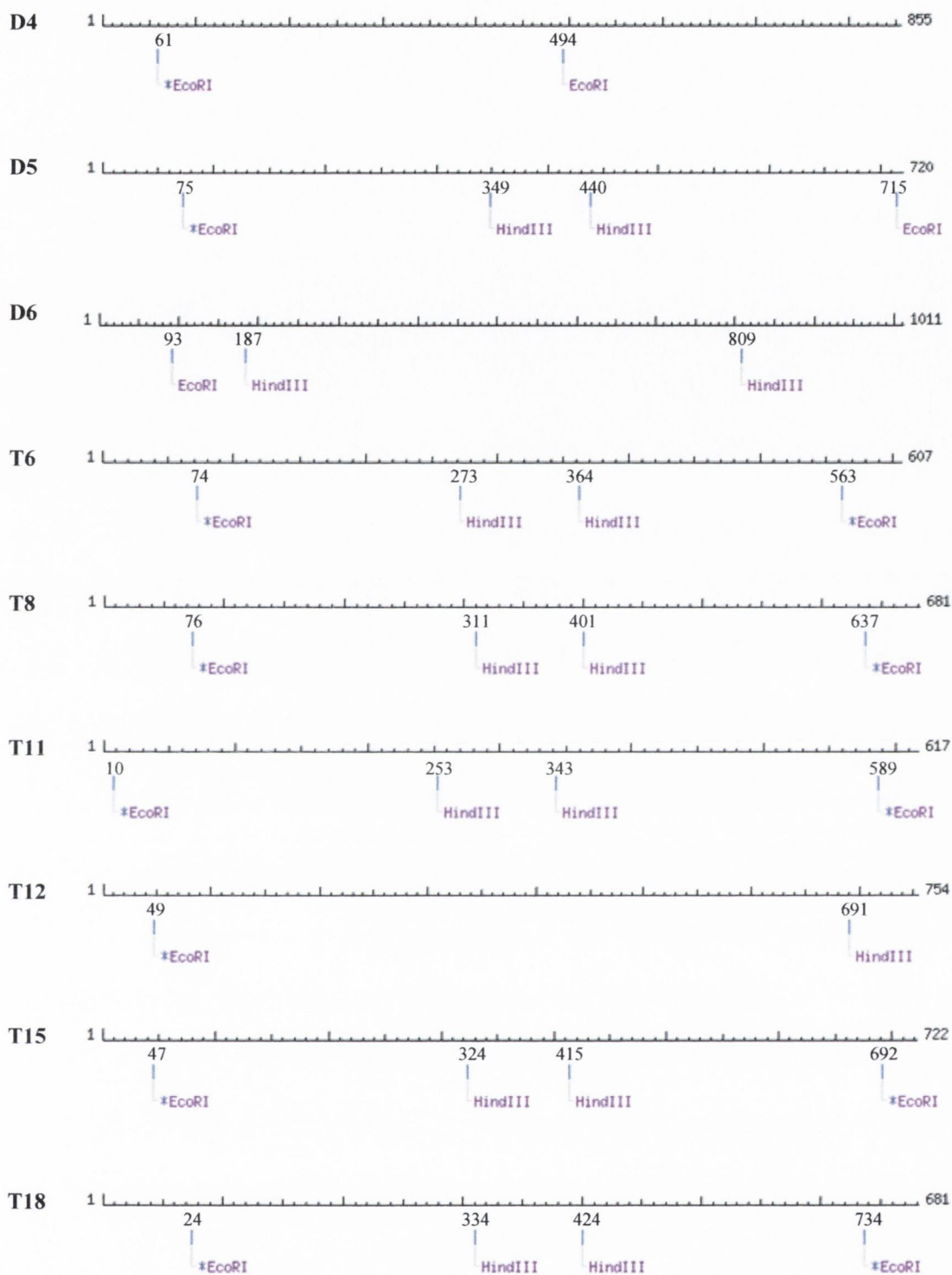
Fig 5.9 Sites for the restriction enzymes *Eco*RI /*Hind*III on the sequenced clones

D4, D5 and D6; phage clones obtained by bio panning against DSP peptide. T6, T8, T11, T12, T15 and T18; phage clones obtained by bio panning against TMG peptide. The sequenced PCR products contain part of the vector arms and the inserted cDNA sequence. Search of the sites for the digestion of the sequences with *Eco*RI /*Hind*III using the program NEB Cutter shows the number of inserts on each clone.

Novagen. Nevertheless, the orientation of the first cDNA insert relative to the encoded coat protein is the correct one as indicated by manufacturer instructions (Fig 5.7) as this is the one that is expressed as a fusion protein on the coat of the T7 phage. When comparing the estimated length of the inserts including the amplified 108bp of vector arms in figure 5.8, and the length of the whole inserted regions in figure 5.9, it can be appreciated they do not match for T6, T8, T11, T15, T18 and D5. These clones were shown to contain two cDNA inserts. Homology search between the region in between the inserts and the primers used for the PCR shown above, indicated the regions are identical and that there is high homology with the T7SelectDown primer (Fig 5.10). The PCR products are the amplification of the smaller sized sequence between the annealing sites for T7SelectUP primer and the T7SelectDown primer, corresponding to the second insert on each clone. The T7SelectUP annealed 61 bp upstream of the second insert and T7SelectDown primer annealed 64bp downstream of the second insert. The sum of the length of the second insert and 125 bp of sequence between the annealing sites and the insert, approximate the length of the PCR products estimated on the agarose gel in figure 5.9. It could be possible that the smaller sized product was amplified in preference to the larger product.

Search of the inserted sequences for matches in the human genome database identified matches for all sequences with the exception of T11 (Table 5.3 and 5.4) which shows no significant similarity with any genome. The sequences D4, D5 and D6, were not annotated in the human genome database, but the analysis of D6 showed it is part of a clone next to an exon of the LARGE gene for like-glycosyltransferase (Table 5.3). Additionally, the sequence D6 (810 bp) corresponds to an unannotated cDNA clone but surprisingly, only up to the base pair numbered 320 (Table 5.3). T8 (89% identities) and T15 sequences also correspond to unannotated cDNA clones in the human genome database (Table 5.4). T6, T12 and T18 sequences were found to correspond to the mRNA sequence of profilin 1, part of the MHC class III region (91% identities) and the 3'end mRNA region of the splicing factor SF1-HL1, respectively (Table 5.4). Another surprising finding is that T12 (643bp) sequence was homologous to an unannotated cDNA clone with 90% identity but only from base pair number 380. Diagrams of the location of the sequences in the human DNA clones are shown in figure 5.11.

Translation of the first inserts in frame with gene 10B indicated by the manufacturer instructions (Fig 5.12) showed that, the expressed inserts do not exceed 22 amino acids in length (Fig 5.13), suggesting the cDNA were inserted in the vector arms

94

```
T6            GCGGCCGCACTCGAGTAACTAGTTAACCCCTTGGGGCCTCTAAACGGGTCTTGAGGGGTT
T8            GCGGCCGCACTCGAGTAACTAGTTAACCCCTTGGGGCCTCTAAACGGGTCTTGAGGGGTT
D5            GCGGCCGCACTCGAGTAACTAGTTAACCCCTTGGGGCCTCTAAACGGGTCTTGAGGGGTT
T11           GCGGCCGCACTCGAGTAACTAGTTAACCCCTTGGGGCCTCTAAACGGGTCTTGAGGGGTT
T15           GCGGCCGCACTCGAGTAACTAGTTAACCCCTTGGGGCCTCTAAACGGGTCTTGAGGGGTT
T18           GCGGCCGCACTCGAGTAACTAGTTAACCCCTTGGGGCCTCTAAACGGGTCTTGAGGGGTT
T7SelectDown  ------------------------AACCCCTCAAGACCCGTTTA----------------
                                      ******  ..*.**   *::*

T6            AACTAGTTACTCGAGTGCGGCCGC
T8            AACTAGTTACTCGAGTGCGGCCGC
D5            AACTAGTTACTCGAGTGCGGCCGC
T11           AACTAGTTACTCGAGTGCGGCCGC
T15           AACTAGTTACTCGAGTGCGGCCGC
T18           AACTAGTTACTCGAGTGCGGCCGC
T7SelectDown  -----------------------
```

61bp

Second insert

Fig 5.10 CLUSTAL W (1.83) multiple sequence alignment of the region between inserts in clones T6. T8, T11, T15, T18, D5 and the T7SelectDown primer

D5; phage clones obtained by bio panning against DSP peptide. T6, T8, T11, T15 and T18; phage clones obtained by bio panning against TMG peptide. The corresponding sequenced PCR products contain two cDNA inserts separated by a region with high homology to the primer T7SelectDown.

Table 5.3 Summary of the sequence analysis on selected clones obtained by bio panning against DSP peptide

CLONES NAMES	CLONES FREQUENCY	LENGTH OF INSERTS (BP)	TWO REVERSE INSERTS	NUCLEOTIDE BLASTS	IDENTITIES (%)
D4	90%	>795	N	Homo sapiens 12 BAC RP11-681J20	98 (693/702)
D5	5%	274	N	Homo sapiens BAC clone RP11-250F14 from 4	100 (272/272)
D6	5%	810	N	ir91h09.y1 HR85 islet Homo sapiens cDNA clone IMAGE:6609858 5'	100 (320/320)
				Human DNA sequence from clone LL22NC01-78G1 on chromosome 22 Contains an exon of the LARGE gene for like-glycosyltransferase (KIAA0609), ESTs, an STS and GSSs	99 (709/712)

Table 5.4 Summary of the sequence analysis on selected clones obtained by bio panning against DSP peptide

CLONE NAMES	CLONES FREQUENCY	LENGTH OF INSERTS (BP)	TWO REVERSE INSERTS	NUCLEOTIDE BLASTS	IDENTITIES (%)
T6	6.6%	200	Y	Homo sapiens profilin 1 (PFN1), mRNA	98 (189/191)
T8	6.6%	236	Y	6002632704F1 NCI_CGAP_ skn3 Homo sapiens cDNA clone IMAGE:4777776 5'	89 (205/228)
T11	6.6%	244	Y	Disseminated	
T12	60%	643	N	UI-H-DP0-avc-i-05-0-UI.s1 NCI_CGAP_Fs1 Homo sapiens cDNA clone UI-H-DP0-avc-i-05-0-UI 3'	90 (218/240)
				Homo sapiens BAC clone 215O12 NG35, NG36, G9A, NG22, G9, HSP70-2,HSP70-1, HSP70-HOM, snRNP, G7A, NG37, NG23, and MutSHgenes, complete cds	91 (218/239)
T15	6.6%	276	Y	K-EST0152252L4SNU368 Homo sapiens cDNA clone L4SNU368-22-E04 5'	100 (271/271)
T18	13.3%	310	Y	*H.sapiens* mRNA for splicing factor, SF1-HL1 isoform	100 (253/253)

Fig 5.11 Diagram of the location of the cDNA sequences selected from the breast tumour T7 library on the human DNA or cDNA cloned sequences submitted to human genome databases D4, D5 and D6; phage clones obtained by bio panning against DSP peptide. T8, T12, T15 and T18; phage clones obtained by bio panning against TMG peptide. The cDNA insert from each phage clone was used to search the human genome databanks using the program BLAST. The boxes correspond to human genomic sequences and the lines to cDNA sequences selected from the breast tumour T7 library.

BAC clone RP11-681520
on chromosome 12
D4

46271bp

3239 —— 3940
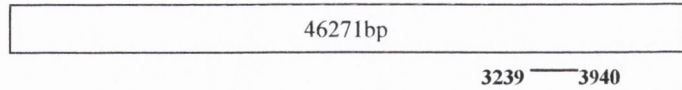
BAC clone RP11-250F14
from chromosome 4
D5
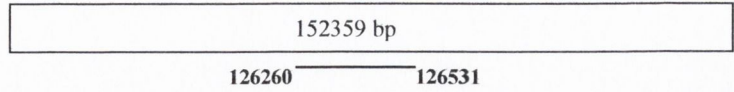
152359 bp

126260 —— 126531

ir91h09.y1 HR85 islet Homo
sapiens cDNA clone
IMAGE:6609858 5'.
D6
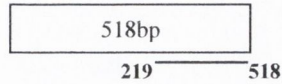
518bp

219 —— 518

LL22NC01-78G1 on
chromosome 22
Contains an exon of the
LARGE gene for like-
glycosyltransferase
D6

29393   29517

36798 bp	exon	

234 —— 945

Homo sapiens profilin 1
(PFN1), mRNA
T6

847bp

388 —— 578
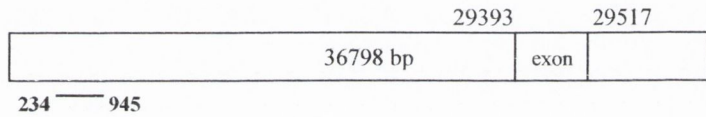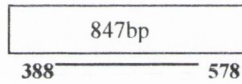
602632704F1 NCI_CGAP
_Skn3 Homo sapiens cDNA
clone IMAGE:4777776 5'
T8

778bp

237 — 464
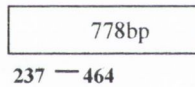
BCA clone 215O12 NG35,
NG36, G9A, NG22, G9,
HSP70-2,HSP70-1, HSP70-
HOM, snRNP, G7A, NG37
NG23, and MutSH5 genes
T12

88287   90212   97494   97793   100222   102608

	Hsp70-1		MHC class III region with disperse repeats AluSg		hsp70-2	

97543 —— 97783
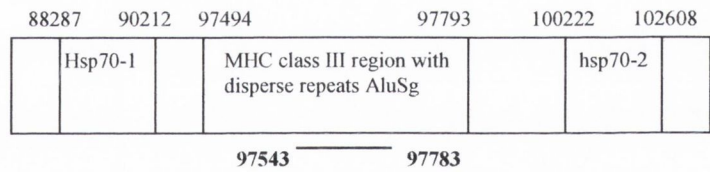
K-EST0152252 L4SNU368
Homo sapiens cDNA clone
L4SNU368-22-E04 5'
T15

444 bp

131 —— 401
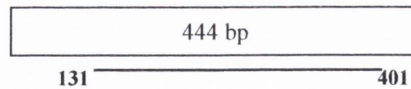
mRNA for splicing factor,
SF1-HL1 isoform
T18

43   1962   3176

	SF1-HL1	3'end	

2643 —— 2895
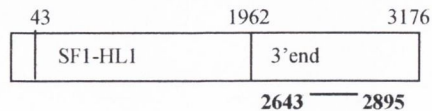
```
gene 10B →                          insert
left arm...GATCCG          AATTXXXX(N)XXXX         AGCTT...right arm
left arm...CTAGGCTTAA          XXXX(N)XXXXTCGA       A...right arm
         ...AspPro         AsnPhe...
                           AsnLeu...
                           AsnSer...
                           AsnTyr...
                           AsnCys...
                           AsnTrp...
```

Fig 5.12   Translation reading frame of inserts cloned into *Eco*R I/*Hin*d III T7Select10-3b vector arms

The inserts require a 5'-AATT "sticky end" on the top strand (amino terminal side) and 5'-AGCT "sticky end" on the bottom strand, either created with oligonucleotides or by *Eco*R I/*Hin*d III digestion. The reading frame requires the AAT (Asn or N) initial codon, followed by a TXX codon (possible second amino acid are shown). Use of *Eco*R I cleavage products places C in the second position, resulting in a TCX codon (Ser or S)

D4 (5aa)

N S S *K Q L T K*

D5 (8aa)

N S K *A D L S R Q K N*

D6 (18aa)

N S S *P Q C I E K S I S E E G S Y I S G N*

T6 (23aa)

N S S *G E G T D E V S T G N A E G G R P F H N I S C*

T8 (2aa)

N S S *H K*

T11

N S

T12 (6aa)

N S R *G E K L E N*

15 (2aa)

N S S *G E*

T18 (19aa)

N S S *V F Q G A I A T P K D V C L K R G V F*

Fig 5.13 Translated cDNA inserts of the phage selected by bio panning of a T7 phage display library against DSP and TMG peptides

The cDNA insert was translated using the 'DNA-protein Translate' tool on the ExPASy proteomics server of the Swiss Institute of Bioinformatics. **Bold;** amino acids that belongs to the cDNA inserts. The rest of the amino acids belong to the junction between the insert and the gene10B coat protein.

out of frame. A search of protein databases did not yield any protein with 100% homology to the polypeptides of 8 or more amino acids long that are fused in frame to the coat protein 10B (Table 5.5). In this situation, the proteins with highest homology to the polypeptides could be understood as possible interactors to TMG or DSP peptides, due to homology between amino acids that could be potentially involved in the interaction with TMG or DSP peptides, but they do not provide the identity of TMG and DSP interactors. Firstly, a search in databases including all organisms was performed to screen for those sequences that were part of the T7 bacteriophage. Then, to narrow the search among the human proteins, the peptides DSP, TMG, the DSP binders D4, D5, D6, and the TMG binders T6, T12, T18 were searched in the *Homo Sapiens* proteinome databases. The search was performed using the tool BLAST for short near exact matches, at the NCBI website. Proteins showing near exact matches are listed in Table 5.5. All of the fused polypeptides as well as the original peptides TMG and DSP show partial similarity with human proteins. However due to their short length and their translation out of frame, firm conclusions cannot be drawn from these results.

5.2.3.2.2 Length of the polypeptides expressed by the selected T7Select10-3 phage clones by western blot

The length of the theoretically predicted coat proteins fusion present in the cDNA clones was further analysed by Western blotting. Phage protein extracts (20µg) were separated by 12% SDS-PAGE. The T7Select10-3 vector is constructed to express the minor capsid protein or gene B which is truncated and fused to a protein encoded by the cDNA insert at its C' terminal with a valency of 5 to 15 copies per virion. T7Select10-3 phage are grown on a complementing host (*E. coli* strain BLT5615) that supplies large amounts of the 10A major capsid protein from a plasmid. Capsids thus contain mostly 10A protein, along with 5-15 copies of 10B fusion protein per virion. Both gene A and truncated gene B protein have 348 amino acids and the same sequence. There is an eleven amino acid leader peptide at the N-terminal of the gene 10 A and B coat proteins termed T7 Tag peptide that is recognised by the T7 Tag antibody. Thus, a western blot could indicate the difference in size between the minor and major coat proteins if the size of the expressed insert is a long polypeptide rather than a short one as it had been predicted by the use of bio informatics.

Table 5.5 Homology search in human protein databases

DSP BINDERS	Identities	Positives
TMG (12AA)		
*Protocadherin 16 precursor (Dachsous 1) (Cadherin-19) (Fibroblast cadherin 1)	7/9 (77%)	7/9 (77%),
ubiquitin specific protease 6; tre-2 oncogene; hyperpolymorphic gene 1	5/6 (83%)	5/6 (83%)
*cadherin 20, type 2 preproprotein Cadherin 18, type 2, preproprotein	6/8 (75%)	8/10 (80%)
Single stranded DNA binding protein 4	7/14 (50%)	8/14 (57%)
receptor tyrosine kinase-like orphan receptor 1	6/7 (85%)	6/7 (85%)
DEAD/H (Asp-Glu-Ala-Asp/His) box polypeptide 5 (RNA helicase, 68kDa) DEAD-box protein p68 belongs to the DDX5/DDX17 subfamily.	6/7 (85%)	6/7 (85%)
D4 (5 AA)		
Glutamate dehydrogenase 1	5/5 (100%)	5/5 (100%)
U3 small nucleolar ribonucleoprotein MPP10 (M phase phosphoprotein 10)	5/5 (100%)	5/5 (100%)
D5 (8 AA)		
*catenin (cadherin-associated protein) delta.	6/7 (85%)	6/7 (85%)
Gastric cancer-related protein VRG107 Small EDRK-rich factor 2	5/7 (71%)	7/7 (100%)
Microtubule-associated protein 4, isoform 3	6/7 (85%)	6/7 (85%)
D6 (18 AA)		
*very large G protein-coupled receptor 1	10/15 (66%)	11/15 (73%)
Protein phosphatase 2 regulatory subunit B	12/21 (57%)	13/21 (61%)
***Ubiquitin**-specific processing protease 29	7/10 (70%)	9/10 (90%)
*Mitosin-associated protein MITAP1	6/6 (100%)	6/6 (100%)
LIS1-interacting protein NUDEL	6/6 (100%)	6/6 (100%)
NY-REN-62 antigen	8/11 (72%)	8/11 (72%)
Similar to kinesin family member 21A	8/11 (72%)	8/11 (72%)

TMG BINDERS	Identities	Positives
DSP(12AA)		
RNA binding motif, single stranded interacting protein 1 isoform c SCR2	7/9 (77%)	7/9 (77%)
single-stranded DNA-binding protein MSSP1	7/9 (77%)	7/9 (77%)
transcription factor TBX5	7/8 (87%)	7/9 (77%)
tau tubulin kinase 2	6/7 (85%)	7/9 (77%)
soluble activin receptor-like kinase 7b	8/13 (61%)	9/13 (69%)
ATPase, Na+/K+ transporting, alpha 1 polypeptide	6/8 (75%)	8/8 (100%)
*FGFR signalling adaptor SNT-1	6/8 (75%)	7/8 (87%)
***ubiquitin** specific protease 26	6/10 (60%)	8/10 (80%)
*Importin-alpha re-exporter (Chromosome segregation 1-like protein) (Cellular apoptosis susceptibility protein)	6/9 (66%)	7/9 (77%)
T6 (23 AA)		
*RAS- responsive element binding protein 1 (RREB-1)	10/14 (71%)	13/14 (92%)
Cleavage and polyadenylation specific factor 1	5/7 (71%)	7/7 (100%)
Dendrin	7/8 (87%)	7/8 (87%)
Tintin; connectin	9/16 (56%)	11/16 (68%)
T12 (6 AA)		
*Kinesin family member 13A; mannose-6-phosphate receptor transporter	5/5 (100%)	5/5 (100%)
Restin; cytoplasmic linker protein-170 alpha-2	5/5 (100%)	5/5 (100%)
*Similar to vascular endhothekial growth factor receptor 1 precursor(VEGFR-1);tyrosine-protein kinase receptor FLT	5/5 (100%)	5/5 (100%)
Tumour potentiating region (translocated promoter region); nucleoprotein TPR	5/5 (100%)	5/5 (100%)
*G1/S-specific cyclin HSPC206 /HSPC232; similar to Periphilin 1 (Gastric cancer antigen Ga50)	5/5 (100%)	5/5 (100%)

T18 (19 AA)		
Amyloid precursor interacting protein type II	10/16 (62%)	11/16 (68%)
Tubby super-family protein; tubulin gamma complex associated protein 6	7/9 (77%)	8/9 (88%)
Complement receptor 1	7/8 (87%)	7/8 87%
*Apoptosis-inhibitor-like protein	8/12 (66%)	9/12 (75%)

Identities; identical amino acids
Positives; homologous amino acids
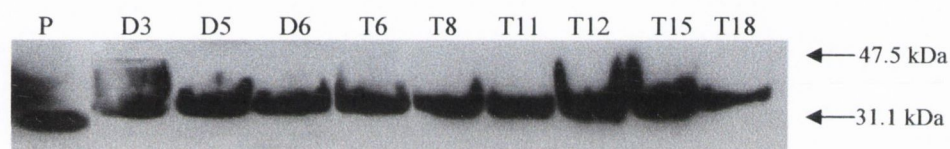
Fig 5.14 Major and minor capsid proteins on the T7 phage clones selected by biopannig against DSP and TMG peptides

D3, D4 and D6; phage clones obtained by bio panning against DSP peptide. T6, T8, T11, T12, T15 and T18; phage clones obtained by bio panning against TMG peptide. P; T7 Tag Positive Control Extract. 20 µg phage extracts and 40 ng T7 Tag Positive Control Extract were separated by 12% SDS-PAGE. The proteins were transferred to a nitrocellulose membrane, blocked and probed with T7 Tag antibody HRP conjugate (1:5,000) as described in *Materials and Methods* section 2.4.8.6. The clone without cDNA insert expression, T11, was used as negative control.

The proteins were transferred from the acrylamide gel to a nitrocellulose membrane. The membrane was blocked for 2 hr with 5% casein PBS, washed and incubated with T7 Tag antibody HRP conjugate. As a positive control a recombinant protein of 31.1 kDa that contains the 11 amino acid T7 Tag peptide was used (T7 Tag Positive Control Extract, Novagen, UK), and the negative control was the clone without cDNA insert expression, T11.

The results in figure 5.14 shows the subtle difference in the migration of the 31.1 kDa positive control (lane P) and 36.73 kDa negative control (lane T11). The difference in size between these two proteins is 5.63 kDa, which correlates to approximately a 56-57 amino acid polypeptide chain. The migration of T6, T8, T12, T15, T18, D3, D4 and D6 shows no difference to the negative control T11, indicating the length of the inserts expressed by these clones is quite small and does not alter the electrophoretic mobility of the fusion protein. This result supports the short length of the predicted peptides displayed by T6, T8, T12, T15, T18, D3, D4 and D6 (Fig 5.13).

5.3 DISCUSSION

The results obtained in chapter 4 indicated that the DSP and TMG peptides interact with cellular components present in the cytoplasm of MDA-MB-231 cells. Several biochemical methods have been used in an attempt to detect, isolate and identify DSP and TMG interacting partners.

When using the Far Western technique in which the MDA-MB-231 cell extracts were separated and transferred to a membrane that was probed with the peptides, the conditions for the interaction proved to be restrictive. However, when the interaction between the peptides and the cells were maintained by cross linking, the Far Western technique allowed the detection of the DSP-binding partner/s but not the TMG-binding partners, supporting the idea proposed in chapter 4, that DSP- binding partner/s are more abundant than TMG-binding partner/s. Total digestion of DSP-binding partners, with trypsin or endoproteinase Glu-C indicated their proteinaceous nature, therefore the experiments included the silver staining technique for protein detection. Once DSP-interacting partner/s were detected, it was decided to proceed to their isolation by using streptavidin-coated beads to retain the biotinylated peptides and their binding partners within the MDA-MB-231 cell extracts. Three bands of 80, 70 and 55 kDa in size were identified in the eluate from both DSP and TMG-bound extracts. The

sum of the three major bands is more than 175 kDa indicating they could be the components of the protein complex observed when using the Far Western technique. However, the fact that the same molecular size bands were obtained with both the DSP and TMG peptides suggests the presence of non-specific binders with affinity to the streptavidin coated beads or that the DSP and TMG peptides are binding to the same peptide binding proteins present in the cell extract. Considering the fact that TMG peptide was formerly identified as a Hsp70-PC interacting molecule, it is quite possible that the 70 kDa protein identified here is Hsp70, a peptide binding protein. To overcome the drawbacks associated with this approach, the biotinylated peptides were incubated with MDA-MB-231 cells and cross linked "ex-vivo" prior to a short incubation of their protein extracts with the streptavidin coated beads. Using this approach, two major bands of about 70 and 40 kDa were identified. Again, the same proteins were identified following cross-linking to either the TMG or the DSP peptide. The major 70 kDa bands were analysed by MALDI-TOF and were shown to contain BSA as a contaminant from the beads storing buffer. Both samples contained some other proteins whose identity could not be deciphered due to low amount of protein sample. Thus, the rest of the bands showing a weaker silver staining were not further analysed. One of the possible TMG-binding partners identified was Hsp70 from Newt. This protein has high similarity to its human counterpart which was initially employed for the generation of TMG and DSP peptides. Its chaperone function would explain its interaction with many peptides including the TMG peptide. A possible DSP- binding partner is the ATP-dependent RNA helicase DEAD-box protein DDX18. Some DEAD-box protein families have been shown to be dysregulated in tumour cells (Abdelhaleem 2004; Abdelhaleem 2004).The DEAD-box protein P68 that belongs to the DDX5/DDX17 subfamily showed 85% identity with TMG peptide which could suggest that DDX18 and P68 proteins could be DSP-binding partners and TMG homologous counterparts in MDA-MB-231 cells.

The finding that the Hsp70 protein was identified through interaction with TMG peptide, could corroborate the earlier findings outlined in chapter 3 regarding the ability of the TMG peptide to mimic the immune response of Hsp70-PC extracted from MDA-MB-231. It had been suggested the possibility that there could be proteins homologous to the DSP/TMG mimic/recogniser pair, similar to for example, a receptor/ligand pair such as EGFR/EGF overexpressed and associated with Hsp70. Moreover, HSP70 due to its chaperone function is able to associate with most peptides in solution (Li 2004) . It would be therefore interesting to immunoblot the cross-linked

proteins associated with the TMG and DSP peptides with anti Hsp70 antibody to confirm or rule out this possibility. In conclusion, the low amount of protein available for analysis by MALDI-ToF Mass Spectrometry was insufficient to allow accurate identification of the proteins and the contamination of the sample with BSA hampered the analysis. The use of cells in suspension rather than growing as monolayers could facilitate the isolation of larger amounts of protein. Furthermore, the contamination by BSA could be avoided by further washing of the streptavidin coated magnetic beads, which are sold in a BSA-containing buffer, prior to their use.

Due to the limitations of the biochemical approach, an alternative method was used for the identification of DSP and TMG interacting partners. A human breast tumour cDNA library expressed on the surface of T7 phage was screened by biopanning with the TMG and DSP peptides. The phage titre at each round of bio panning and the plaque lift results indicated that the technique worked. Unfortunately, analysis of the cDNAs isolated revealed that there were several problems with this cDNA library. Firstly, a large number of their clones contained multiple inserts and secondly the cDNA inserts were out of frame with the gene10B minor coat protein, therefore the polypeptides identified by this approach could only give information about amino acids taking part in the interaction but not about the identity of the binding partner(s). As a result, the translated cDNA inserts fused with the coat protein from the selected clones did not correspond to any human protein in the databases. A western blot employed for the detection of the gene 10 proteins A and B confirmed the expression of short fusion proteins by the DSP and TMG-binding T7 clones. Nevertheless, the peptides displayed by the selected clones are peptides that interact with DSP or TMG peptides, therefore human proteins that shared homology with the selected peptides could have peptide motifs with binding affinity for DSP or TMG peptides. Interestingly, among the proteins from databases that showed over 50% homology for the peptides displayed by the selected clones many are involved in regulation of growth and cell survival. Proteins identified included the fibroblast growth factor receptor (FGFR) signalling adaptor SNT-1, the very large G protein-coupled receptor 1, a protein similar to vascular endothelial growth factor receptor (VEGFR), the protein E-cadherin and E-cadherin-interacting protein catenin, a protein involved in apoptosis inhibition, cellular apoptosis susceptibility protein, G1/S-specific cyclin HSPC206 /HSPC232 and the Mitosin-associated protein MITAP1 (Fig 4.5 *). However, it must be borne in mind the possibility that the "in vitro" conditions used for the interaction between peptides DSP

and TMG might not be appropriate. These results are not conclusive, however, the protein β-catening plays an important role in cell-cell adhesion as it links E-cadherin, a transmembrane glycoprotein, to the actin cytoskeleton through the protein α-catenin. E-cadherin expression is frequently lost or significantly reduced in malignantly transformed cells with an invasive phenotype that show a low degree of cell contact growth inhibition. Down regulation of E-cadherin allows EGRF activation by its ligand and liberates β-catenin, bound to its intracellular domain, into the cytoplasm. This accumulation drives its interaction with members of LEF/TCF family of nuclear transcription factor resulting in altered gene expression (Takahashi and Suzuki 1996). This could explain the results obtained when detecting DSP and TMG peptides binding to cells at different confluence stages by cytochemistry and histochemistry. The use of random primers for the construction of the inserts of the breast tumour cDNA T7Select10-3 library by Novagen, led to double inserts in a high portion of the clones and to their expression out of frame, impeding its use as a means of identifying the proteins interacting to TMG and DSP peptides.

In conclusion, the approaches taken for the identification of DSP and TMG binding partners in MDA-MB-231 cells failed to generate fully reliable results. The limitations in the amounts of cells available for protein purification, the strict required intracellular conditions in which the interaction takes place and the intrinsic error in the cDNA library used were the major impediments for the success of the aims intended in this chapter. However the tentative finding of Hsp70 as a TMG interacting partner by two separate techniques warrants further investigation.

GENERAL DISCUSSION

6 GENERAL DISCUSSION

One of the major goals in cancer research is the identification of tumour-specific antigens with immuno-stimulatory activity that can be used for the development of tumour vaccines. The pioneering studies of P.K. Srivastava and colleagues discovered that Hsps isolated from tumours are a rich source of antigenic peptides. Subsequent research has led to the development of a vaccine strategy in which Hsp96 or Hsp70, purified from resected solid tumours, are used in autologous cancer treatment. Thus far, the vaccine has shown encouraging results in melanoma patients. The active component in this vaccine is not the heat shock proteins per se but the peptides associated with them. Hsp70 depleted from its associated peptides proved to be not immunogenic.

The fact that the immunogenic potential of Hsp preparations lies in their associated peptides, allows the development of a novel approach to produce tumour vaccines through the generation of mimic epitopes (mimotopes) of these peptides. Mimotopes of the breast tumour antigen HER-2/neu and the gastric cancer antigen MG7 have been obtained through the screening of phage display libraries. The selection of mimotopes with high affinity and stringent specificity for the antibody idiotype indicates that the synthetic peptides, displayed on the phage, mimic the antibody-binding domain of HER-2/neu and MG7 antigens. Significantly, the selected mimotopes elicited highly specific humoral immune responses against the peptides and/or the original tumour antigen.

The peptide pool associated with Hsp70 from the breast tumour cell line MDA-MB-231 was used as a source of antigenic molecules, to screen a 12-mer phage display library to identify, initially, peptides that bind to the Hsp70 peptide pool (Arnaiz et al., submitted for publication). Phage recognising the Hsp70 moiety were selected against through a negative selection process. Using this approach, a number of potential peptide 'Recognisers' were identified.

The advantages of this approach is that no prior knowledge of the antigenic peptides are required and the fact that the isolated mimotope would not be recognised as 'self' by the immune system. However, several drawbacks became apparent during the experimentation. For example, the phage bio-panning selects recogniser peptides based on selective binding to the displayed peptide. Strength of interaction may not necessarily correspond to antigenic capacity and thus potentially good antigens may be

missed in the selection process. Additionally, a large reportoire of recogniser phages were identified during the process. Therefore, a high throughput screening process needs to be developed to test the immunogenic potential of all the isolated peptides. This proves expensive and would require financial input from commercial organisations. Nevertheless, certain peptides were repeatedly identified in the bio-panning experiments and in particular, the TMG recogniser peptide was identified in three independent experiments. The novelty of the approach taken was the use of these Recogniser peptides in a second reverse bio-panning experiment to identify potential mimics of the original pool of antigenic peptides associated with Hsp70. Using this approach, a single peptide was identified when the TMG peptide was used in the reverse bio-panning experiment. The fact that only a single peptide sequence was identified indicates that the interaction of the TMG peptide with the DSP peptide is highly specific.

The aims of the research presented in this thesis was to develop a method to validate the 'proof of concept' that a two step reverse bio-panning, using Hsp70 associated peptides as a starting material, can be used for novel antigen discovery. Specifically the approach taken was to characterise further the immunogenic properties of the DSP mimic peptide. The development of a sequential T-cell stimulation assay, employing immature dendritic cells as antigen presenting cells, showed that DSP peptide was capable of stimulating T-cells to secrete IFN-γ. Using the sequential T-cell stimulation assay, I was also proved that the DSP peptide resembles antigens present in the breast tumour cell line MDA-MB-231 and more specifically resembles antigens associated with Hsp70 from these cells. However, the mimic peptide DSP also appeared to resemble antigens present in the non-tumour breast cell line MCF-12A. Thus, the results generated in this thesis validated the approach of a two-step reverse bio-panning against Hsp-PCs as a means of identifying immunogenic antigens. In the future, changes in the bio panning such as the use of a subtracting incubation step with normal cell extracts, or the employment of human tissue sections or HSP70-PCs from tumours could improve the chances of generating tumour specific antigens. Another alternative approach would be to analyse the peptides associated with Hsp70 by mass spectrophotometry to determine the most common peptide sequences binding to Hsp70. These identified sequences could then be synthesised and used directly as bait in the bio-panning experiment, thus reducing the complexity of the peptide reportoire used for the selection process.

Surprisingly, the T-cell stimulation assay showed that the TMG peptide also possessed antigenic potential. This led to the hypothesis that the TMG/DSP peptide pair may resemble a natural protein/protein interacting pair in the cell in a manner similar to a receptor/ligand pair. Receptor/ligand pairs, for example the EGF/EGFR pair, have been identified where both partners are oncogenic. Therefore it is not implausible that such protein pairs may be upregulated in cancer cells and could thus contribute to the antigenic pool.

The hypothesis that the TMG/DSP peptide pair resembled a natural protein/protein pair in the cell was further explored using cytochemistry and histochemistry tests on several tumour and normal cell lines and human breast tissue sections. These studies showed that both the TMG and DSP peptides interacted with a number of cell lines and breast tumour tissue and that this interaction was dependent on the confluency state of the cells. In particular maximum interaction was observed in cells lacking cell-cell contact. This interaction was diminished with those cell lines with a slower growth rate. One of the properties of invasive tumour cells is unresponsiveness to cell-cell contact growth inhibition signals, correlating with a high proliferation rate. Although the type of staining seemed to be intracellular and cytoplasmic, confocal microscopy would have showed it more clearly. The employment of tissue sections of different types could have further support the results obtained with the different cell lines. Another improvement that could be made in the use of tissue sections would be the staining with H&E and fluorescence of consecutive sections, thus the type of cells stained with fluorescence could be more clearly localised on the section stained with H&E. The prove of the existence of DSP and TMG interacting partners on MDA-MB-231 cells at the subconfluent stage of 50-65%, set the conditions under which the cells had to be harvested for their use in biochemical approaches to identify the peptides interacting partners. The identification of the TMG peptide-binding partner could lead to the identification of the molecule/s that DPS mimics in MDA-MB-231 cells. The definition of mimics, as explained above, was initially purely immunological, however, the mimic peptide was selected for its binding affinity to TMG peptide, thus, it is possible that the TMG peptide interacting partners in MDA-MB-231 cells could also resemble DSP and share its immunogenic features. In this study, several approaches were taken to identify the natural binding partners of the TMG and DSP peptides in human cells. Two major biochemical approaches were used that involved the use of mass spectrometry analysis of proteins cross-linked to the

peptides and the screening of a breast tumour cDNA library. For the biochemical purification of the peptide binding partners, the protocol employed was one in which the peptides were cross linked to components of permeabilised MDA-MB-231 cells. The proteins were extracted and the peptide-protein complexes retained through the biotin-streptavidin tag system. The proteins were separated by SDS-PAGE. Surprisingly, the most abundant protein present in extracts cross-linked with either the DSP or the TMG peptide had a molecular weight of 70kDa. The 70kDa protein band was excised and analysed by analysis by MALDI-Tof mass spectrophotometry. Homology search in databases of the mass spectra of the peptides obtained following trypsin digestion of the bands failed to give a fully conclusive result due to the low amount of protein present in the bands. The spectrum did however identify Hsp70 as a potential candidate. It is interesting therefore to speculate that the 70kDa band identified as cross-linking to both DSP and TMG is in fact Hsp70, especially as Hsp70 has peptide binding properties. Immunoblotting with anti-Hsp70 antibodies after isolation of cross-linked complexes may provide proof of this hypothesis.

An alternative approach employed was the screening of a breast tumour cDNA T7 library for those clones displaying polypeptides with affinity for DSP or TMG peptides. The results, while inconclusive, provided some tantalising clues to the nature of the peptide binding partners. The bio-panning approach selected a small number of phage displaying cDNAs which interacted with the peptides. However, an interesting finding was that the peptides selected phage in which the cDNAs were expressed out of frame. It is interesting to note that a large number of identifed tumour antigens have been shown to be dervived from the out of frame translation of cellular proteins. Therefore, further examination of the isolated sequences is certainly warranted. The reason these artefactual clones were generated relies on the employment of random primers for the cloning of the cDNA library in the T7Select10-3 vector. Ideally, vectors that can take a full cDNA as inserts could be a more reliable expression library. The use of a breast tumour cDNA T7 library for the initial bio panning experiment using HSP70-PC as ligands could more easily allow the identification of the cellular protein counterparts of the selected polypeptides with affinity for DSP or TMG in the cells. However, more attention should be directed to the selection of the library as its manufacturing process could lead to the display of polypeptides that are not part of the human proteome. A breast tumour cDNA phage display library that displays longer polypeptides could also allow the identification of the human protein counterpart of the

selected tumour antigen, thus eliminating the necessity for biochemical approaches that offer very limited success when working with non-recombinant mammalian systems. In conclusion, the studies presented here portray a novel approach to the identification of mimics of tumour antigens and provide new approaches to characterise such mimics.

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