



## **WestminsterResearch**

<http://www.westminster.ac.uk/westminsterresearch>

### **Characterisation of the *Helix pomatia* agglutinin binding glycoproteins of colorectal cancer cell lines and tissue samples.**

**Julien Saint-Guirons**

School of Life Sciences

This is an electronic version of a PhD thesis awarded by the University of Westminster. © The Author, 2007.

This is a scanned reproduction of the paper copy held by the University of Westminster library.

---

The WestminsterResearch online digital archive at the University of Westminster aims to make the research output of the University available to a wider audience. Copyright and Moral Rights remain with the authors and/or copyright owners.

Users are permitted to download and/or print one copy for non-commercial private study or research. Further distribution and any use of material from within this archive for profit-making enterprises or for commercial gain is strictly forbidden.

---

Whilst further distribution of specific materials from within this archive is forbidden, you may freely distribute the URL of WestminsterResearch: (<http://westminsterresearch.wmin.ac.uk/>).

In case of abuse or copyright appearing without permission e-mail [repository@westminster.ac.uk](mailto:repository@westminster.ac.uk)

**Characterisation of the *Helix pomatia* agglutinin binding glycoproteins of colorectal cancer cell lines and tissue samples.**

**Julien Saint-Guirons**

A thesis submitted in partial fulfilment of the requirements of the University of  
Westminster for the degree of Doctor of Philosophy

September 2007



## **DEDICATION**

À papa

## ACKNOWLEDGEMENTS

First I wish to express my sincere thanks to my supervisor, Dr Miriam Dwek for her guidance throughout the four years spent under her supervision and for tirelessly correcting my spelling mistakes during the writing. I am also most grateful for her support during bad times.

Thanks to my colleagues Anupama, Loveleen, Clare, Kushen and Anatoliy for their help and friendship. As well, thanks to Dr Marilena Loizidou for helping me with the cell culture and thanks to the technicians of the University of Westminster for their assistance. Thanks to Dr Mark Odell for useful discussions and Elton Zeqiraj for his help with the confocal microscopy work. I also would like to acknowledge the financial support that I have received from the School of Biosciences of the University of Westminster.

I would like to thank my friends from Toulouse for their endless friendship throughout my time in London and thanks to Rym for her support.

Finally I would like to express a special thanks to my mum, my dad and my little sister for their love and their faith in me.

## ABSTRACT

Colorectal cancer (CRC) is one of the most common malignancies in the US and Western Europe and metastatic dissemination after CRC is a leading cause of mortality. New markers predictive of cancer cell behaviour are actively sought both as targets and as a means of predicting patient prognosis. The lectin from the Roman snail *Helix pomatia* (HPA) has attracted interest as tool for the detection of metastatic colorectal cancer but the HPA binding partners have remained poorly characterised.

We established an *in vitro* model using human colorectal cancer cell lines ranging from HPA negative, non metastatic, to HPA positive and metastatic. Confocal microscopy was used to assess the HPA binding pattern in the cell lines and the monosaccharides N-acetylgalactosamine, N-acetylglucosamine, and sialic acid were used to inhibit the interaction between the lectin and the cancer cells.

A proteomic approach based on cell membrane isolation, pre-fractionation using lectin affinity chromatography, followed by 2-dimensional electrophoresis and MALDI-TOF-MS enabled the identification of the HPA binding proteins in the metastatic cancer cell line HT29. The proteins that eluted in the HPA binding fraction were present either by virtue of their ability to bind directly to HPA or as protein complexes of HPA binding partners and included molecules involved in cell adhesion / migration (integrin  $\alpha 6$ , integrin  $\alpha V$ , annexins) re-modeling (filament proteins including  $\alpha$  tubulin,  $\beta$ tubulin, cytokeratins, actin) and anti-apoptotic pathways (Hsp-70, Hsp-90, Hsp-96 and TNFR-1). Although many of these proteins have previously been described as altered in cancer, we are not aware of a single reagent like HPA which will concurrently bind all of these molecules.



# TABLE OF CONTENTS

	Page Numbers:
Dedication	i
Acknowledgement	ii
Abstract	iii
Table of content	iv
List of figures	viii
List of tables	xi
Abbreviations	xii
<b>CHAPTER 1: Introduction</b>	<b>1</b>
1.1 Cancer	2
1.1.1 Incidence	2
1.1.2 Hallmarks of cancer	2
1.1.3 Angiogenesis and metastasis	4
1.1.3.1 Angiogenesis	4
1.1.3.2 Metastasis	5
1.2 Colorectal cancer	7
1.2.1 Colon anatomy and histopathology of CRC development	
1.2.2 CRC staging	8
1.2.3 Incidence and mortality	10
1.2.4 Risk factors	11
1.2.5 CRC screening and treatments	13
1.2.5.1 Screening	13
1.2.5.2 Treatments	14
1.2.5.3 CRC biomarkers	15
1.2.6 Genetic factors of CRC development	15
1.3 Glycosylation and cancer	18
1.3.1 Function of oligosaccharides on glycoproteins	18
1.3.2 N-linked oligosaccharides	21
1.3.3 O-linked oligosaccharides	22
1.3.4 Important extensions of N-and O-linked glycans-blood group sugars and Lewis antigens	26
1.3.5 Altered glycosylation in cancer	26
1.3.5.1 Altered O-glycans	26
1.3.5.2 Alteration of Lewis sugars and blood group antigens	29
1.3.5.3 Altered N-glycans	30
1.3.5.4 Sialylation	32
1.4 Glycoproteins and cancer	32
1.4.1 Cell adhesion molecules (CAMs)	33
1.4.1.1 Cadherins	33
1.4.1.2 Selectins	33

1.4.1.3	Immunoglobulin-like CAMs	34
1.4.1.4	Hyaluronate receptor: CD44	35
1.4.1.5	Integrins	36
1.5	Lectins and cancer	38
1.5.1	History of lectins	38
1.5.2	HPA	40
1.6	The work described in this thesis	44
 <b>CHAPTER 2: Materials and methods</b>		 45
2.1	Cell lines	46
2.2	Confocal microscopy	47
2.3	Specificity of HPA binding assessed by confocal microscopy	48
2.4	Confocal microscopy image capture	49
2.5	Image 3D reconstructions and 2D models	49
2.6	Data analysis of images from confocal microscope	50
2.7	HPA binding assay using microtitre plate format	50
2.8	Assessing the specificity of HPA binding using the microtitre plate assay	51
2.9	Cell lysate preparation	52
2.10	Preparation of membrane-enriched fraction	52
2.11	Protein solubilisation	54
2.12	Protein assay	54
2.13	1D Electrophoresis (1-DE)	55
2.14	SDS-PAGE	55
2.15	Data analysis	56
2.16	2D Electrophoresis (2-DE): sample preparation	56
2.17	In-gel rehydration	56
2.18	Isoelectric focussing	57
2.19	Equilibration of IPG strips	58
2.20	Second dimension: SDS-PAGE	58
2.21	Protein staining using Coomassie Brilliant Blue	58
2.22	Fluorescent staining of proteins separated by 2-DE	59
2.23	Data analysis from 2-DE experiments	60
2.24	Protein transfer by Western blotting	60
2.25	Ponceau S staining of Western blots	60
2.26	Immuno/lectin detection on Western blots	60
2.27	Lectin blotting with HPA	61
2.28	Probing Western blots with anti-blood group A antibody	61
2.29	Probing Western blots with Anti-integrin $\alpha 6$ (Anti CD49f) antibody	62
2.30	Inhibition of HPA binding to HT29 proteins	62
2.31	HPA affinity chromatography	62
2.32	Improved method for the purification of the HPA binding partners from HT29	63
2.33	Spot picking from 2-DE separated HPA binding proteins	64
2.34	Protein identification by MALDI-TOF mass spectrometry	64
2.35	Selection of colorectal tissue samples for evaluation of HPA binding glycoproteins	65



2.36	Protein preparation	66
2.37	Protein separation	67
2.38	Analysis of HPA binding proteins separated by 1-DE	67
2.39	Analysis of HPA binding proteins separated by 2-DE	67
2.40	Probing Western blots of CRC samples with antibody to integrin $\alpha 6$	67
<b>CHAPTER 3: Lectin binding to colorectal cancer cells. A focus on <i>Helix pomatia</i> agglutinin (HPA)</b>		<b>68</b>
3.1	Introduction	69
3.2	Lectin binding to HT29 and SW480	72
3.3	Localisation of HPA binding in HT29 and SW480	74
3.4	Quantification and localisation of the binding	76
3.5	2D and 3D reconstruction	76
3.6	Evaluation of the specificity of HPA binding at the cellular level	77
3.7	The development of a microtitre plate assay to evaluate HPA binding to SW480, SW620 and HT29 cells	79
3.8	HPA binding using the microtitre plate assay system	79
3.9	Inhibition of the binding of HPA in the microtitre plate assay	80
3.10	Discussion	83
<b>CHAPTER 4: Method development for the separation of membrane-enriched proteins via a proteomic approach</b>		<b>84</b>
4.1	Introduction	85
4.2	Compatibility of solubilisation buffers with proteins assay reagents	86
4.3	Membrane preparation	89
4.4	Solubilisation of membrane-enriched proteins for separation by 2-DE	90
4.4.1	T buffer	93
4.4.2	U buffer	94
4.4.3	U7M/T buffer	95
4.4.4	U7M/T2 buffer	96
4.5	Reproducibility of the 2-DE system	97
4.5.1	Analytical reproducibility using two replicates of HT29 membrane-enriched proteins	97
4.5.2	Comparison of HT29/SW480 membrane-enriched proteins using a 2-DE system	100
4.6	Discussion	102
<b>CHAPTER 5: Identification of HPA binding glycoproteins in HT29 and SW480 cells</b>		<b>105</b>
5.1	Introduction	106
5.2	Overview of the HPA binding proteins of HT29, SW620 and SW480 by SDS-PAGE and lectin blotting	107
5.3	Binding of a blood group A antibody to Western blots of HT29, SW620 and SW480 proteins	109

5.4	Inhibition study of HPA binding to membrane-enriched proteins from HT29 at the protein level	111
5.5	HPA binding: 2-DE and lectin blotting analysis	112
5.6	Inhibition of the HPA binding to GP130 in HT29	114
5.7	Analysis of GP130	115
5.8	Purification of the HPA binding proteins of HT29	117
5.9	Improvement to the HPA affinity chromatography purification of HT29 proteins	119
5.10	Anti integrin $\alpha 6$ antibody binding to HT29, SW620 and SW480 proteins	121
5.11	Protein expression analysis of HT29 and SW480	123
5.12	Discussion	126
<b>CHAPTER 6: Preliminary work on colorectal tissue samples</b>		<b>130</b>
6.1	Introduction	131
6.2	Separation of tissue sample proteins by 1-DE	132
6.3	HPA binding glycoproteins	133
6.4	Reactivity of CRC tissue samples with antibody directed against the blood group A antigen	135
6.5	HPA binding glycoproteins: 2-DE and lectin blotting analysis	137
6.5.1	Separation of tissue sample proteins by 2-DE	137
6.5.2	Analysis of the HPA binding proteins separated by 2-DE	139
6.5.3	Integrin $\alpha 6$	143
6.6	Discussion	144
<b>CHAPTER 7: General discussion</b>		<b>147</b>
7.1	HPA as a tool for cancer prognostication	148
7.2	The binding of HPA to CRC cells	148
7.3	HPA-binding glycoproteins in CRC cell lines	151
7.4	HPA-binding glycoproteins in CRC tissue samples	153
7.5	Conclusions and future directions	154
<b>APPENDICES</b>		<b>155</b>
<b>REFERENCES</b>		<b>173</b>
<b>PUBLICATION</b>		<b>218</b>



## LIST OF FIGURES

Figure 1.1 Proportion of the four main cancers in the UK	2
Figure 1.2 The six hallmarks of cancer development	4
Figure 1.3 The steps involved in the distant spread of cancer cells	6
Figure 1.4 Representation of the cancer progression through the colon wall	9
Figure 1.5 Adenoma-carcinoma sequence	17
Figure 1.6 Monosaccharides associated with human glycoproteins and glycolipids	20
Figure 1.7 Monosaccharide binding via alpha and beta bonds	20
Figure 1.8 The glycosylated oligosaccharide lipid intermediate	22
Figure 1.9 Biosynthesis pathways of the most represented cores 1-4 and their extensions	24
Figure 1.10 Integrin family members	36
Figure 1.11 Integrin Structure	38
Figure 1.12 HPA structure	41
Figure 2.1 HPA binding to HT29, SW620 and SW480: experiment layout	51
Figure 2.2 Experimental layout for inhibition experiments of HPA binding to cell lines	52
Figure 2.3 Preparation of a membrane-enriched fraction	53
Figure 3.1 Lectins binding to HT29 and SW480	73
Figure 3.2 Confocal microscopy view of the binding of HPA-TRITC to HT29 and SW480	75
Figure 3.3 Linear profiling of HPA binding to HT29	76
Figure 3.4 2D and 3D representation of HPA binding to HT29 cells	77
Figure 3.5 Specificity of the binding of HPA to HT29 cells	78
Figure 3.6 Assessment of the HPA binding using a microtitre plate assay	80
Figure 3.7 Assessment of the inhibition of the HPA binding to HT29, SW620 and SW480 using a microtitre plate assay	82



Figure 4.1 Bio-Rad DC protein assay	87
Figure 4.2 Bio-Rad RC/DC protein assay	88
Figure 4.3 Bio-Rad Bradford protein assay	88
Figure 4.4 Evaluation by Western blotting of the HPA binding glycoproteins content of the three fractions from the membrane preparation	90
Figure 4.5 2-DE profiles of the HT29 membrane proteins solubilised in four different buffers	92
Figure 4.6 Evaluation of T buffer	93
Figure 4.7 Evaluation of U buffer	94
Figure 4.8 Evaluation of U7M/T buffer	95
Figure 4.9 Evaluation of U7M/T2 buffer	96
Figure 4.10 HT29 membrane proteins separated using 2-DE with pH 3-10 IPG strip	97
Figure 4.11 Zoom view of area 1, 2, 3 in two replicates of HT29 membrane proteins	99
Figure 4.12 Comparison of HT29 and SW480 membrane proteins separated by 2-DE	100
Figure 4.13 Zoom view of area 1 from Figure 4.12	101
Figure 4.14 Zoom view of area 2 from Figure 4.12	102
Figure 5.1 HPA lectin blotting after separation by 1-DE	108
Figure 5.2 HT29, SW620 and SW480 membrane-enriched proteins detected by HPA and blood group A antibodies	110
Figure 5.3 Inhibition of HPA binding to HT29 proteins	112
Figure 5.4 Comparison of HPA binding proteins of HT29 and SW480	113
Figure 5.5 Inhibition of HPA binding to GP130 in HT29	114
Figure 5.6 Separation of GP130 using a zoom 2D gel strategy	116
Figure 5.7 Affinity purification of HPA binding proteins from HT29, SW620 and SW480	118
Figure 5.8 Improved strategy to purify the HPA binding proteins of HT29	119
Figure 5.9 Proteomic analysis of the affinity purified HPA binding proteins of HT29	120

Figure 5.10 Integrin $\alpha$ 6 antibody and HPA binding to Western blots of HT29, SW620 and SW480 proteins	122
Figure 5.11 Comparison of the proteins from HT29 and SW480 using 2-DE	124
Figure 5.12 Overlay of 2-DE separation from HT29 and SW480	125
Figure 6.1 Protein composition of C1, N1, C2 and N2 analysed by SDS-PAGE	133
Figure 6.2 Lectin blotting to pools of C1, C2, N1 and N2	134
Figure 6.3 Reactivity of tissue samples with blood group A antibody	136
Figure 6.4 Separation of proteins from samples C1, N1, C2 and N2	137
Figure 6.5 3D views of proteins showing altered expression using 2-DE	139
Figure 6.6 Detection of the HPA-binding proteins of C1, N1, C2 and N2	140
Figure 6.7 3D views of HPA-binding proteins of C1 and C2	142
Figure 6.8 Western blotting using an antibody against integrin $\alpha$ 6	143

## LIST OF TABLES

Table 1.1 Frequency and five-year survival rate of Duke's stage A, B, C and D CRC	10
Table 1.2 Relation between the three types of classification used to characterise colorectal tumours	11
Table 1.3 Types of O-linked oligosaccharides found on glycoproteins and mucins	25
Table 1.4 O-glycans associated with cancer	29
Table 2.1 The characteristics of the three cell lines HT29, SW620 and SW480	47
Table 2.2 The lectins used in this study and their putative binding sugars	48
Table 2.3 Spectra parameters of the fluorophores used in this study	49
Table 2.4 Solubilisation buffers	54
Table 2.5 Buffers used for proteins assay	55
Table 2.6 Isoelectrofocussing	57
Table 2.7 Coomassie blue staining	59
Table 2.8 Fluorescent staining of proteins using Sypro Ruby	59
Table 2.9 Patient samples characteristics	66
Table 4.1 Buffer composition for the preparation of the BSA solution	87
Table 5.1 Proteins recognised by HPA in HT29, SW620 and SW480	108
Table 5.2 Identification of the HPA binding protein of HT29	121
Table 6.1 Description of the tissue samples C1, N1, C2 and N2	132



## ABBREVIATIONS

1-DE	one dimension electrophoresis
2-DE	bidimensional electrophoresis
5-FU	5-fluorouracil
BSA	bovine serum albumin
CAM	cell adhesion molecule
CEA	carcinoembryonic antigen
CHAPS	3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate
CID	collision induced dissociation
ConA	concanavalin a
CRC	colorectal cancer
DAB	diamino benzidine
DBA	<i>Dolichos biflorus</i> agglutinin
DMEM	dulbecco's modified eagle's medium
DNA	deoxyribonucleic acid
DTT	Dithiothreitol
ECA	<i>Erythrina cristagalli</i> agglutinin
ECM	extra cellular matrix
EcoRA	<i>Erythrina corallodendron</i> agglutinin
EDTA	ethylenediamine tetraacetic acid
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
FAP	familial adenomatous polyposis
FITC	fluorescein isothiocyanate
FOBT	faecal occult blood test
GAG	Glycosaminoglycans
Gal	Galactose
GalNAc	n-acetylgalactosamine
Glc	Glucose
GlcNAc	n-acetylglucosamine
GnT	n-acetylglucosaminyltransferase
GPI	glycosylphosphoinositol anchor
GTases	Glycosyltransferases
GTases	Glycosyltransferases
H+E	haematoxylin and eosin
HA	hyaluronic acid
HAA	<i>Helix aspersa</i> agglutinin
HNPCC	hereditary non-polyposis colorectal cancer
HPA	<i>Helix pomatia</i> agglutinin
HPLC	high performance liquid chromatography
IGF	insuline-like growth factor
IL	Interleukin
IPG	immobilised ph gradient
kDa	kilo dalton
LOH	loss of heterozygosity
L-PHA	l-phytohaemagglutinin

MALDI/TOF MS	matrix-assisted laser-desorption ionisation / time of flight mass spectrometry
Man	Mannose
MIDAS	metal-ion dependent adhesions site
MW	molecular weight
N/A	not available
NS	not significant
PBS	phosphate-buffered saline
PDGF	platelet-derived growth factor
pI	isoelectric point
Pro	Proline
RCA-1	<i>Ricinus communis</i> agglutinin 1
SA	sialic acid
SBA	soybean agglutinin
SCID	severe combined immunodeficiency
SD	standard deviation (of the mean average)
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SEM	standard error of the mean
Ser	Serine
SNA	<i>Sambucus nigra</i> agglutinin
TBS	tris-buffered saline
TFA	trifluoroacetic acid
TGF $\alpha/\beta$	transforming growth factor $\alpha/\beta$
Thr	Threonine
TME	total mesorectal excision
TRITC	tetramethylrhodamine isothiocyanate
TWEEN	polyoxyethylene (20) sorbitan monolaurate
UEA-1	<i>Ulex europeaus</i> agglutinin 1
VNTR	variable number tandem repeats
WGA	wheat germ agglutinin
N/D	non detectable

# **CHAPTER ONE:**

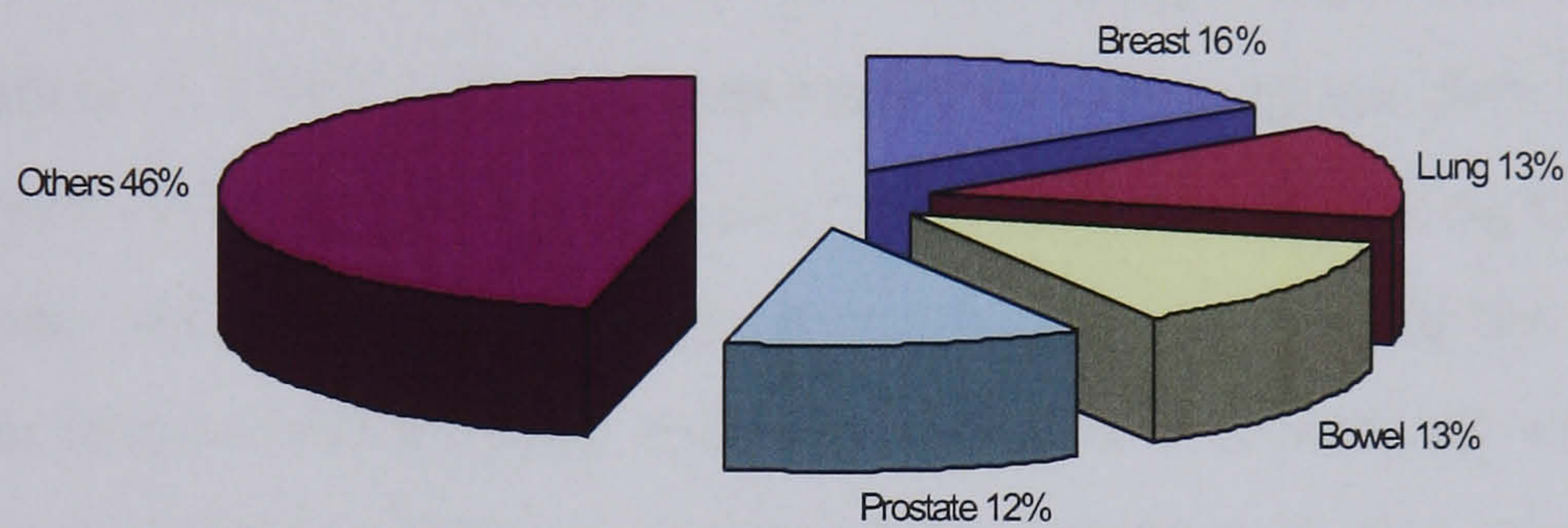
## **Introduction**



# 1.1 Cancer

## 1.1.1 Incidence

Cancer is a leading cause of death in the developing world with 1 in 3 people affected in their lifetime. The worldwide incidence is dramatic with about 10 million new cases every year. In the UK 276,678 persons were diagnosed with cancer in 2003 with four types of cancer accounting for more than half of the cases. The pie chart Figure 1.1 shows the proportion of breast, lung, bowel and prostate cancers recorded in the UK population.



**Figure 1.1:** Proportion of the four major cancers, breast, lung, prostate and bowel in the UK, Office for National Statistics 2005.

## 1.1.2 Hallmarks of cancer

The critical event for many patients with solid tumours is the spread of tumour cells from their primary site, through the circulatory system (blood and lymph) to form secondary tumours in a distant organ, this process is known as metastasis. Although treatment and diagnosis of cancers has improved in the past two decades with corresponding increases in survival rates of patients, the prognosis remains poor for patients with distant metastases.

Cancers develop from a single cell, which undergoes genetic changes in three types of genes: oncogenes, tumour-suppressor, and stability genes, this leads to a characteristic dysregulation in cell growth. The transformation of the normal cells commonly occurs via a process which favours clones of cancer cells in response to natural selection pressure, thereby paralleling the process of evolution described by Darwin. The cells with genetic alterations conferring a substantial growth advantage are selected eventually leading to a cancerous phenotype. Over 100 histological types of cancer have been classified and those often differ by their tissue of origin. Carcinomas



occurring in epithelial cells, such as the colon, represent 85% of all cancers. Cancers of different tissue may have distinct features and different molecular mechanisms may be responsible for their development, nevertheless, the general progression in development of the cancer is similar and is composed of six essential steps described by Hanahan and Weinberg (2000). Transformed cells first acquire the ability to grow in the absence of mitogenic growth signal yet required for the proliferation of normal cells. Several strategies are used by the cancer cells to achieve that goal. They may synthesise their own growth factor such as PDGF/TGF $\alpha$  (Fedi *et al.*, 1997), overexpress growth factor receptors such as EGF-R/erbB (Slamon *et al.*, 1987) or synthesise constitutively activated receptors. Next the cancer cells escape regulation of the cell cycle via disruption of TGF $\beta$  pathways responsible for arresting the cells in the G1 phase of the cell cycle (Weinberg, 1995; Moses *et al.*, 1990). The following step in the development of cancer is the evasion of the programmed cell death. Here the importance of the P53 protein that normally blocks apoptotic effectors is recognised with an observation that greater than 50% of human cancers show mutations in this gene (Harris, 1996). Also survival factors such as IGF-1/2 and IL3 are activated during tumour progression (Evan and Littlewood, 1998) Although cancer cells exhibit deregulated growth, studies have shown that mammalian cells carry independent mechanisms of senescence that limit the potential of the cells to divide by progressive shortening of the telomeres through cell replication cycle eventually causing cell death. The neoplastic cells maintain the length of their chromosomes by expressing telomerase enzymes (Shay and Bacchetti, 1997) or by interchromosomal sequence exchange (Bryan *et al.*, 1995) therefore acquiring unlimited replication potential. The fast-growing abnormal cells require an increasing need for oxygen and nutrients, these are supplied by vascularisation induced by the neoplasia (Folkman, 1997; Bouck *et al.*, 1996; Hanahan and Folkman, 1996). Genetic alterations of angiogenic activators and inhibitors occur during neoplasia. For example, the angiogenic activator VEGF and FGF are often increased and the angiogenic inhibitors such as thrombospondin-1 or  $\beta$ -interferon are decreased in developing tumours (Singh *et al.*, 1995; Volpert *et al.*, 1997). The final step in the development of cancer development is an invasion of surrounding tissues and settlement of cells that form metastatic deposits, metastases are responsible for approximately 90% of cancer-related death (Sporn, 1996).



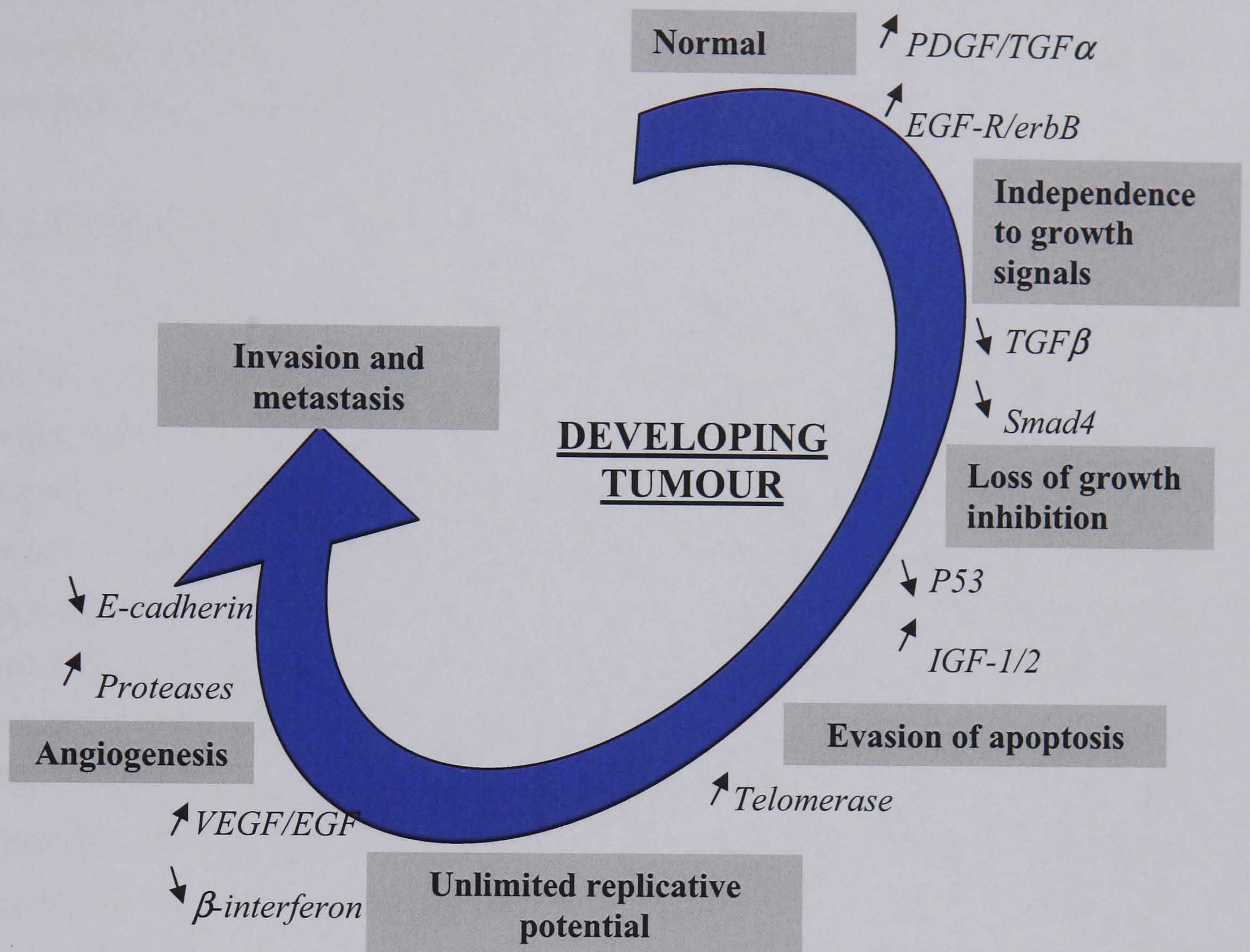


Figure 1.2: The six hallmarks of cancer development.

## 1.1.3 Angiogenesis and metastasis

### 1.1.3.1 Angiogenesis

As discussed in the previous paragraph, the mutation of oncogenes and tumour suppressor genes leads to uncontrolled proliferation of cancer cells, a corresponding increase in size correlated with an increased demand for oxygen and nutrients (Folkman, 1986). It has been shown that an adequate supply of oxygen, nutrients and the removal of toxic molecules is necessary for the survival and growth of tumour cells. Moreover oxygen diffusion from capillaries extends to only 150  $\mu\text{m}$ -200  $\mu\text{m}$ , therefore beyond this distance oxygen has to be supplied by new blood vessels. Expansion of tumour

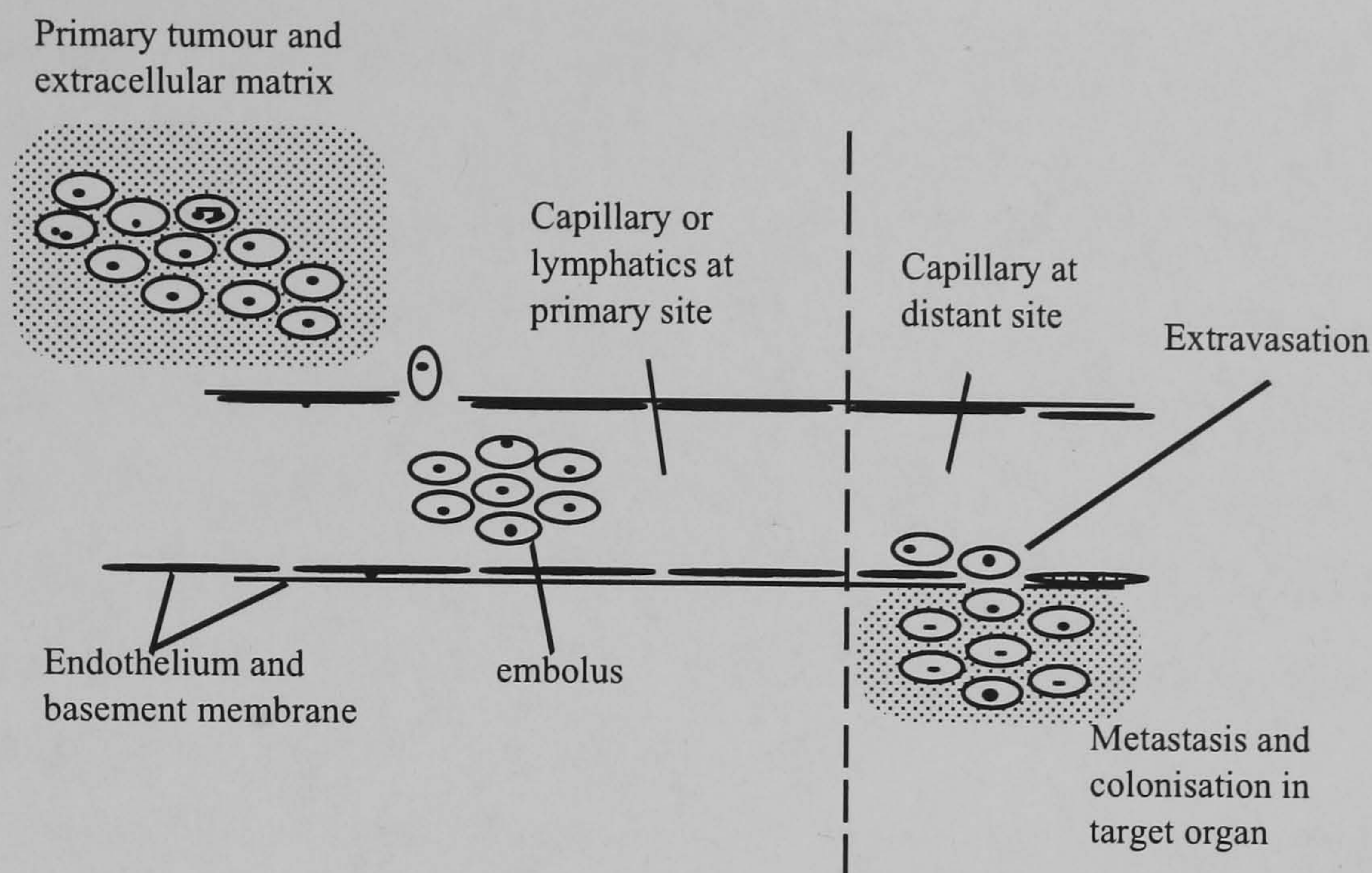


larger than 1 mm of diameter depends on the formation of new blood vessels via the process of angiogenesis (Fidler, 1994; Folkman and Klagsbrun, 1987). The establishment of a neocapillary network from the host tissue not only provides the appropriate supply to allow the tumour to grow, it constitutes an entry into the circulation for metastasing cells.

### **1.1.3.2 Metastasis**

The metastatic spread of tumour cells is a selective process which consists of a series of interrelated steps that leads to the establishment of micrometastasis in a host organ, these steps were reviewed by Fidler (1991) and summarised in Figure 1.3. Tumour cells invade the surrounding tissue and penetrate into newly formed blood vessel or lymphatic channels in a step named intravasation. The tumour cells circulate as a single cell or as clumps in order to escape the immune system and survive before arrest of circulating cells and extravasation (Fidler and Bucana, 1977). Metastatic cells eventually proliferate with the target organ parenchyma and form a secondary tumour. The metastatic spread of tumour cell follows the theory of “seed” and “soil” first established by Paget (1889). Based on post mortem observations, Paget suggested that certain tumours cells with metastatic ability, “seed”, would develop on the right “soil”, organ that provides a growth advantage to the seeds, these ancestral observations were recently supported by studies showing site-specific metastasis of solid tumours (Fidler, 1995).





**Figure 1.3:** Schematic diagram illustrating the steps involved in the distant spread of cancer cells

Tumour cells are composed of a heterogeneous population of cancer cells, exhibiting a wide range of genetic, biochemical, immunological and biological characteristics (Poste and Fidler, 1979; Fidler and Hat, 1982; Nicolson, 1987; Fidler and Balch, 1987). During metastasis, specific subpopulations of cells present in the parent tumour appear to be selected for their ability to invade and grow to a distant secondary tumour by interacting with host factors (Fidler, 1995). Adhesion molecules play a central role in this process as they are involved at each step by attaching to neighbouring cells or matrix molecules and the ability of tumour cells to form transient attachments is necessary. Tumour cells involved in metastasis show alteration in their cell adhesion receptors and ligands (Nicolson, 1988; Yeatman and Nicolson, 1993).

The first step in the metastatic process is the invasion of the surrounding tissues by cancer cells. The invasion through the ECM is achieved by the combined action of degradative enzymes and by the mechanical pressure exerted by the expanding mass of cells. It is a common feature of tumours to find higher level of lytic enzymes such as cathepsin B2, plasminogen activator and matrix metalloproteases in malignant cells (Jones and Declerck, 1980; Ray and Stetler-Stevenson, 1994). The migration of cells through the matrix is a dynamic process, the leading edge must express activated proteinases in order to give rise to a proteolytic zone. Transient attachment mediated by integrins such as  $\alpha 5\beta 1$ ,  $\alpha 6\beta 1$ ,  $\alpha 4\beta 1$ ,  $\alpha 2\beta 1$  at the attachment site, along with other cell



adhesion molecules (CAM), occurs at the leading edge and rear of the cell therefore enabling the metastatic cell to crawl through the ECM (Lauffenburger and Horwitz, 1996) eventually heading towards original or angiogenic blood vessels or lymph channels. By this process cancer cells enter the circulation as a single cell or more often as clumps of cells in order to improve their survival within the blood stream. The cancerous cell may also form complexes with blood-borne cells (Gasic, 1984), this step appears to be essential to allow cells to survive both mechanical pressure and immune system surveillance until they attach to the endothelial cells at the site of metastasis, extravasate and colonise the organ of metastasis.

The arrest of circulating cancer cells occurs by attachment of cancer cells to endothelium, in particular at the endothelial cell junctions (Kramer *et al.*, 1982) or sub-endothelial matrix (Nicholson, 1982a). The attachment of cancer cells involves the binding of sialyl Lewis x and sialyl Lewis a sugars expressed by the cancer cells, to P-selectin and E-selectin expressed by the activated endothelial cells (Miller *et al.*, 1996; Takada *et al.*, 1993; Dejana *et al.*, 1992, Lauri *et al.*, 1991). After attachment to endothelial cells, cancer cells break through the basement membrane and invade the tissue in a process named extravasation in a similar manner to the intravasation process described earlier. The tumour grows and in doing so may induce the formation of new blood vessel (neoangiogenesis) allowing the metastatic cancer to develop further.

## **1.2. Colorectal cancer**

### **1.2.1 Colon Anatomy and histopathology of CRC development**

The colon starts on the right hand side of the body at the Caecum and ends at the rectum on the left hand side of the body. It consists of the ascending colon, transverse colon, descending colon and sigmoid colon.

The colon wall is composed of several layers from the inside to the lumen, the serosa, muscle, submucosa and mucosa. The colonic epithelium is characterised by a monolayer (mucosa) of specialised cells forming invaginations known as crypts. The epithelial layer is renewed every 3-8 days in a dynamic process (Altman and Enesco, 1967; Wright and Irwin, 1982; Schmidt *et al.*, 1965) and is composed of four cell types;



columnar absorptive cells, mucus secreting cells, neuro epithelial cells and the paneth cells (Le Blond and Cheng, 1976). They all originate from intestinal stem cells located at the base of the crypt which proliferate and become fully differentiated as they move toward the upper crypt region (Gordon *et al.*, 1992).

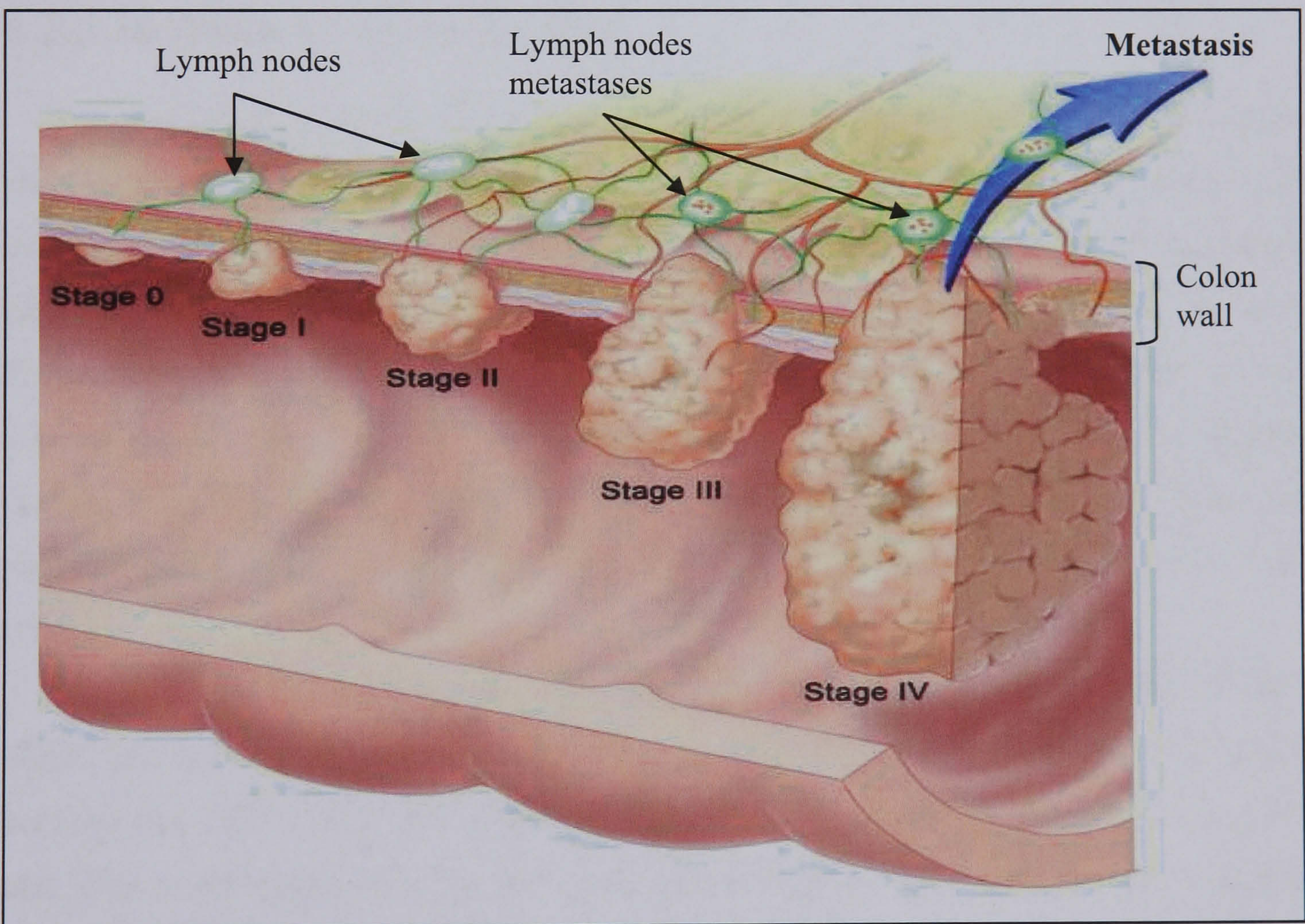
Colorectal cancer begins to develop as hyperplastic or dysplastic growth named aberrant crypt foci (ACF). The accumulation of epithelial cells is the result of a deregulation of the crucial birth/loss balance. It may result in formation of a protrusion in the epithelial cells, called benign polyps. There are mainly two types of polyps which predispose to variable outcome with respect to CRC development. Dysplastic or adenomatous polyps, similar to those found in people with the genetic disorder familial adenomatous polyposis, are characterised by abnormal cells organised in several layers on the lamina propria. These lesions are also called carcinoma *in situ* and are confined to the epithelial layer but may eventually progress through the muscularis mucosae and invade the surrounding tissue forming a malignant carcinoma.

### **1.2.2 CRC staging**

CRC staging characterises the extent of the primary tumour lesion in order to facilitate prognostication and is a key element in helping to determine appropriate patient management strategies. The Dukes classification system was the first to establish categories of tumour stages and these were termed Dukes A, B and C (Dukes, 1932). This system was subsequently modified by Astler-Coller (1954) to include a fourth stage (Stage D). These staging systems were based entirely on the extent to which the primary tumour had spread locally as well as regionally to the lymph nodes and was based on histo-pathological examination of tissues from resected specimens taken from the bowel. Although Dukes staging was originally described for rectal cancer, it has also subsequently been shown to be of value in staging colon cancer. Dukes stages A, B and C correlate with the disease-free (and overall) patient survival. Subsequently, the American Joint Committee on Cancer introduced the TNM (Tumour, Node, Metastasis) staging system, which sought to classify all cancer patients into one of four stages (Stage I-IV) (AJCC, 2002). The TNM system combined a variety of staging systems and is now the method that is most often used to describe the extent of a primary cancerous lesion and metastases at the time of diagnosis. The TNM system



describes the size of the primary tumour (T), metastases to regional lymph nodes (N) and distant metastases (M) and is shown in detail in Appendix 1. Figure 1.4 depicts the steps in CRC progression. At TNM stage 0, also known as tumour *in situ* (Tis), the tumour is confined to the mucosa and represents the earliest stage of CRC cancer. At TNM stage I the cancer has infiltrated the submucosa and may also have invaded the muscle layer (T1-2). Cancer at TNM stage II has migrated through the colon wall and invaded nearby tissues (T3-4). At stage 0, I and II there is neither lymph node involvement (N0) nor metastases in distant organs, also known as secondary spread of the tumour (M0). At stage III, the cancer cells have spread to nearby lymph nodes (N1-N2) but distant metastases are not observed (M0) whilst at stage IV the cancer cells have spread to secondary organs (M1) and represent the most advanced stage of CRC. Patients presenting at stage IV have the poorest 5 year survival rate.



**Figure 1.4:** Schematic representation of the cancer progression through the colon wall and invasion of the surrounding tissues associated with each stage (0-4) of the disease. (adapted from NCI, <http://www.cancer.gov/cancertopics/pdq/treatment/colon/Patient>)



The different TNM stages from I to IV and the correlation with the Dukes and Astler-Coller staging system are described in Table 1.1

<b>Stages</b>	<b>TNM status</b>	<b>Dukes stage</b>	<b>Astler-Coller</b>
0	Tis, N0, M0		
I	T1-2, N0, M0	A	A, B1
IIA	T3, N0, M0	B	B2
IIB	T4, N0, M0	B	B3
IIIA	T1-2, N1, M0	C	C1
IIIB	T3-4, N1, M0	C	C2, C3
IIIC	T1-4, N2, M0	C	C1, C2, C3
IV	T1-4, N0-2, M1		D

**Table 1.1:** Relation between the three types of classification used to characterise colorectal tumours.

### 1.2.3 Incidence and mortality

Colorectal cancer is the second leading cause of cancer-related death in Europe and was the cause of more than 16000 deaths in the UK in 2005. In the UK around 100 new cases are diagnosed each day and CRC is ranked as the third most common cancer after breast and lung. In 2003, 35000 new cases of CRC were registered in the UK, with the men (around 19000) being more affected than women (around 16000). Colon cancers are distributed with 2/3 in the colon and 1/3 in the rectum and reports suggest that the left side of the bowel is more often affected than the right side. In particular the sigmoid colon, rectosigmoid junction and the rectum represent more than 50% of all cases.

There have been important improvements in the diagnosis and treatment of CRC which has resulted in an increase of the five-year survival rate in men and women, between the 1970's and 1990's the average five-year survival has doubled from 22% and 23% to 47% and 48% for men and women respectively. The average five-year survival rate has increased further and is currently 51% and 52% for men and women. These rates are average values but the five-year survival rate is very dependent of the Dukes stage at the time at which the cancer was diagnosed and therefore important

variations in survival are observed. The Table 1.2 shows the average five-year survival rate of people diagnosed with CRC.

<b>Dukes stage of the CRC</b>	<b>Frequency at diagnosis</b>	<b>Five year survival rate</b>
A	<10%	93%
B	30%	82%
C	30%	55-60%
D	20%	5-8%

**Table 1.2 :** Frequency and five-year survival rate of Duke's stage A, B, C and D CRC. Adapted from Nicholson *et al.* 2005.

### **1.2.4 Risk factors**

The impact of the environment and lifestyle on the development of CRC was first proposed by Burkitt in the 1960's who suspected a relation between diet and bowel disease. Burkitt's hypothesis was that the colonic transit is slower in low fiber-diet and that this would facilitate the action of carcinogens on colon cells. In the mid 1970's the first large multicentre studies showed that lower fat intake reduced the incidence of human cancers (Walker and Burkitt, 1976). Studies based on population migrating from low risk to high risk areas showed a dramatic increase in incidence (Parkin *et al.*, 1992) and it has since been estimated that environmental and lifestyle factors contribute to approximately 70-80% of all CRC (Doll and Peto, 1981; Willett, 1995).

The diet is one of the most important lifestyle factors linked to the incidence of CRC. For instance dietary fat and meat intake have been linked to CRC and studies have postulated that a correlation between high fat intake and CRC was via bile salts. Bile salts produced by the liver to facilitate the absorption of fat in the small intestine are eventually reabsorbed and redirected to the liver but 1-2% of residual bile salt are metabolised by the colonic microflora into mutagenic secondary bile salts (Van Munster and Nagengast, 1993). Although early studies linked dietary fat intake with CRC incidence (Walter and Burkitt, 1976; Pwynder, 1975), more recent studies failed to find an association between fat intake with risk of CRC (Giovannucci *et al.*, 1992; Howe *et*



*al.*, 1997; Stemmermann *et al.*, 1984). Red meat intake has been linked to CRC risk, several studies have shown an increased risk of CRC with high intake of red meat (Giovannucci *et al.*, 1992, Willett *et al.*, 1990) and an even higher risk with processed meats (Chao *et al.*, 2005; LeMarchand, 2002). More over there is evidence that there is a reduced incidence of CRC in populations that consume a high-fibre diet. A large European study, European Prospective Investigation into Cancer and Nutrition (EPIC), is currently being conducted. This is the largest prospective study yet involving more than half million people in 10 countries, and the first results of this study found a dose dependent protective effect of a high-fibre diet on CRC development (Bingham *et al.*, 2003), and a dose dependent association between red meat intake and colorectal cancer incidence was observed (Norat *et al.*, 2005). The sedentary lifestyle of the Western countries, where the energy balance is displaced (more energy intake than energy consumption), is characterised by an increase of obesity which has been identified as a risk factor for CRC in human and animal models. Indeed people with active lifestyles have increased five-year survival as compared to inactive people (Haydon *et al.*, 2006). Cigarette smoke has been linked to lung cancer development however cigarette smoke has also been consistently linked to CRC in several epidemiological studies (Reviewed in Franco *et al.*, 2005; IARC, 2004).

Age is a major risk factor as the chance of developing CRC dramatically increases after the age of 50 (Quinn *et al.*, 2001). In the UK there is also a geographic component as London and south east England have lower incidence and mortality than the rest of the UK and Ireland (Office for National Statistics, 2005).

Other factors linked to CRC formation include personal or familial history of CRC, personal history of adenomatous polyps and history of inflammatory bowel disease. Inherited genetic predisposition also increases the chance of developing CRC and the two major factors are Familial Adenomatous Polyposis (FAP) and Hereditary Non Polyposis Colorectal Cancer (HNPCC). FAP is a genetic disorder where people develop hundreds of polyps in their colon and rectum throughout their life. Virtually all the people with this disorder develop a colorectal cancer by the age of 40 but they represent only 1% of all the CRC cases. HNPCC is another genetic predisposition that increases the chance of young people developing CRC, together these syndromes account for no more than 2-6% of all CRC (Kemp *et al.*, 2004).



## 1.2.5 CRC screening and treatments

### 1.2.5.1 Screening

Colorectal cancer has a high incidence (see section 1.2.3), and the mortality rate is elevated in Europe but when detected at early stage (Dukes A) the five year survival is greater than 93% (Nicholson *et al.*, 2005). The problems with early detection are that colorectal cancers remain asymptomatic and when the symptoms appear the cancer cells have already invaded the neighbouring tissues with a corresponding drop in the five year survival. For an individual without a family history of CRC the life time incidence for CRC is 6% and the risk doubles every ten years after the age of 40 (Nicholson *et al.*, 2005), therefore the population screening is likely to reduce the mortality but the best screening tool has yet to be identified.

The double contrast barium enema has good sensitivity of 83% (Winawer *et al.*, 2000) for colorectal cancer but it is only used if colonoscopy has failed. Sigmoidoscopy is a quick, sedation-free approach. A long-term follow-up study over a 13 year period has shown a reduction in incidence of CRC of 80% and a reduction in mortality of 50% however studies show that missed CRC lesions in the right colon occur when sigmoidoscopy is used as a diagnostic tool (Lewis *et al.*, 2003; Harewood and Lieberman, 2003). Colonoscopy is an invasive but sensitive method for the detection of neoplastic lesions in the colon. A large multi centre study over a follow-up period of 10 years was conducted in the Italian population (Citarida *et al.*, 2001). 1693 patients, male and female of 40-69 years old which had undergone colonoscopic polypectomy of at least one adenoma >5mm were studied and the incidence ratio for CRC was compared to the expected age/sex-specific incidence of the general population. Six cases of CRC were observed in the screened population over a period of 10 years whereas 17.7 cases would have been expected in the general population. This result is in accordance with the results of the National US Polyp Study (NPS) and confirm that the removal of adenomatous polyps is associated with a decrease in incidence of CRC. Flexible sigmoidoscopy and colonoscopy are both invasive screening tools which are not popular with an asymptomatic patient. Moreover there are risks associated with these two procedures such as perforation, bleeding for sigmoidoscopy or complication and risk of death with colonoscopy. The Faecal occult blood test (FOBT) is a non invasive



technique which consists in detecting traces of blood in stools. The sensitivity is not as high as colonoscopy but the acceptance of asymptomatic patients is much higher and this enables repeated testing currently recommended at 1-2 yearly intervals, this increases the sensitivity for CRC detection to 90% (Mandel *et al.*, 1993). Also a new immunochemical FOBT has shown better sensitivity (66%-90%) than the original Guaiac-based FOBT. However it seems that endoscopy is becoming the method of choice as it can be used to take a tissue sample in order to evaluate the stage of the cancerous lesion and hence plan treatment. New imaging techniques such as computed tomography scans and magnetic resonance imaging are now used to stage a tumour at the time of diagnosis and to plan the optimum treatment.

#### **1.2.5.2 Treatments**

CRC is highly treatable and curable when diagnosed at early stage when the tumour has not penetrated through the bowel wall. The characteristic of the cancer is defined by TNM staging, described in section 1.2.5, and taken together with the serum levels of carcinoembryogenic antigen (CEA) have an impact on the treatment decisions. About 50% of CRC are cured by surgery but recurrence of the tumour at loco-regional or distant sites occur and are a major cause of patient mortality.

Surgery remains the main treatment and about 80% of patient with CRC undergo surgery, 50% of which have cancer recurrence within 24 months. This rate may be lowered in rectal cancer by using total mesorectal excision (TME). However chemotherapeutic drugs such as 5-FU and folinic acid are often used after surgery and reduce cancer recurrence therefore increasing the 5 years survival rate by 5-6% (Dube *et al.*, 1997). In particular patients diagnosed with stage III cancer (see 1.2.5.2) are treated by chemotherapy and stage IV patients require palliative chemotherapy and/or radiotherapy. New approaches are also being investigated such as the use of monoclonal antibody (Bevacizumab and Cetuximab) for first and second line treatment (NICE, 2005).



### **1.2.5.3 CRC biomarkers**

Over the past few decades the early detection of cancers has become a focus. An ideal tumour marker would be a protein or peptide that can be detected in blood or urine of cancer patients but not in healthy persons. The first biomarker to be used was the carcinoembryonic antigen (CEA), which was found in the blood of patients with colon cancers (Gold and Freedman, 1965). Later serum tests have been developed for a variety of cancers such as the detection of CA19-9 for colorectal and pancreatic cancers, CA15-3 for breast cancers, CA-125 for ovarian cancer and Prostate Serum Antigen (PSA) for prostate cancers (reviewed by Chatterjee and Zetter, 2005). However most cancer clinical diagnosis still rely on a pathological tissue examination and biomarkers such as CEA are used to monitor cancer recurrence or evaluate treatment efficacy rather than for the detection of early stage cancers. There is still a need for highly sensitive and specific biomarkers in CRC and other cancers. In more recent years several markers have been identified for their ability to predict the tumour outcome which would improve the therapeutic approach for each patients. In CRC, mutations in mismatch-repair genes are associated with poor prognosis and their detection could be useful to predict the outcome of several colon tumour (Bubb *et al.*, 1996; Halling *et al.*, 1999). Also increases in level of D-dimer has been proposed as a prognostic biomarkers for invasive CRC (Blackwell *et al.*, 2004). However the discovery of sensitive and specific biomarkers remain a challenge and new technologies such as gene microarray or mass spectrometry-based proteomics could enable the identification of such molecules.

### **1.2.6 Genetic factors in CRC development**

The majority of CRC are sporadic (85%) whilst hereditary factors account for the minority (15%) of CRC cases. FAP is an autosomal dominant condition which affects both men and women, it is characterised by the development of thousands of adenomatous polyps in the colon. Most cases of FAP carry a germ line mutation of the APC gene located on chromosome 5q21. HNPCC, also named Lynch syndrome (Lynch *et al.*, 1993) accounts for approximately 3% to 5% of all CRC and is an autosomal dominant condition caused by mutation of one of the DNA mismatch repair genes



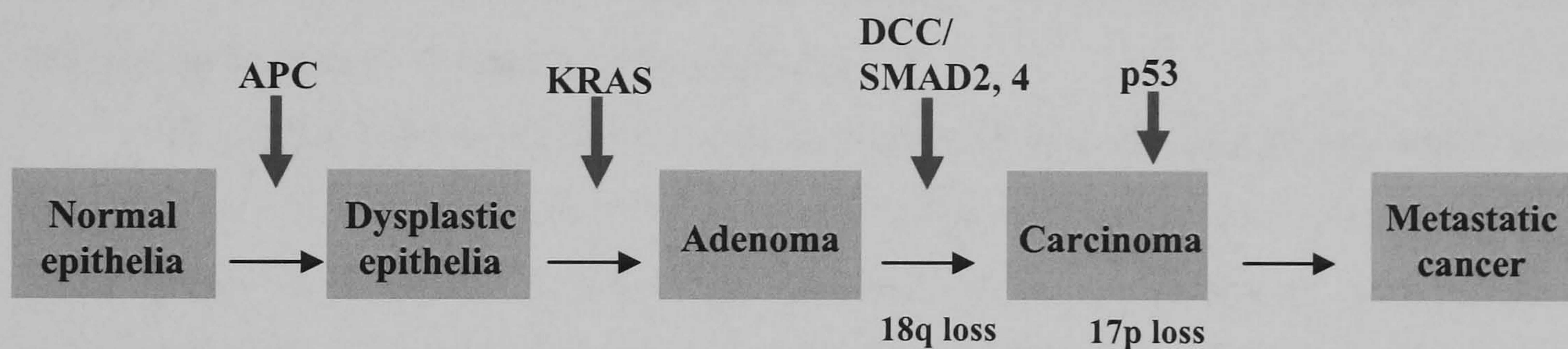
(MLH1, MSH2, PMS2, MSH6). The average age for developing sporadic CRC is 64 years whereas it is 55-57 years for individuals with a mutation in MSH6 and individuals with HNPCC have an 80% chance of developing CRC in their lifetime (Vasen *et al.*, 1996).

The most common genetic event in CRC is a mutation in the APC gene, this occurs in approximately 80% of CRC cases. APC is a tumour suppressor gene whose mutation is necessary and occurs at very early stage of the development of CRC. Fearon and Vogelstein (1990) have described a model of CRC tumour progression which is a multistep process involving genetic alterations leading to the progressive transformation of normal colonic epithelial cells into malignant carcinoma (Kinzler and Vogelstein, 1996; Fearon and Vogelstein, 1990). This multistep process is illustrated in Figure 1.5. The earliest alteration described in this model is the mutation of APC. The genetic instability observed in CRC is necessary for the malignant progression of such lesions, two main mechanisms are observed, chromosomal instability or loss of heterozygosity in 85% of CRC and microsatellite instability in 15% of CRC.

Chromosomal instability or loss of heterozygosity (LOH) ensures a fast and efficient accumulation of other cancerous mutations. A recent study has linked the loss of function of APC with chromosomal instability (Fodde *et al.*, 2001). In HNPCC and 15% of sporadic CRC's chromosomal instability does not occur, instead these tumours present a hypermutable phenotype (microsatellite instability) to facilitate the accumulation of mutations necessary to progress to a malignant phenotype. Microsatellite instability occurs by loss of DNA repair mechanisms and the mismatch repair genes mentioned earlier play a crucial role in microsatellite instability. The genes mutated during the progression from adenoma to invasive carcinoma mainly belong to four signalling pathways: Wnt signalling pathway, KRas pathway, P53 pathway and TGF $\beta$  pathway. APC mutations appear to be the rate-limiting genetic event in CRC initiation (Powell *et al.*, 1992). The tumour suppressor activity of APC resides in its capacity to regulate intracellular levels of  $\beta$ catenin, a key member of the Wnt pathway. Other members of this pathway such as  $\beta$ catenin, axin-1 and axin-2 have also been shown to be mutated during CRC progression. The activation of KRas represents the second step in the cancer progression model proposed by Fearon and Vogelstein. KRas mutations are found in at least 50% of CRC larger than 1cm (Vogelstein *et al.*, 1988).



The mutation of KRas often affects the guanine triphosphate activity leading to a constitutive activation of Ras, Raf, MEK and ERK signal transduction pathway, therefore it induces proliferation and inhibition of apoptosis and thus malignant transformation. On the way to malignancy tumours undergo two other genetics events. LOH occurs on the long arm of chromosome 18 (18q) (Vogelstein *et al.*, 1988, Vogelstein *et al.*, 1989) involving the possible alteration of the “deleted in colorectal cancer” (DCC) genes, SMAD2 and SMAD4 (TGFβ pathway). Mutated SMAD2 and SMAD4 prevent cellular functions such as growth inhibition, apoptosis, differentiation and matrix production (Heldin *et al.*, 1997; Duff and Clarke, 1998). Mutations in TGFβ are also frequent in microsatellite unstable tumours and are observed on chromosome 17 (17p) where the p53 gene is located. p53 is known as the “guardian of the genome” and blocks cell proliferation to allow DNA repair or induce apoptosis (Lane, 1992; Burns and El-Deiry, 1999), the loss of p53 has a central role in the adenoma to carcinoma transition.



**Figure 1.5:** Adenoma-carcinoma sequence. This diagram depicts the major genetic alterations occurring at each step of colorectal cancer development. Adapted from Fearon and Vogelstein 1990.

The model described by Vogelstein and Fearon had a major impact on CRC research as it provided new insights into understanding the genetic modifications associated with colorectal cancer progression. However, several studies have proposed a more complicated system in the development of CRC. The existence of two categories of CRC according to their location in the proximal or distal colon was proposed by several research groups (Bufill, 1990; Knudson, 1989; Rothberg *et al.*, 1985; Distler and Holt, 1997; Gervaz *et al.*, 2001). The two main hereditary CRC, FAP and HNPCC, are characterised by different changes in signal transduction pathways and arise at different sites in the colon. Almost all FAP develop in the distal colon and rectum and



approximately 70% of HNPCC develop in the proximal colon (Lynch *et al.*, 1988). The anatomic differences in the right and left colon and clinical differences of CRC development in the proximal and distal colon have led to the idea that the formation of a CRC may follow different molecular pathways (Iacopetta, 2002).

## **1.3. Glycosylation and cancer**

### **1.3.1 Functions of oligosaccharides on glycoproteins**

Glycoconjugates are essential in a vast number of biological functions including blood clotting, lubrication, structural support, immunological protection, hormone activation and recognition, storage of bioactive molecules, cell-cell and cell-matrix interactions and adhesion (reviewed in Varki, 1993). In contrast to peptides and oligonucleotides which form linear polymers, oligosaccharides are arranged as branched macromolecules and can exhibit a large number of structures. This potentially vast variation in oligosaccharide structures confers considerable opportunity for carbohydrate-protein recognition and adhesion.

The carbohydrate-protein and carbohydrate-carbohydrate interactions which are known to occur during cell-cell, cell-matrix and cell-pathogen interaction (Liotta, 1992), (for example sialylated- $\text{Le}^x$  and  $-\text{Le}^a$ ) are important in the recognition of, for example, endothelial cells by leukocytes. Oligosaccharides are involved in compaction of the embryo at the 16-cell stage of development and this occurs via  $\text{Le}^x$ - $\text{Le}^x$  interactions (Eggens *et al.*, 1989). At the protein level, oligosaccharides have diverse functions such as protection of proteins from enzymatic degradation (Homans *et al.*, 1987) or as cell adhesion molecules (Lasky, 1992, Springer, 1990). Plasma membrane proteins have three main roles, as enzymes, channels or cell / protein receptors and most of these proteins undergo glycosylation as they traffic through the secretory pathway. The glycans confer additional properties such as shape, hydrophobicity, charge and may interact with carbohydrate binding proteins.

Carbohydrates have been shown to play an important role in cancer and metastasis. Lectins, plant or animal carbohydrate binding proteins, provided the first evidence of aberrant glycosylation in cancer. The changes in carbohydrate composition of cancer cells have since been shown to play a critical role in the cell-cell and cell-

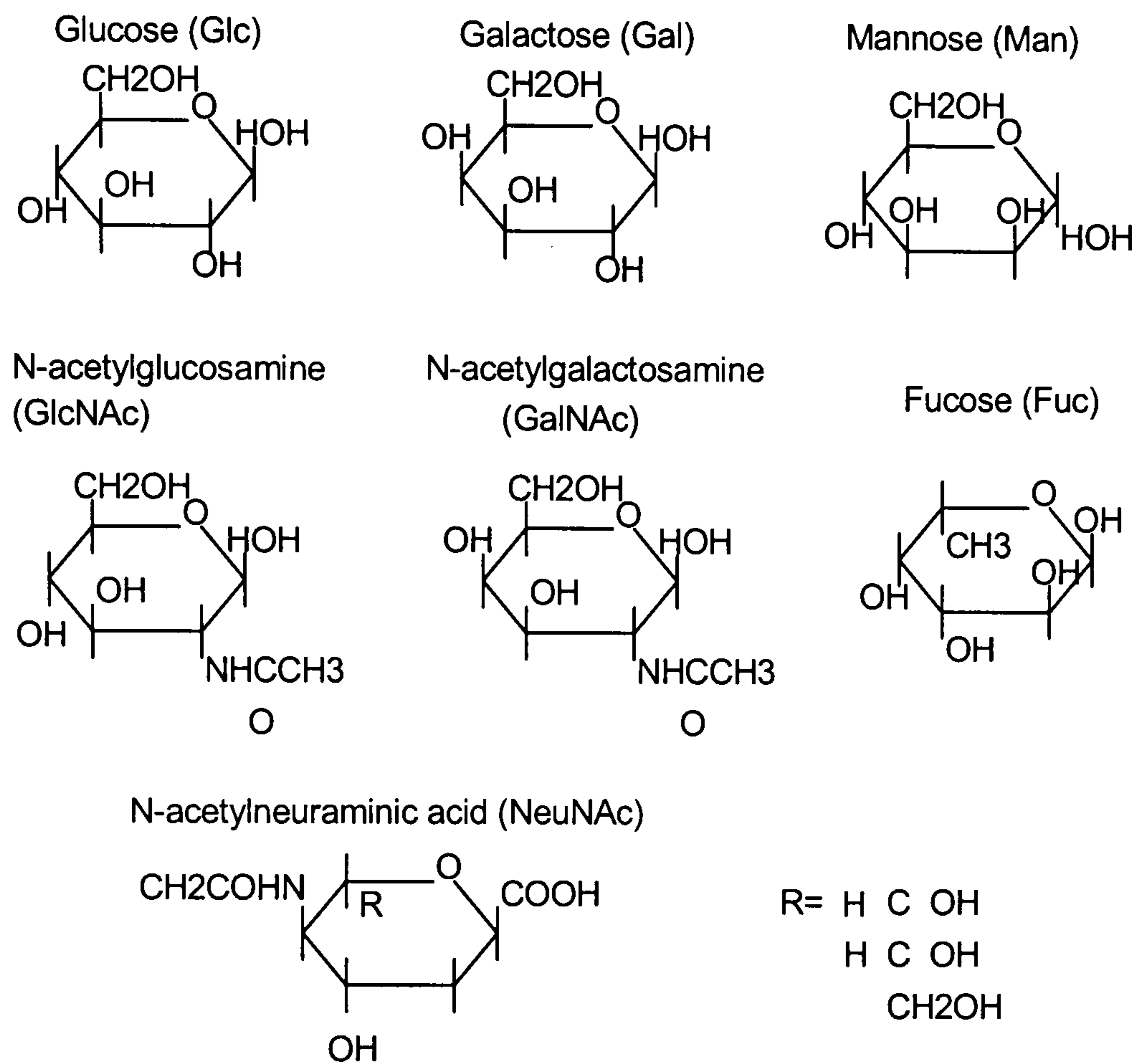


matrix interactions necessary for cancer cell survival, invasion and metastasis. When cells are oncogenically transformed they often express foetal carbohydrates called oncofoetal antigens. A variety of oncofoetal changes in cancer glycosylation have been described, these include increased N-linked carbohydrate size due to extensive branching, truncated carbohydrates as observed on mucin-type glycoconjugates, exposing structures such as the T and Tn antigens and increased expression of the Lewis sugars Lewis x and Lewis x.

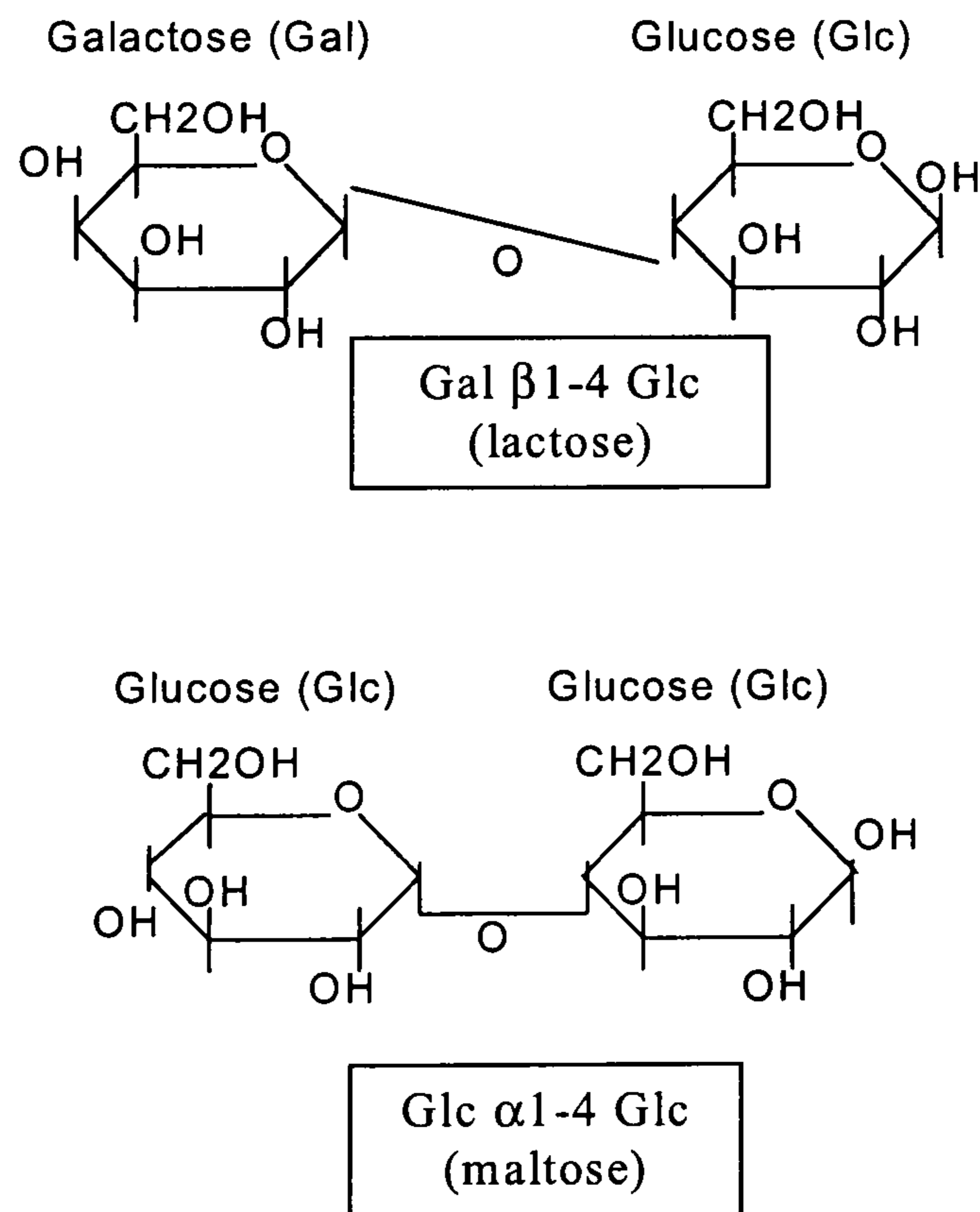
In normal human cells there are four main groups of glycoconjugates: glycoproteins, glycolipids, glycoaminoglycans (GAGs) and glycosphosphoinositol (GPI) anchors. GPI anchors are exclusively membrane bound and are implicated in cell signalling (Low *et al.*, 1986; Merida *et al.*, 1990). Glycolipids are mainly located on cell membranes (Hakomori, 1986) and glycoproteins are found in various cellular locations such as the intracellular compartment, plasma membrane (integral or membrane-associated glycoproteins) or in the sera (Lis and Sharon, 1993). Finally GAGs play a structural role in the extracellular matrix.

In humans seven monosaccharides are commonly found attached to glycoproteins or glycolipids, these entities are shown in Figure 1.6. The potential diversity of structures, for example in the formation of a simple disaccharide from two monosaccharides, is shown in Figure 1.7. Two types of oligosaccharides are most commonly found attached to the protein backbone. These are classified by the nature of their linkage to the proteins: N-linked glycans by the attachment of an N-acetylglucosamine (GlcNAc) residue on the nitrogen atom of an asparagine residue and O-linked glycans are mainly characterised by a GalNAc residue attached to an oxygen atom of the hydroxyl group of serine or threonine on the protein. The importance of glycosylation in human biology is evidenced by the number of gene products involved in the biosynthesis of glycans with about 1% of the translated genome being implicated in glycosylation reactions (Varki and Marth 1995).





**Figure 1.6:** Monosaccharides associated with human glycoproteins and glycolipids



**Figure 1.7:** Monosaccharide binding via alpha and beta bonds.



### 1.3.2 N-linked oligosaccharides

Biosynthesis of all N-linked oligosaccharides begins in the rough ER and can be viewed as four distinct phases associated with different compartments of the secretory pathway. Firstly glycosyltransferases specifically and sequentially add monosaccharides to a dolichol molecule anchored in the ER membrane eventually generating the large lipid-bound oligosaccharide intermediate of general formula  $\text{Glc}_3\text{Man}_4\text{GlcNAc}_2$ -dolichol presented in Figure 1.8 (Hubbard and Ivatt, 1981; Kobata and Takasaki, 1992). The oligosaccharide is eventually transferred by the enzyme dolichylpyrophosphoryl oligosaccharide-polypeptide oligosaccharyltransferase (Das and Heath, 1980) to an accessible asparagine residue within the sequence Asn-X-Ser/Thr, where X can be any amino acid except proline. After attachment of the oligosaccharide intermediate to the nascent polypeptide chain in the ER the glycan structure is recognised by the calreticulin and calnexin system and if the protein is correctly folded it is transported via vesicles to the Golgi apparatus where the oligosaccharide is trimmed and processed by a series of glycosidase and transferase enzymes (Kornfeld and Kornfeld, 1985). The structural variation in glycans is due to tissue-specific variations in expression of glycosyltransferases genes, sugar nucleotide availability in the Golgi apparatus and competition between enzymes during glycan elongation. In the Golgi apparatus further residues are added to the oligosaccharide, these most commonly include galactose, sialic acid and fucose and these residues form extensions and “branches” eventually generating a diverse range of mature N-glycans. N-linked oligosaccharides possess a common feature,  $\text{Man}_3\text{GlcNAc}_2$  (Kobata and Takasaki, 1992) but the wide range of variability in branches results in structural heterogeneity. Furthermore these glycoproteins often undergo O-glycosylation within the Golgi apparatus (Kornfeld and Kornfeld, 1985) and are then transported via vesicles to the cell membrane (Zubay, 1993).



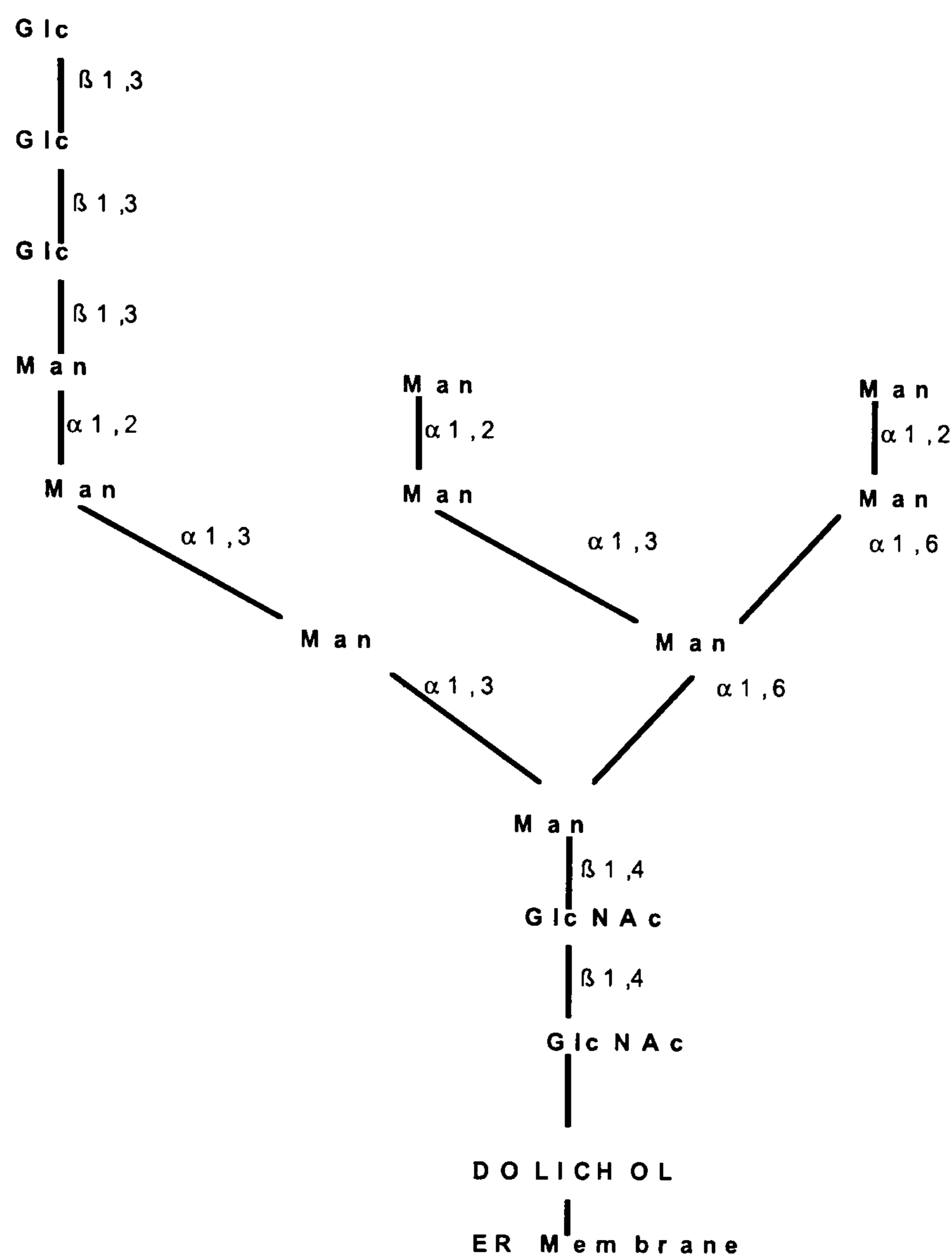


Figure 1.8: The glycosylated oligosaccharide lipid intermediate.

### 1.3.3 O-linked oligosaccharides

O-linked oligosaccharides are typically characterised by the addition of N-acetylgalactosamine (GalNAc) to serine or threonine of a polypeptide backbone, this occurs in the cis Golgi. GalNAc is transferred to the serine or threonine from the nucleotide donor substrate UDP-GalNAc by the action of one of several UDP-GalNAc-polypeptide  $\alpha$ -N-acetylgalactosaminyl transferase (ppGalNAc-T) enzymes. The presence of proline residues in the region surrounding the glycosylation site is necessary for effective transfer of  $\alpha$  GalNAc by ppGalNAc-T. The attachment of the  $\alpha$  GalNAc glycan to the glycosylation site of the polypeptide gives rise to the Tn antigen, this is then processed by glycosyltransferases to generate O-linked glycan



motifs termed “core” structures which can be further modified to create O-linked glycans (Brockhausen, 2000).

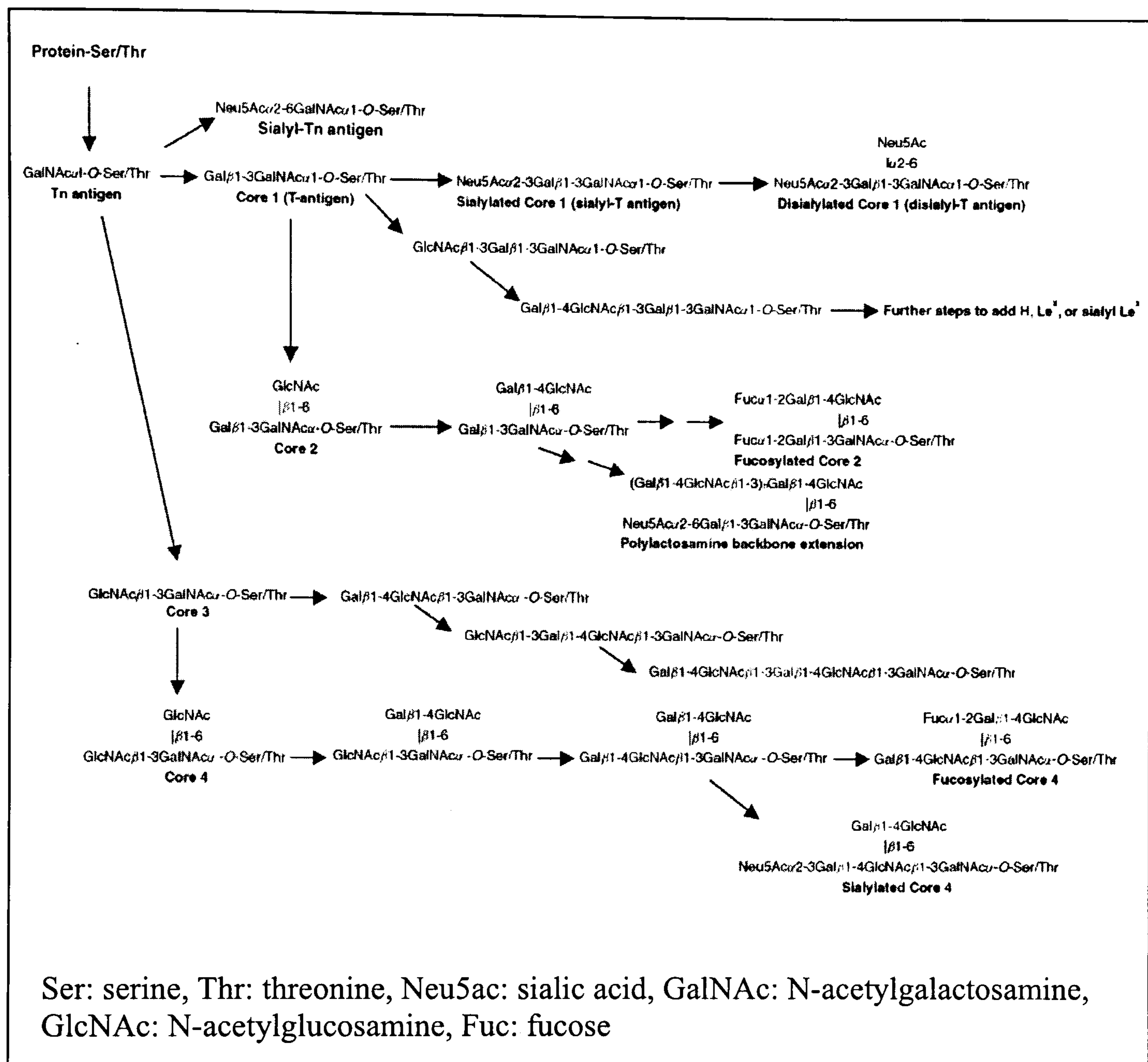
The oligosaccharides attached to Ser/Thr vary widely in size from a single GalNAc (Tn antigen) up to greater than 18 sugar residues. The structure of large O-glycans may include:

- A core unit linked to the Ser/Thr of a polypeptide
- A backbone, not always present
- Peripheral structures

Eight core O-linked structures have been identified (core 1-8) but cores 1 and 2 are the most common in normal breast and colorectal tissues whilst core 3 and 4 have a lower occurrence, cores 5-8 occur rarely. Figure 1.9 depicts the biosynthetic pathways of core 1-4 O-linked glycans and their extensions. The type of core has a direct influence on the final structure of the O-glycans. The peripheral structures decorating the core and backbone units are responsible for the huge variation in O-glycan structures. O-linked glycans play a variety of biological roles, for example as cell adhesion molecules (sialyl Le<sup>x</sup>/sialyl Le<sup>a</sup>) and as blood group antigens. The three parts of the O glycans, mentioned earlier are shown in Table 1.3, the structure of the Tn antigen, cores 1-8 and the backbone units and selected peripheral branches are presented.

Glycosidases and glycosyltransferases (GTases) enzymes are responsible for the elaboration of the glycan extensions on the newly synthesised protein. Their sequential and ordered action leads to the addition of defined glycans to proteins. The GTases are found in the organelles of the secretory pathway and in particular in the ER and Golgi. The GTases are themselves glycoproteins attached to the ER/Golgi membrane via a transmembrane domain (Paulson and Colley, 1989). The diversity of protein glycosylation relies on the specificity of GTases and recent developments in molecular biology have shown that there is a great diversity of enzymes with, potentially, several hundreds of genes coding for enzymes involved in glycosylation reactions. In humans, more than 200 glycosyltransferase (GTase) sequences have been reported. The various GTases compete with each other to react with polypeptides and monosaccharides to form glycopeptide or oligosaccharide structures. Factors such as the level and activity of an enzyme and the presence of appropriate nucleotide donors influence the final glycan structure produced (Breton *et al.*, 2001; Brockhausen, 2006).





**Figure 1.9:** Biosynthesis pathways of the most represented cores 1-4 and their extensions. The addition of an  $\alpha$ 1 linked GalNAc to Ser/Thr constitute the Tn antigen. The addition of Gal to the Tn antigen in a  $\beta$  1-3 linkage form the Core 1 structure. GalNAc in a  $\beta$ 1-6 linkage added to Core 1 constitute the Core 2 structure. The formation of Core 3 consists of the addition of a GlcNAc residue to the Tn antigen. A GlcNAc residue added to Core 3 in a  $\beta$ 1-6 linkage form the Core 4 structure. Cores 1-4 can be extended by peripheral structures as shown. From Corfield, 2005 (Encyclopaedia of genetics, genomics, proteomics and bioinformatics).



<b>Core classes</b>	<b>Structures</b>
Core 1	Gal $\beta$ 1-3GalNAc $\alpha$ -O-Ser/Thr
Core 2	Gal $\beta$ 1-3GalNAc $\alpha$ -O-Ser/Thr   GlcNAc $\beta$ 1-6
Core 3	GlcNAc $\beta$ 1-3GalNAc $\alpha$ -O-Ser/Thr
Core 4	GlcNAc $\beta$ 1-3GalNAc $\alpha$ -O-Ser/Thr   GlcNAc $\beta$ 1-6
Core 5	GalNAc $\alpha$ 1-3GalNAc $\alpha$ -O-Ser/Thr
Core 6	GlcNAc $\beta$ 1-6 GalNAc $\alpha$ -O-Ser/Thr
Core 7	GalNAc $\alpha$ 1-6GalNAc $\alpha$ -O-Ser/Thr
Core 8	Gal $\alpha$ 1-3GalNAc $\alpha$ -O-Ser/Thr
<b>Backbone units</b>	
Type 1	Gal $\beta$ 1-3GlcNAc
Type 2	Gal $\beta$ 1-4GlcNAc
Poly N-acetyl lactosamine type 2	(Gal $\beta$ 1-4GlcNAc $\beta$ 1-3) <sub>n</sub>
Branched N-acetyl lactosamine 2	Gal $\beta$ 1-4GlcNAc $\beta$ 1-6Gal $\beta$ 1-   Gal $\beta$ 1-4GlcNAc $\beta$ 1-3
<b>Peripheral Structures</b>	
Blood Group H	Fuc $\alpha$ 1-2Gal $\beta$ 1-3GlcNAc $\beta$ 1-
Blood Group A	GalNAc $\alpha$ 1-3Gal $\beta$ 1-4GlcNAc $\beta$ 1-   Fuc $\alpha$ 1-2
Blood group B	Gal $\alpha$ 1-3Gal $\beta$ 1-3GlcNAc $\beta$ 1-   Fuc $\alpha$ 1-2
Sialyl Lewis <sup>a</sup>	Neu5Ac $\alpha$ 2-3Gal $\beta$ 1-3GlcNAc $\beta$ 1-   Fuc $\alpha$ 1-4
Sialyl Lewis <sup>x</sup>	Neu5Ac $\alpha$ 2-3Gal $\beta$ 1-4GlcNAc $\beta$ 1-   Fuc $\alpha$ 1-3
Sialyl Tn	Neu5Ac $\alpha$ 2-6GalNAc $\alpha$ -O-Ser/Thr
Sialyl T antigen	Neu5Ac $\alpha$ 2-3Gal $\beta$ 1-3GalNAc $\alpha$ -O-Ser/Thr

**Table 1.3:** Types of O-linked oligosaccharides found on glycoproteins and mucins. Adapted from Brockhausen, 1999.



### **1.3.4 Important extensions of N- and O-linked glycans— blood group sugars and Lewis antigens**

The complexity of glycan structures on glycoproteins relies on the ability of GTases to form the various branched extensions of oligosaccharides. The classical core structures of N- and O-linked glycans often comprise a GlcNAc residue at the non-terminal end; this is usually extended with a Gal residue in either a  $\beta$ 1-3 or  $\beta$ 1-4 linkage resulting in type 1 or 2 chains respectively.

The blood group ABO system was first described by Landsteiner in 1902. ABH determinants (Table 1.3) are attached to type 1 or type 2 O-linked glycan chains of glycolipids of erythrocytes, on glycoproteins and in secretions such as mucus (reviewed by King, 1994). The Lewis blood group antigens are fucosylated antigens found mainly in glycolipids but also glycoproteins. Lewis a and Lewis b are based on the type 1 chain and Lewis x and Lewis y are based on the type 2 chains, shown in Table 1.3. Sialylation of these antigens can also occur conferring a negative charge to the glycan chain. Modification of the Lewis determinants has been shown in cancer and will be described further. The type 2 chain can contain repeating units known as polylectosamine repeats and these have been found to occur on both N- and O-linked carbohydrates.

### **1.3.5 Altered glycosylation in cancer**

There is a considerable body of evidence detailing abnormal glycosylation of glycoproteins in cancer development. There are four main types of glycosylation changes in solid tumours (reviewed by Dwek and Brooks, 2004); synthesis of truncated glycans, inappropriate synthesis of blood group determinants, changes in polylectosamine synthesis and increased  $\beta$ 1-6 branching and finally alterations in sialylation.

#### **1.3.5.1 Altered O-glycans**

Mucin glycoproteins are highly glycosylated in specific domains termed variable number tandem repeats (VNTR), these regions are rich in the serine and threonine residues to which O-linked oligosaccharides are attached. Mature mucins typically



comprise between 50% and 90% carbohydrate (Kim, 1992). Mucins also contain N-linked oligosaccharides but these are a minor constituent although they have been reported to play an important role in the oligomerisation of these glycoproteins (Dekker and Strous, 1990). The first gene sequence for a mucin was obtained for MUC1. Many other genes coding for mucins have since been identified and these have been named MUC1 to MUC17. Mucins are classified into two main groups according to their structural and functional characteristics: secreted mucins (MUC2, MUC5AC, MUC5B, MUC6), transmembrane mucins (MUC1, MUC3A, MUC3B, MUC4, MUC11, MUC12, MUC17) and mucins that belong to neither of these groups, for example MUC7, MUC8, MUC9, MUC13, MUC15 and MUC16 (reviewed in Byrd and Bresalier, 2004). Initial studies suggested that mucin expression was organ-specific (Yonezawa *et al.*, 1991) but it now seems that most organs express more than one type of mucin although there are predominant mucin types in a particular organ. The loss of organ specific expression of mucins is a common feature of gastrointestinal tumours.

In CRC MUC1 expression is an independent prognostic factor (Balduş *et al.*, 2002) and an indicator of high risk of death in Caucasians, (Manne *et al.*, 2000). Early studies used an immuno-histochemical approach to evaluate MUC1 expression in normal colon and CRC (Nakamori *et al.*, 1994) but it was suggested that differences in binding between normal and cancerous cells observed with antibodies directed towards MUC1 were not due to differences in the level of the mucin but due to differences in glycosylation, affecting antibody interaction with the mucin protein backbone. mRNA based studies showed that there is stable expression of the MUC1 gene product in normal and cancerous colon and further work showed that in CRC there is a decrease in glycosylation which leads to unmasking of the MUC1 protein backbone (Ajioka *et al.*, 1996; Cao *et al.*, 1997). MUC1 with abnormal glycosylation becomes antigenically distinct from its normal form and this may render it a favourable target for immunotherapy of CRC (Singh and Bandyopadhyay, 2007; Tarp and Clausen, 2007).

It has been reported that, in cancer, the oligosaccharides on mucins and other glycoproteins are truncated or show structural differences compared with the glycans of mucins and proteins derived from matched normal tissues (Kim *et al.*, 1974). Alterations in the enzymes active in glycosylation pathways (Boland and Deshmukh, 1990; Yang *et al.*, 1994) leads to the altered glycan structures. In colon cancer there is



an increase in Le<sup>a</sup> and Le<sup>x</sup> sugars and exposure of the Tn (GalNAc $\alpha$ 1-3-O-Ser/Thr), sialyl Tn and T antigens (Springer, 1984; Itzkowitz *et al.*, 1986b; Itzkowitz *et al.*, 1989; Kim, 1992).

Increased expression of T antigen is partially due to an increase in core 1  $\beta$ 3 galactosyl transferase and decrease in core 3  $\beta$ 3 GlcNAc transferase (Yang *et al.*, 1994) and correlates with an increased risk of liver metastases (Cao *et al.*, 1995). The presence of T antigen has also been correlated with the presence of lymph node metastases in patients with breast and gastric cancer (Mustac *et al.*, 1996, Yamashita *et al.*, 1995). Leathem and Brooks (1987) reported a correlation between an increased ratio of Tn/T and the degree of differentiation of breast tumour cells, malignancy and survival. Over-expression of  $\alpha$ 2,6-sialyltransferase-I, responsible for addition of  $\alpha$ 2,6 linked sialic acid residues, has been shown to lead to the abnormal sialylation of the Tn antigen (STn) (Yang *et al.*, 1994). Increased levels of STn have been reported at an early stage of CRC formation and STn expression correlates with the formation of metastases and poor prognosis (Itzkowitz *et al.*, 1989).

Sialyl Lewis<sup>x</sup>, sialyl Lewis<sup>a</sup> and sialyl-dimeric Lewis<sup>x</sup> have all been shown to be elevated in metastatic cancer cells as compared to primary tumours (Hanski *et al.*, 1996; Ito *et al.*, 1997; Nishihara *et al.*, 1999; Petretti *et al.*, 2000). Elevated levels of sialyl Lewis<sup>x</sup> can be attributed to an increase in the activity of  $\alpha$ 3-sialyltransferase and correlates with lymph node invasion by colorectal cancer cells. The enzyme  $\alpha$ 3-fucosyltransferase which controls the formation of sialyl-dimeric Lewis<sup>x</sup> is increased in colorectal cancer and is related to poor prognosis (Kudo *et al.*, 1998).

In summary, the incomplete glycosylation of O-linked glycans observed in cancer results in exposure of glycan structures normally masked by further glycosylation. Some of the most commonly reported cancer associated glycan motifs are shown in Table 1.4



Cancer-associated glycans	Increased/decreased in cancer
Tn antigen	↑
STn antigen	↑
Core 1, T antigen	↑
Sialyl-T antigens	↑
Core 2	↑ ↓
Core 3	↓
Core 4	↓
Type 1 chain	↓
Type 2 chain	↑
Sialyl Lewis <sup>a</sup>	↑
Sialyl Lewis <sup>x</sup>	↑
Sialyl-dimeric Lewis <sup>x</sup>	↑

**Table 1.4:** O-glycans associated with cancer. Adapted from Brockhausen, 2006

### 1.3.5.2 Alteration of Lewis sugars and blood group antigens

The role of ABH determinants in normal epithelia or neoplasia is unknown although it has been suggested that blood group A antigen may be involved in regulation of cell proliferation (Feizi and Childs, 1987). It is now widely recognised that the normal pattern of tissue-associated blood group antigens are modified in cancerous lesions (Bloom *et al.*, 1990). Aberrant expression of antigens and/or deletion of antigens normally expressed often occurs during cancer formation. The loss of the blood A and B antigens by removal of GalNAc and Gal residues respectively is a common feature of carcinoma development (Hakomori, 1999; LePendou *et al.*, 2001). This uncovers the common precursor of blood A and B determinants termed the blood group H antigen which is often seen to be increased in carcinomas such those of the breast, colon and stomach (Marionneau *et al.*, 2001; Idikio and Manickavel, 1992). Decreased ABH expression in lung and bladder cancer is associated with metastatic potential and poor prognosis (Matsumoto *et al.*, 1993; Orntoft *et al.*, 1996) and patients with gastric carcinoma and increased blood group A antigen / GalNAc containing oligosaccharides (as detected using the lectin HPA) exhibit a lower survival rate (Kakeji *et al.*, 1991).



The changes in glycosylation of blood group antigen may occur at the invasive front of the tumour and may be associated with the degree of differentiation of the tumour. The Lewis A and Lewis B ( $\text{Le}^b$ ) and the sialylated form of the Lewis antigens have been shown to be altered during cancer formation with decreased levels in cancer cells as compared to the normal surrounding tissues. The loss of  $\text{Le}^b$  correlates with the grade of the lesion (Idikio and Manickavel, 1991).  $\text{Le}^x$  is an oncofetal antigen (Feizi, 1985) found in higher quantities in breast, colon and renal carcinomas and  $\text{Le}^y$  is elevated in various carcinomas such as colon and liver cancer (reviewed by Dwek and Brooks, 2004). In a manner similar to the ABH antigen, the abnormal occurrence of Lewis antigens is a marker of de-differentiation and therefore a marker of malignancy.

Sialylated forms of Lewis antigens have attracted much interest as they are binding partners for selectins and are implicated in metastatic mechanisms. Hoff *et al.* (1989) showed that elevated levels of  $\text{SLe}^x$  are found on the surface of liver metastases confirming a significant role of this antigen in the metastatic spread of the cancer cells.  $\text{SLe}^x$  and  $\text{SLe}^a$  on cell surface glycoconjugates assists in the attachment of cells to E-, P- and L-selectins present on activated endothelia, leukocytes and platelets. This ability of cancer cells to bind to blood-borne cells and endothelia is an important factor in enabling the metastatic spread of tumour cells. Expression of  $\text{SLe}^x$  is an early marker of metastasis and has been inversely correlated with survival in CRC and other carcinomas (Nakamori *et al.*, 1997, Baldus *et al.*, 1998). A recent study conducted using cell lines and a murine model by Fuster *et al.* (2003) established that a disaccharide which inhibits the synthesis of  $\text{SLe}^x$  results in a decrease in the metastatic potential of colon and lung cancer cell lines and cancer cells treated with this disaccharide showed a reduction in binding to Selectin, thrombin-activated platelets and  $\text{TNF}\alpha$ -activated endothelium and a reduced ability of the colon cancer cell lines to metastasise to the lung in SCID mice.

### 1.3.5.3 Altered N-glycans

It is a common feature that malignant cells display increased glycosylation in particular the acquisition of large N-linked glycans as a result of the presence of polylactosamine extensions  $(\text{Gal}\beta 1-4\text{GlcNAc}\beta 1-3)_n$ . The presence of polylactosamine repeats has been associated with malignant transformation and metastatic competence



(Dennis, 1986; Pierce and Arango, 1986; Yamashita *et al.*, 1984). Polylactosamine repeats may form on N-glycans but also on O-glycans to a lesser extent. Studies have demonstrated that polylactosamine extensions are preferentially added to the GlcNAc $\beta$ 1-6Man $\alpha$ 1-6 $\beta$  branch of N-linked oligosaccharides as a result of an increased activity of  $\beta$ 1-3GnT which is responsible for the synthesis of polylactosamine (van den Eijnden *et al.*, 1988). For instance in colon cancer  $\beta$ 1-3GnT activity is increased and this results in an increased synthesis of the polylactosamine structures. These structures may bear blood group determinants which may be functionally involved in malignancy. For example SLe<sup>x</sup> on polylactosamine extensions of colon cancer cells exhibits a more invasive phenotype when implanted in nude mice (Saitoh *et al.*, 1992).

The acquisition of large N-glycan structures in cancer development due to polylactosamine extensions is the result of increased  $\beta$ 1-6 branching and increased levels of polylactosamine extensions in rodent cells have been correlated with increased levels of the enzyme responsible for the synthesis of  $\beta$ 1-6 branching structures, GlcNAc transferase V (GnTV, Yousefi *et al.*, 1991). Several studies have reported increased  $\beta$ 1-6 branching in human carcinomas of the breast and colon (Dennis and Laferté 1989; Fernandes *et al.* 1985) and the overexpression of GnTV was associated with increased invasion and metastasis in various tumour types (Yagel *et al.*, 1989).

The lectin L-PHA recognises  $\beta$ 1-6 branched structures and has been used to study the involvement of  $\beta$ 1-6 branched structures in cancer development and metastasis. The mutant lymphoma cell line MDA-D2 which displays large amounts of polylactosamine and  $\beta$ 1-6 branches (as recognised by L-PHA lectin) was highly metastatic in nude mice whereas mutant cells without GnTV activity which did not synthesise these glycan structures were resistant to L-PHA-mediated cell killing. Moreover the L-PHA resistant cells were no longer metastatic and this provided the first evidence of the importance of polylactosamine extensions and  $\beta$ 1-6 branching in metastasis (Dennis *et al.*, 1987).

Inhibitors of N-linked glycans synthesis which result in abrogation of biosynthesis of polylactosamine extensions and  $\beta$ 1-6 branching have been used in numerous studies. For instance melanoma cells treated with swainsonine display a decreased metastatic potential in the nude mouse model (Dennis *et al.*, 1987). Changes in N-linked glycosylation play a key role in cancer progression and could be used as



biological markers, for example the N-linked oligosaccharide on prostate specific antigen (PSA) is altered in the serum from patients with prostate cancer (Tajiri *et al.*, 2007). This alteration in glycosylation may allow discrimination between patients with benign prostatic hyperplasia and prostate cancer both of who may present clinically with elevated levels of PSA.

#### **1.3.5.4 Sialylation**

The negative charge conferred to the polypeptide by sialic acid residues may modulate cell-cell and cell-environment interactions. Alterations in quantity, distribution, the type of sialic acid and bonding to neighbouring sugars have been reported in cancer (Bhavanadan and Furukawa, 1995). The use of the lectin SNA to detect sialic acid on colorectal cancer cells has shown an increase in N-acetylneuraminic acid containing glycans in this type of cancer (Dall'olio and Trere, 1993) and a study using an immunodeficient murine model has shown the role of N-acetylneuraminic acid in metastasis (Saitoh *et al.*, 1992).

The extent of sialylation appears to be an important factor in cancer formation (and metastasis) and a loss of cancer cell adhesion has been attributed to the cells expressing increased levels of sialic acid (Dennis *et al.*, 1989; Dennis, 1986).

The observations of several studies, taken together with the importance of sialylation on the Lewis determinants in tumourigenesis, suggests that sialylation may play an important role in malignancy.

### **1.4. Glycoproteins and cancer**

Cell-cell and cell-substratum interaction have long been established as essential for the survival of the human cells. During development and cell migration, the contacts established by epithelial cells with their surroundings, such as the ECM or neighbouring cells, plays an essential role in cellular behaviour. The integrity of the epithelia relies on the connections established between cells and between cells and the ECM, these interactions are mediated by glycoproteins and proteoglycans. Cell adhesion molecules (CAMs) are cell surface glycoproteins with a large extracellular domain, a membrane spanning region and a cytoplasmic domain. Although their primary function is to



maintain tissue structure via cell-cell and cell-substratum interactions, they are also involved in cell migration, differentiation cell signalling and gene transcription (Goodison *et al.*, 1999). CAMs involved in cell-cell and cell-matrix interactions are altered during progression to tumour malignancy. Alterations in CAMs modify adhesion processes and may modulate intracellular signalling (reviewed by Christofori, 2003). More than 50 CAMs have been identified and can be organised in superfamilies. The largest superfamilies will be treated in this chapter; mucins, cadherins, immunoglobulin-like CAMs, selectins, integrins and proteoglycans.

## **1.4.1 Cell adhesion molecules**

### **1.4.1.1 Cadherins**

Many human cancers originate in epithelial cells which are, in normal conditions, tightly organised and maintained by a number of specific junctions such as tight junctions, adherens-type junctions and desmosomes. These are connected to actin and intermediate filaments of the cytoskeleton and constitute a tightly bound structure. Cell-cell adhesion is often mediated by cadherins, a family of  $\text{Ca}^{2+}$  dependent glycoproteins and in epithelia, they mediate cell-cell adhesion at adherens junctions via homophilic protein-protein interactions.

E-cadherin expression is lost during tumourigenesis. Birchmeier and Behrens (1994) suggested that loss of E-cadherin function is a prerequisite for tumour cell invasion and several other studies have shown that loss of E-cadherin expression is accompanied by the acquisition of an invasive phenotype. Further studies on cell lines have demonstrated that re-expression of E-cadherin by invasive cell lines (which had previously lost E-cadherin) resulted in a reversion from an invasive to benign cellular phenotype (Vleminckx *et al.*, 1991).

### **1.4.1.2 Selectins**

The selectin family is composed of E-selectin, L-selectin and P-selectin. E-selectin is expressed solely on endothelial cells, P-selectin is expressed on platelets and endothelia and finally L-selectin is present on leukocytes. Selectins are calcium-dependent type 1 transmembrane glycoproteins. Structurally, they consist of a short



intracellular C-terminal domain and a single transmembrane domain. The extracellular domain is composed of varying numbers of short consensus repeats (SCRs) which act as spacers between the N terminal domain and the plasma membrane. In humans L-, E-, P-selectins possess respectively 2, 6 and 9 SCRs. The N terminal domain of selectins is a C type ( $\text{Ca}^{2+}$  dependent) lectin-like domain which mediates interaction with small sialylated, fucosylated carbohydrate ligands such as  $\text{SLe}^x$  and  $\text{SLe}^a$  (Bresalier *et al.*, 1998). In normal conditions monocytes and neutrophils express  $\text{SLe}^a$ - and  $\text{SLe}^x$ -containing carbohydrates and their binding to selectins has been shown to participate in the recruitment of leukocytes to sites of tissue damage binding to the selectins on activated endothelial cells (reviewed in Nangia-Makker *et al.*, 2002).  $\text{SLe}^x$  is an oncofetal antigen that has been shown to be expressed in various cancers including colon cancer (Itzkowitz *et al.*, 1986). The amount of  $\text{SLe}^x$  expressed by cancer cells has been linked to metastasis and patient survival (Ono *et al.*, 1996; Nakamori *et al.*, 1993).  $\text{SLe}^x$  plays a role in metastasis via its ability to interact with selectins. Selectins have been involved in several aspects of carcinogenesis. E and P selectins have been shown to be related to angiogenesis by inducing neovascularisation (Koch *et al.*, 1995). L selectin binding to tumour cells may facilitate metastasis in a murine model (Qian *et al.*, 2001). Moreover when colorectal cancer cells were implanted into P-selectin deficient mice, a reduced number of lung metastases was observed and this correlated with a decrease in the number of tumour cell-platelet aggregates (Kim *et al.*, 1998). It has been suggested that the tumour cell-platelet complex occurs via adhesion to P- selectins and this is one of the mechanisms by which tumour cells escape the host immune system during the metastatic process (Honn *et al.*, 1992). The selectins mainly bind to mucin-type glycoproteins or O-linked glycoproteins that exhibit glycan structures  $\text{SLe}^a$ ,  $\text{SLe}^x$ . Such glycans are often the result of altered glycosylation in cancer cells (described earlier) and their overexpression has been linked to increased metastatic potential and poor patient prognosis.

#### **1.4.1.3 Immunoglobulin-like CAMs**

The Immunoglobulin-like CAMs (Ig-CAMs) possess Ig-like folds in their extracellular domain and are involved in homophilic interaction (Tang and Honn, 1995). Carcinoembryonic antigen (CEA) is an Ig-CAM and has been used as a marker of



malignancy and for measuring response to treatment in colorectal cancer (Fearon *et al.*, 1991; Benchimol *et al.*, 1989).

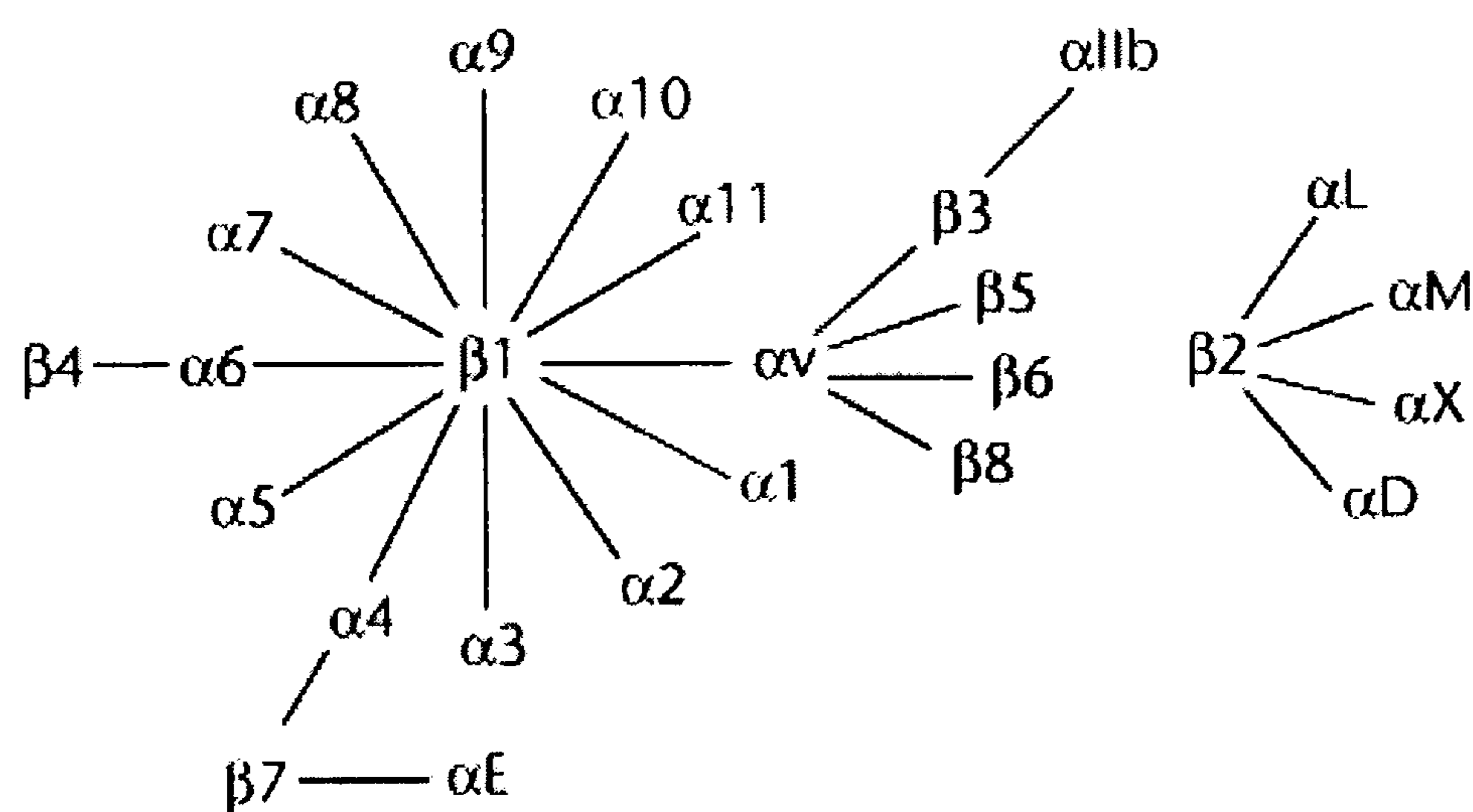
#### **1.4.1.4 Hyaluronate receptor CD44**

CD44 is a cell surface proteoglycan and is a member of the hyaluronate receptor family. There are multiple CD44 isoforms that originate from splice variants of a single CD44 gene (Goodison *et al.*, 1999). CD44 is a single polypeptide chain oriented with its large N terminus domain on the outside of the cell, and C terminal domain in the cytosol. Its major ligand is hyaluronic acid (HA) an extracellular polysaccharide component of the ECM. CD44 is the most commonly expressed isoform in mammalian cells although some epithelial cells express the larger isoform CD44E. CD44 is involved in a variety of functions such as maintenance of the structure of organs and tissues, cell movement and cell aggregation of macrophages, lymphocytes and fibroblasts via its HA binding site. CD44 exists as several isoforms which enables binding to a variety of ligands, for example, osteopontin, serglycin, collagen, fibronectin and laminin. All CD44 isoforms contain the HA binding site and it has been shown that loss of CD44 N-glycosylation abrogates HA binding (Lesley *et al.*, 1995). CD44 molecules which bind to HA may play a role in cancer development and invasion (Sy *et al.*, 1991). Tumour cells express multiple unusual isoforms of CD44 as compared to normal tissues and this abnormal expression may result in changes in HA binding and may influence tumour growth (Stamenkovic *et al.*, 1989). Studies in animal models have shown that increased expression levels of CD44 proteins results in a more invasive phenotype (Birch *et al.*, 1991). Gunthert *et al.* (1991) first investigated the impact of increased expression of CD44 in non-metastatic rat pancreatic cancer cells with respect to cancer progression and invasion. Over expression of CD44v conferred metastatic potentiality to the cells whereas CD44s did not. Variant v6 and v7 modulated the invasive behaviour of cancer cells independently of their ability to bind HA (Gunthert *et al.*, 1991; Seiter *et al.*, 1993). More recent studies have used the metastatic colorectal cancer cell line HT29 and silenced CD44v6 by an antisense approach. Down-regulation of CD44v6 did not abrogate the capacity of HT29 cells to bind to hyaluronan but did diminish their ability to form liver metastases in nude mice (Reeder *et al.*, 1998).



### 1.4.1.5 Integrins

The integrin superfamily is a family of extensively glycosylated glycoproteins which emerged in metazoa over 600 million years ago (Hynes, 1992; Hynes and Zhao, 2000). It is a large family of at least 24  $\alpha\beta$  heterodimeric type I transmembrane glycoproteins. Integrins are composed of two subunit  $\alpha$  and  $\beta$  noncovalently attached together. To date there have been 18  $\alpha$  and 8  $\beta$  chains characterised and these have been shown to form 24 different heterodimers. The 24  $\alpha\beta$  combinations are presented in Figure 1.10, the  $\alpha$  subunit has one  $\beta$  subunit partner with the exception of  $\alpha_6$ ,  $\alpha_4$  and  $\alpha_V$  which form heterodimers with several  $\beta$  subunits.



**Figure 1.10:** Integrin family members.

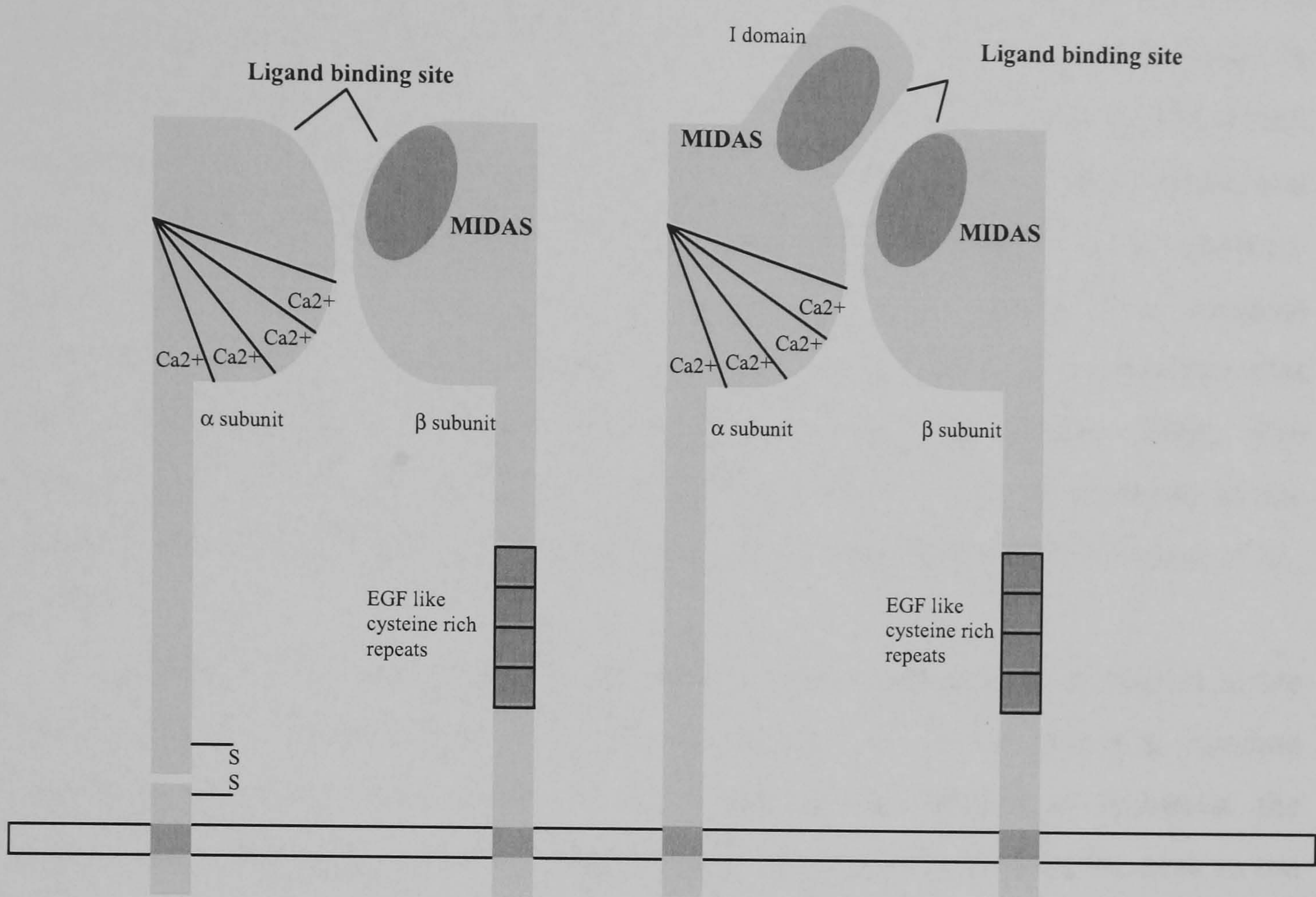
The  $\alpha$  and  $\beta$  subunits possess an extracellular domain and, by noncovalent interaction, these form the ligand binding site (Figure 1.11). The  $\alpha$  and  $\beta$  subunits also have a small hydrophobic transmembrane region and a cytoplasmic domain (Loftus *et al.*, 1990, Vogel *et al.*, 1990). The extracellular domain of the integrins plays a central role in cell-cell, cell-ECM recognition and adhesion. An inserted domain of  $\sim 200$  residues, termed the I domain is present in several  $\alpha$  subunits ( $\alpha_1, \alpha_2, \alpha_{10}, \alpha_{11}, \alpha_E, \alpha_L, \alpha_M, \alpha_X$  and  $\alpha_D$ ). The I domain and the  $\beta$  subunit of the integrins have a ligand binding activity via the metal-ion dependent adhesions site (MIDAS). Integrins are essential for cell migration, cell differentiation, proliferation and survival. These functions also confer important roles for the integrins in the development of an adult organism (Hynes 1992). Integrins function as receptors for ECM but also as cell surface counter receptor and as receptors for some soluble plasma proteins. The expression of



integrins is cell type dependent, for example  $\alpha$ II $\beta$ 3 is platelet-specific and recognises fibrinogen and fibronectin, similarly in leukocytes all  $\beta$ 2 integrins and  $\alpha$ E $\beta$ 7 are involved in cell-cell interactions. Epithelial cells express mainly integrins that bind to the ECM and include the followings,  $\alpha$ 1 $\beta$ 1,  $\alpha$ 2 $\beta$ 1,  $\alpha$ 3 $\beta$ 1,  $\alpha$ 5 $\beta$ 1,  $\alpha$ 6 $\beta$ 1,  $\alpha$ 9 $\beta$ 1,  $\alpha$ 6 $\beta$ 4,  $\alpha$ V $\beta$ 5 and  $\alpha$ V $\beta$ 6.  $\alpha$ 1 $\beta$ 1 and  $\alpha$ 2 $\beta$ 1 recognise principally collagen, in particular collagen I and collagen IV.  $\alpha$ 9 $\beta$ 1 is a receptor for tenascin C and osteopontin,  $\alpha$ 3 $\beta$ 1 and  $\alpha$ 6 $\beta$ 1 recognise laminin isoforms.  $\alpha$ 6 $\beta$ 4 is laminin receptor with a specificity for laminin-5 as well as a component of hemidesmosomes.  $\alpha$ V $\beta$ 1,  $\alpha$ V $\beta$ 6 and  $\alpha$ 5 $\beta$ 1 are vitronectin receptor which are expressed in response to injury (reviewed in Gilcrease, 2007). Alterations in integrin structure and expression are associated with cancer and metastasis (Ruoslahti and Giancotti, 1989). The loss of polarized integrin expression is a common feature of malignant cells, for instance integrin  $\alpha$ 6 $\beta$ 4, is mainly localised in the hemidesmosome of the basal surface of epithelial cells and sustains changes in expression in cancer. Here  $\alpha$ 6 $\beta$ 4 becomes more localized at the leading edge of the invading carcinoma cells (Rabinovitz *et al.*, 1999; Chao *et al.*, 1996; Rabinovitz and Mercurio, 1996; Mercurio *et al.*, 2001). Loss of polarisation of  $\alpha$ 6 subunit has also been shown in ovarian cancer (Bottini *et al.*, 1993) and in breast carcinoma an up regulation of  $\alpha$ 6 has been correlated with reduced survival (Friedrichs *et al.*, 1995) although other studies have found  $\alpha$ 6 $\beta$ 4 to be down regulated in aggressive breast cancer (Natali *et al.*, 1992; D'ardenne *et al.*, 1991). Integrin  $\alpha$ 2 $\beta$ 1 has been associated with malignant transformation and its down regulation is the most common type of alteration in integrin expression that has been associated with malignant transformation (Pignatelli and Stamp, 1995). Integrin  $\alpha$ 2 $\beta$ 1 has been shown to be down-regulated in several types of cancer for example colon, breast, pancreatic and lung carcinoma (reviewed in Gilcrease, 2007).

The integrins are important molecules in CRC and it has been suggested that  $\alpha$ 6 $\beta$ 1 and  $\alpha$ 6 $\beta$ 4 play a role in invasion and migration through the ECM via adhesion to laminin. It seems that although there is a tendency towards decreased integrin levels during tumour progression, numerous reports suggest a great heterogeneity of integrin expression which depends on the tissue from which the cancer derives and its molecular environment.





**Figure 1.11:** The two types of Integrin structures: The  $\alpha$  and  $\beta$  subunit of an integrin associate by noncovalent interaction to form a ligand binding site via the metal-ion dependent adhesion binding site (MIDAS) as shown on the left-hand side of the diagram. Several  $\alpha$  subunits possess an inserted domain (I domain) which comprise the MIDAS domain and form a ligand binding site when associated with the appropriate  $\beta$  subunit (right-hand side of the diagram). All  $\beta$  subunits possess four EGF-like cysteine rich repeats and  $\alpha$  subunits comprise  $\text{Ca}^{2+}$  binding sites.

## 1.5. Lectins and cancer

### 1.5.1 History of lectins

Lectins are sugar-binding proteins of non-immune origin that agglutinate cells or precipitate glycoconjugates and were first identified in the 19<sup>th</sup> century by Peter Herman Stillmark (1888) who described a protein extract from the seeds of the castor tree (*Ricinus communis*) which had the ability to agglutinate erythrocytes and this was termed a haemagglutinin (Reviewed in Franz, 1988). The first lectins to be described were plant lectins although Mitchell observed agglutination activity in snake venom before 1860 but this was described as an “antibody-like factor”. In the 1940’s the ability



of plant lectins to specifically agglutinate A, B and O erythrocytes was discovered and later, specificity towards other blood group substances and antigens such as T and Tn were also described. The lectin HPA was one of the first blood group A specific lectins of animal origin to be described (Uhlenbruck and Prokop, 1966; Hammarstrom and Kabat 1969) and HPA remains one of the most specific blood group A lectins described. Lectins have played a crucial role in determining the sugar composition of antigens associated with the ABO blood group system. Their use also led to the observation that cells possess glycans on their surfaces (reviewed in Morgan and Watkins, 2000). The discovery of the first mammalian lectin occurred in 1974 with the isolation of the galactose specific hepatic asialoglycoprotein receptor from rabbit liver (Stockert *et al.*, 1974).

In animals the main function of lectins are the recognition of molecules in the immune system. In invertebrates lectins are thought to act as primitive immune molecules directed against pathogens by virtue of their ability to recognise the extracellular oligosaccharides of microorganisms. In humans several lectins such as the mannan-binding lectin, ficolins and the membrane-bound macrophage mannose receptor mediate elimination of pathogens (reviewed in Kilpatrick, 2002). Several cell adhesion molecules with lectin activity, for example selectins and CD44 have been identified in humans and these are involved in cell recognition and cell trafficking. Galectins-1 have immuno-regulatory properties in animals (Levi *et al.*, 1983; Offner *et al.*, 1990; Santucci *et al.*, 2000) and cytokines play a role in immune-regulation via lectin-like interactions (Fukushima and Yamashita, 2001).

Several findings have led to an interest in lectins in cancer research, in particular the ability of some lectins to bind preferentially to malignant cells. The first observation concerning recognition of malignant cells by lectins was by Aub *et al.* (1965) who discovered that wheat germ agglutinin (WGA) recognised cells with a malignant phenotype. Since that time many lectins have been tested to evaluate their ability to bind differentially to normal and cancerous cells. The differential binding of lectins to normal and cancer cells has shown that alterations in glycosylation occur during malignant transformation. For instance in colon cancer the lectins from *Erythrina cristagalli* (ECA) and *Erythrina corallodendron* (EcorA) display altered binding as compared to normal colonic epithelia (Baldus *et al.*, 1996). Other lectins such as

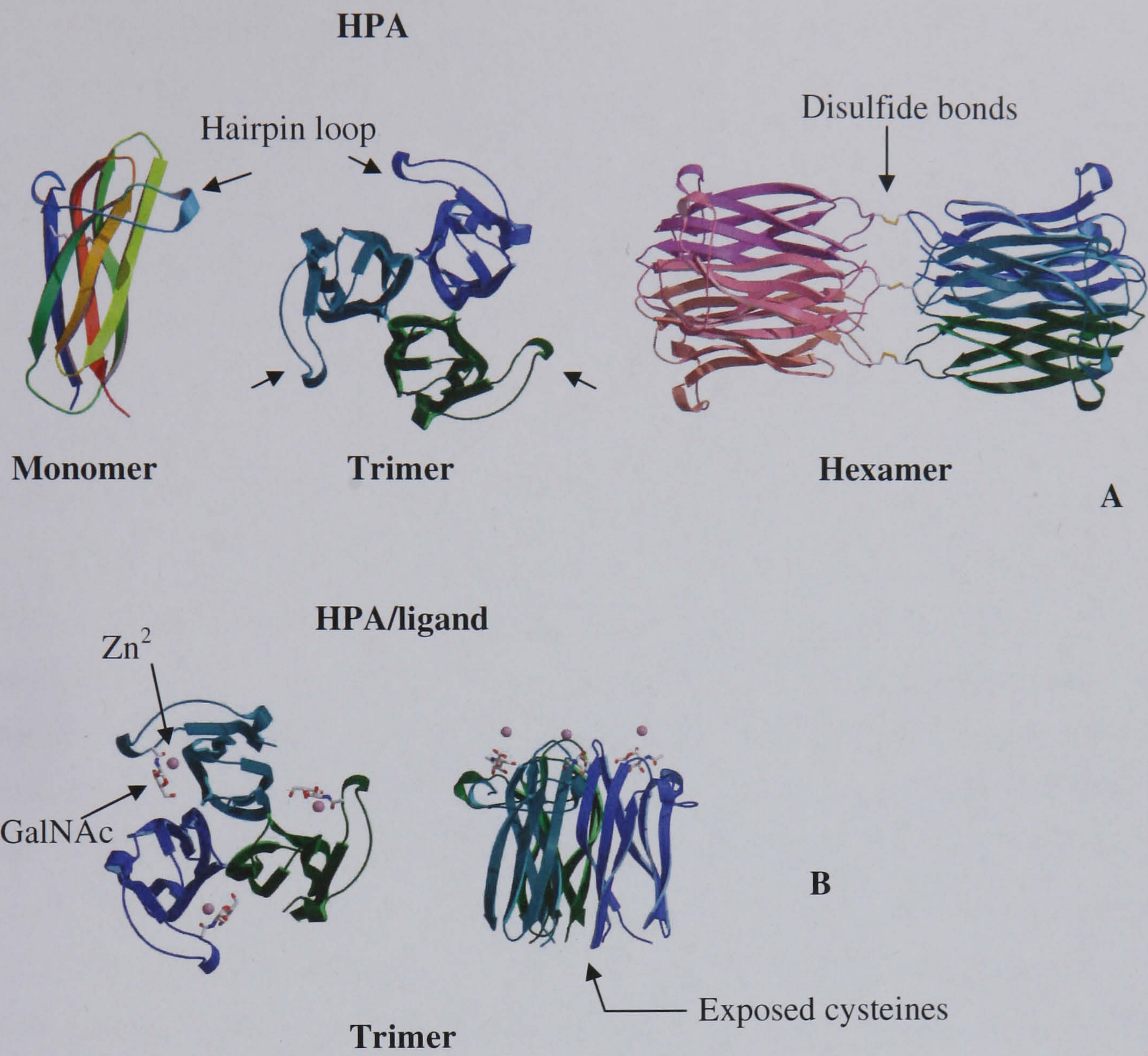


*Dolichos biflorus* agglutinin (DBA), *Ulex europaeus* agglutinin-1 (UEA-1) and WGA have shown differential binding between normal and cancerous colorectal cells (Iwakawa *et al.*, 1996; Sams *et al.*, 1990). The lectin HPA extracted from the Roman snail *Helix pomatia* has attracted interest as its binding has been associated with poor patient prognosis and the lectin has been suggested to be a useful tool for identifying aggressive cancer including colorectal cancer (Ikeda *et al.*, 1994; Schumacher *et al.*, 1994a, 1994b).

## 1.5.2 HPA

*Helix pomatia* agglutinin (HPA) is a lectin extracted from the albumen gland of the Roman snail. The lectin is thought to function in the innate immune system of the snails by conferring protection for the fertilized eggs. The lectin has been shown to be capable of aggregating bacterial pathogens and Herpes virus (Kholer *et al.*, 1973; Patchett *et al.*, 1991; Slifkin and Cumbie, 1989). HPA has a binding specificity for the Forssman antigen ( $\alpha$ GalNAc1-3GalNAc-R), the blood group A antigen ( $\alpha$ GalNAc1-3[ $\alpha$ Fuc1-2]Gal), Cad antigens (Baker *et al.*, 1983), the Tn antigen ( $\alpha$ GalNAc-Ser/Thr) (Springer, 1989), GalNAc, GlcNAc (Hammarstrom and Kabat, 1969) and also sialic acid (Saint-Guirons *et al.*, 2007). HPA is a 79kDa hexamer which contains 7% carbohydrate and is composed of identical monomers present in at least 12 glycoforms (Vretlab *et al.*, 1979), the lectin is a trimer of covalently-bound dimers (Hammarstrom and Kabat, 1969; Hammarstrom *et al.*, 1972 Vretlab *et al.*, 1979). Mass spectrometry data indicate that the HPA monomer, dimer and hexamer are 12.7 kDa, 24.6 kDa and 76 kDa respectively. The crystal structure of HPA has been recently solved by Sanchez *et al.* (2006). The HPA monomer is formed of six anti-parallel  $\beta$ -sheets connected by short loops, which form a  $\beta$ -sandwich structure stabilised by a disulphide bridge between cysteine 9 and 80. The dimers then associate together to form trimers. There is one GalNAc and  $Zn^{2+}$  binding site in each monomer at the opposite side of the dimerisation site, as shown on Figure 1.16. The sugar binding site is formed in the hairpin-like loops that connect the strands at the extremities of each monomer.





**Figure 1.12:** HPA structure. Panel A shows the structure of HPA alone obtained with a resolution of 2.5 Å. Panel B shows the HPA structure in the presence of GalNAc and Zinc molecules (From Sanchez *et al.*, 2006).



In addition to the ability of HPA to specifically agglutinate human blood-group A erythrocytes, it has been demonstrated that HPA binds preferentially to cancer cells with a metastatic phenotype. Leathem *et al.* (1983) first observed the preferential binding of HPA to breast cancer cells as compared to normal breast cells. These findings were later corroborated by Leathem and Brooks (1987a) who reported an association between the expression of HPA-binding glycans in breast cancer cells and metastatic phenotype. Numerous studies (Fenlon *et al.*, 1987, Fukutomi *et al.*, 1989, 1991; Alam *et al.*, 1990; Noguchi *et al.*, 1993 a, b; Thomas *et al.*, 1993) have confirmed the prognostic significance of the lectin including a large study carried out by Leathem and Brooks (1991) using 373 primary breast cancers with 24-year patient follow-up. They demonstrated that the HPA staining of primary tumours and the presence of lymph node metastases were significantly related although no correlation was made with tumour size, histological grade or age at diagnosis. The prognostic significance of HPA has also been demonstrated in other types of cancers such as oesophageal (Yoshida *et al.*, 1994; Takahashi *et al.*, 1994), gastric (Kakeji *et al.*, 1991, 1994), prostatic (Shiraishi *et al.*, 1992), lung (Kawai *et al.*, 1991) and colorectal (Ikeda *et al.*, 1994; Schumacher *et al.*, 1994 a,b) cancers. Ikeda *et al.* (1994) have studied the HPA staining pattern in 117 CRC tissue samples and showed a positive correlation between HPA-staining and the occurrence of lymph-node metastases. This was confirmed by Schumacher *et al.* (1994a) in a study of 130 CRC tissue samples. In this study they found that the binding of HPA to primary CRC cells was associated with poor prognosis, with similar outcome of patients with HPA positive tumours as those of Dukes stage C. The observations made on cell lines and tumours from SCID mice with respect to HPA binding corroborate the previous statement about the prognostic utility of the lectin. However it has been shown that the local environment modulates the expression of carbohydrates associated with metastasis (Schumacher *et al.*, 1994b; Schumacher and Adams, 1997).

Several groups have failed to establish a relationship between HPA binding and poor cancer prognosis (Galea *et al.*, 1991; Taylor *et al.*, 1991; Gusterson *et al.*, 1993). It has been argued that the reasons for the differences in the results were methodological, in particular that an indirect method of detection for histochemistry-based studies is preferred (Brooks *et al.*, 1996).



HPA has the ability to preferentially bind to metastatic cancer cells and may be useful as a prognostic marker in a range of solid tumours. Approximately 80% of metastatic tumours contain HPA binding epitopes (Brooks and Leathem 1998-1999) and an association between HPA binding to cancer cells and metastasis *in vivo* has been described (Schumacher *et al.*, 2005). Despite the evidence of its utility HPA does not yet have a role in clinical decision-making for any tumour type, and the glycoproteins of HPA binding cancer cells have similarly never been defined. It was initially hypothesized that HPA simply detects cancer cells over-expressing blood group A substance but this was discounted (Brooks and Leathem, 1995). Extraction of cancer-associated HPA-binding glycans revealed that they contain both N-acetylgalactosamine (GalNAc) as well as sialic acid and the predictive power of HPA was lost when cancer tissue sections were pretreated with neuraminidase (Fenlon *et al.*, 1987). A seemingly diverse range of glycan structures bind to HPA including GalNAc $\alpha$ 1-3GalNAc, GalNAc $\alpha$ -Ser/Thr (Tn antigen) and the Forssman antigen. With the exception of the Tn antigen (Chen *et al.*, 1995; Piller *et al.*, 1990), the relevance to cancer of these antigens and the proteins to which they are attached has not been established. However the HPA binding partners found on the cancer cells have been incompletely characterised. Their further characterisation (elucidation) will provide new insights into the understanding of the role of HPA binding partners in cancer progression.

The aim of this research project were to establish a robust, reproducible *in vitro* model of HPA binding to CRC cells and, using a proteomic approach, to identify the cell surface glycoproteins recognized by HPA in the metastatic CRC cell lines. The secondary aim of the project was to evaluate whether the proteins identified in the metastatic CRC cell lines were also present in tissue specimens collected from patients with CRC.



## 1.6. The work described in this thesis

The work described here is in four parts:

1) The development of an approach using confocal microscopy and fluorescently labelled HPA was undertaken in order to localise with precision the HPA-binding glycoproteins on the membrane of HT29. A new type of microtitre plate assay was developed to evaluate the binding of HPA on a large population of cell (>100000 cells) and hence provide statistically significant data. This new method aimed to evaluate the intensity of the HPA binding to the three cell lines HT29, SW620 and SW480 as well as providing insights into the sugar composition of the glycotope recognised by the lectin (Chapter 3).

2) A method to prepare a membrane-enriched fraction from HT29 was developed in order to concentrate the HPA-binding proteins, which are mainly localised in the membrane of HT29. The conditions of solubilisation and separation of these proteins were also optimised in order to obtain a robust 2-DE based method suitable to analyse the composition of the HPA-binding proteins as well as their changes of expression (Chapter 4).

3) The characterisation of the HPA binding glycoproteins of the metastatic cell line HT29 was undertaken using western blotting with HPA. The proteins were separated by 1-DE to obtain an overview of the HPA binding glycoproteins, which are localised in two main bands, GP130 and GP80. 2-DE was used to provide insights into the composition of the main HPA binding protein band GP130. A strategy using affinity chromatography and 2-DE was developed to concentrate and separate the HPA-binding proteins into single polypeptide in order to be identified by MS MALDI-TOF and hence understand the clinical significance of HPA (Chapter 5).

4) Finally a preliminary study was undertaken on human tissue samples in order to evaluate and validate our previous findings in an *in vivo* model of metastasis (Chapter 6).



## **CHAPTER TWO: Materials and methods**



## 2.1. Cell lines

The three cell lines, HT29, SW620 and SW480 have been extensively studied as models of metastasis in relation with HPA binding. In particular HT29 and SW480 have been studied *in vitro* and in animal model and proved to be consistently metastatic and non-metastatic respectively when implanted in SCID mice (Schumacher *et al.*, 1994a; Schumacher and Adams, 1997). An other interesting feature resides in the common origin of SW480 and SW620, which constitute a unique model for studying the later stage of CRC progression ( Zhao *et al.*, 2007; Liang *et al.*, 2007; Kim *et al.*, 2004). SW480 was derived from a Duke's B primary tumour of the colon and SW620 was derived from mesenteric lymph node metastases of the same patient six months later and has been shown to be more metastatic than SW480 *in vitro* (Hewitt *et al.*, 2000). Also shared marker chromosomes were seen (Gagos *et al.*, 1995) and confirmed a monoclonal origin of the two cell lines. The model used in our study is hence composed of a highly metastatic cell line (HT29), a moderately metastatic cell line (SW620) and a non-metastatic cell line (SW480). More over the phenotypic difference of SW620 and SW480 are likely to be associated with malignant progression and hence confirm the suitability of our model to study the process of metastasis. An interesting feature of this model is the clonal origin of SW480 and SW620 which confer important similarities and the phenotypic differences of SW620 and SW480 are likely to be associated with malignant progression and constitute a suitable model to study the process of metastasis. However this remains an *in vitro* model and it does not reflect the heterogeneity of an *in vivo* tumour and the importance of the cellular environment in the complex phenomenon of metastasis.

Human colorectal cancer cell lines HT29, SW480 and SW620 were kindly provided by Dr M Loizidou (Department of Surgery Royal Free and University College Medical School). The details of the three cell lines are given in Table 2.1. All the cell lines were grown in either 75 cm<sup>3</sup> or 175 cm<sup>3</sup> flasks (Falcon) maintained in Dulbecco's Modified Eagle's Medium, DMEM (Cambrex, Berkshire, UK) supplemented with 10% v/v foetal calf serum (Biosera) and 0.1% v/v gentamycin (Cambrex). All the cell lines were grown at 37°C with 5% CO<sub>2</sub> (Hera Cell incubator 240), maintained by changing the media every two days and passaging every 5 to 6 days using standard trypsinisation protocols as follows: Briefly, cells were grown to approximately 80% confluence and



washed once with phosphate buffered saline, PBS (Sigma). Cells were then incubated for 3 to 5 min at 37°C with 0.5 g/l trypsin, 0.2 g/l EDTA (Sigma) made up in PBS until they rounded up and were then removed by gentle agitation and pipetting. Cells were pelleted at 400 g for 5 min, resuspended in media and diluted at a ratio of 1:6 in a new flask.

Cells used for protein extraction were grown to near confluence, washed in PBS, mechanically detached in PBS (Sigma) using a sterile plastic cell scraper, centrifuged at 400 g in Centaur I MSE centrifuge and stored at -80°C until cell membrane preparation (Lehner *et al.*, 2003) and protein extraction were performed.

<b>Cell lines</b>	<b>ATCC number</b>	<b>Derived from</b>	<b>Dukes stage</b>	<b>Reference</b>
HT29	HTB-38	Moderately differentiated primary colon cancer. 44 year old female	N/A	Fogh , 1975
SW620	CCL-227	Lymph-node metastasis from colon adenocarcinoma. 51 year old male	C	Leibowitz <i>et al.</i> , 1976
SW480	CCL-228	Poorly differentiated primary colon cancer. Adult male	B	Leibowitz <i>et al.</i> , 1976

**Table 2.1:** The characteristics of the three cell lines HT29, SW620 and SW480 used in this study.

## 2.2. Confocal microscopy

Cells were grown in 6 well plates for 24 hours after passaging and fixed for 30 minutes in 10% formalin in PBS (pH 7.4). After washing away the formalin with PBS, the fixed cells were incubated for 20 min at 37° C with 1 mg/ml trypsin (type II from porcine pancreas; Sigma). Trypsinisation was used as an antigen retrieval technique. For fluorescent lectin staining, cells were fixed and washed as above, and blocked in 5% w/v BSA for 30 minutes. Fluorescein isothiocyanide (FITC) and tetramethylrhodamine isothiocyanide (TRITC) conjugated lectins encompassing a range of binding sugars were used to stain the cells (Table 2.2). Cells were incubated in the dark after washing 3



times for 5 min in PBS and each lectin was used at 10 $\mu$ g/ml for one hour in lectin buffer (0.05 M TBS, 1 mM CaCl<sub>2</sub>, 1 mM MgCl<sub>2</sub> pH 7.6). All lectins (except HPA which was obtained from Sigma), were purchased from Vector Laboratories, Burlingame, CA. The nuclei were counterstained using To-Pro-3 (Molecular Probes, Eugene, USA) at 1  $\mu$ M in PBS for 20 min after treatment with 100  $\mu$ g/ml Ribonuclease A (Sigma) for 20 min at 37°C in PBS. A DNase free Ribonuclease A stock solution was prepared by boiling for 10 min in 10 mM Sodium Acetate buffer (pH 5.2). As a negative control for lectin staining cells were incubated with buffer alone.

<b>Lectins</b>	<b>Abbreviations</b>	<b>Nominal binding sugars</b>	<b>Fluorophores</b>
Concanavalin A	Con A	$\alpha$ -mannose	FITC
Wheat germ agglutinin	WGA	GlcNAc	FITC
Soybean agglutinin	SBA	$\alpha/\beta$ GalNAc	FITC
<i>Ulex europeaus</i> agglutinin 1	UEA-1	$\alpha$ fucose	FITC
<i>Ricinus communis</i> agglutinin 1	RCA-1	Gal/GalNAc	FITC
<i>Helix pomatia</i> agglutinin	HPA	GalNAc/GlcNAc	TRITC
<i>Dolichos biflorus</i> agglutinin	DBA	$\alpha$ GalNAc	FITC
Peanut agglutinin	PNA	Galactose	FITC

**Table 2.2:** The lectins used in this study and their putative binding sugars.

## 2.3 Specificity of HPA binding assessed by confocal microscopy

HPA-positive HT29 cells were grown in 6 well plates, fixed and blocked with BSA as described in section 2.2. The specificity of the lectin binding to HT29 was evaluated by assessing the intensity of the HPA staining (10 $\mu$ g/ml) after pre-incubation for 30 min with increasing concentrations of GalNAc (25mM, 50 mM and 150 mM). D-mannose (50 mM) was included in the experiment as a negative control. The cells were incubated in the dark with the HPA/sugar mixture for 1h and counterstained with To-Pro-3. The intensity of the HPA staining was evaluated in 10 cells for each condition.



The average and standardised value for fluorescence intensity (representing the intensity of the HPA binding) were used to evaluate the specificity of interaction between HPA and its cellular binding partners in HT29.

## 2.4 Confocal microscopy image capture

Images were acquired by sequential scanning using a Leica TCS SP2 confocal system (Leica Microsystems, Milton Keynes, UK) and a X63 ceramic dipping objective at 1024x1024 format and scanning speed of 400 *Hz* with a line average of 2. A 488 nm (Intensity 25%) laser was used for the excitation of FITC, a 543 nm laser (Intensity 100%) was used for TRITC, and a 633 nm laser (Intensity 35%) was used for To-Pro-3. Emission was recorded over the bandwidth of 500-550 nm (FITC), 553-630 nm (TRITC) and 650-720 nm (To-Pro-3). These parameters are summarised in Table 2.3. the background was compensated by adjusting the Gain and Offset commands in order to minimise the noise. For 3D images, Z-stacks with increments of 1  $\mu\text{m}$  were scanned.

Fluorophore	Excitation	Emission	Laser
	Wavelength (nm)	Bandpass (nm)	Intensity (%)
FITC	488	500-550	25
TRITC	543	550-630	100
To-Pro-3	633	650-720	35

**Table 2.3:** Spectra parameters of fluorophores and the intensity of lasers used for confocal imaging.

## 2.5 Image 3D reconstructions and 2D models

3D reconstructions were produced using the Imaris<sup>®</sup> 4.0 software from Bitplane<sup>®</sup>-AG to combine the Z-stacks obtained by confocal microscopy. 2D models were made using the Imaris Surpass<sup>®</sup> tool from Imaris<sup>®</sup> 4.0 after baseline correction.



## **2.6 Data analysis of images from confocal microscope**

Relative quantification of lectin binding was performed using the Confocal Analysis Package (Leica Microsystems, Milton Keynes, UK). The method developed previously by Kerrigan and Hall, (2005) was used as follows. For repeatable and unbiased analysis, two linear profiles through the centre of each cell were drawn avoiding regions of cell-cell contact, where possible. The fluorescence along each line was determined using the Leica software. The mean data acquired every 0.0125  $\mu\text{m}$  for 22 cells (per cell line) was calculated using Microsoft Excel<sup>®</sup>. The background fluorescence signal was determined by drawing and averaging at least 10 individual linear profiles from areas with no cells and was used to subtract from each value calculated from the linear cell profile.

The data was expressed as mean maximal fluorescence  $\pm$  standard error of the mean using SigmaPlot<sup>®</sup> (Jandel Scientific, Ekrath, Germany).

## **2.7 HPA binding assay using microtitre plate format**

HT29, SW620 and SW480 cells were harvested and pelleted by centrifugation at 400g as before and resuspended in 1ml PBS. 100  $\mu\text{l}$  of the cell solution was mixed with 100  $\mu\text{l}$  of 0.2% Trypan blue (Cambrex) and a cell count was performed using a haemocytometer and a light microscope. 100 000 cells were seeded into each well of a 96 well microplate (Falcon) and allowed to settle for 12 hours. The lectin staining procedure is similar to the one described earlier. Briefly, cells were fixed for 30 min in 10 % formalin prepared in PBS. Cells were washed 3 times each for 5 min in PBS to remove the formalin solution and incubated for 20 min at 37°C with 1mg/ml trypsin. The cells were washed again and incubated for 30 min in 5% w/v BSA as a blocking step. The cells were then incubated with 10  $\mu\text{g}/\text{ml}$  biotinylated-HPA for 2 h and 4  $\mu\text{g}/\text{ml}$  FITC-conjugated streptavidin for 1 h. As a negative control cells were incubated only with FITC-conjugated streptavidin. Fluorescence readings were acquired using a fluorescent microplate reader (Fluostar Optima, BMG Labtech). The fluorescence emission was read from the bottom of each well at 544 nm after excitation at 485 nm. The fluorescence intensity was calculated from an average of 81 readings taken from



each well. A negative control without lectin was included in each experiment and the resulting average fluorescence was used to normalise the data. The experiments were performed in six replicates. The experimental layout is presented in the Figure 2.1.

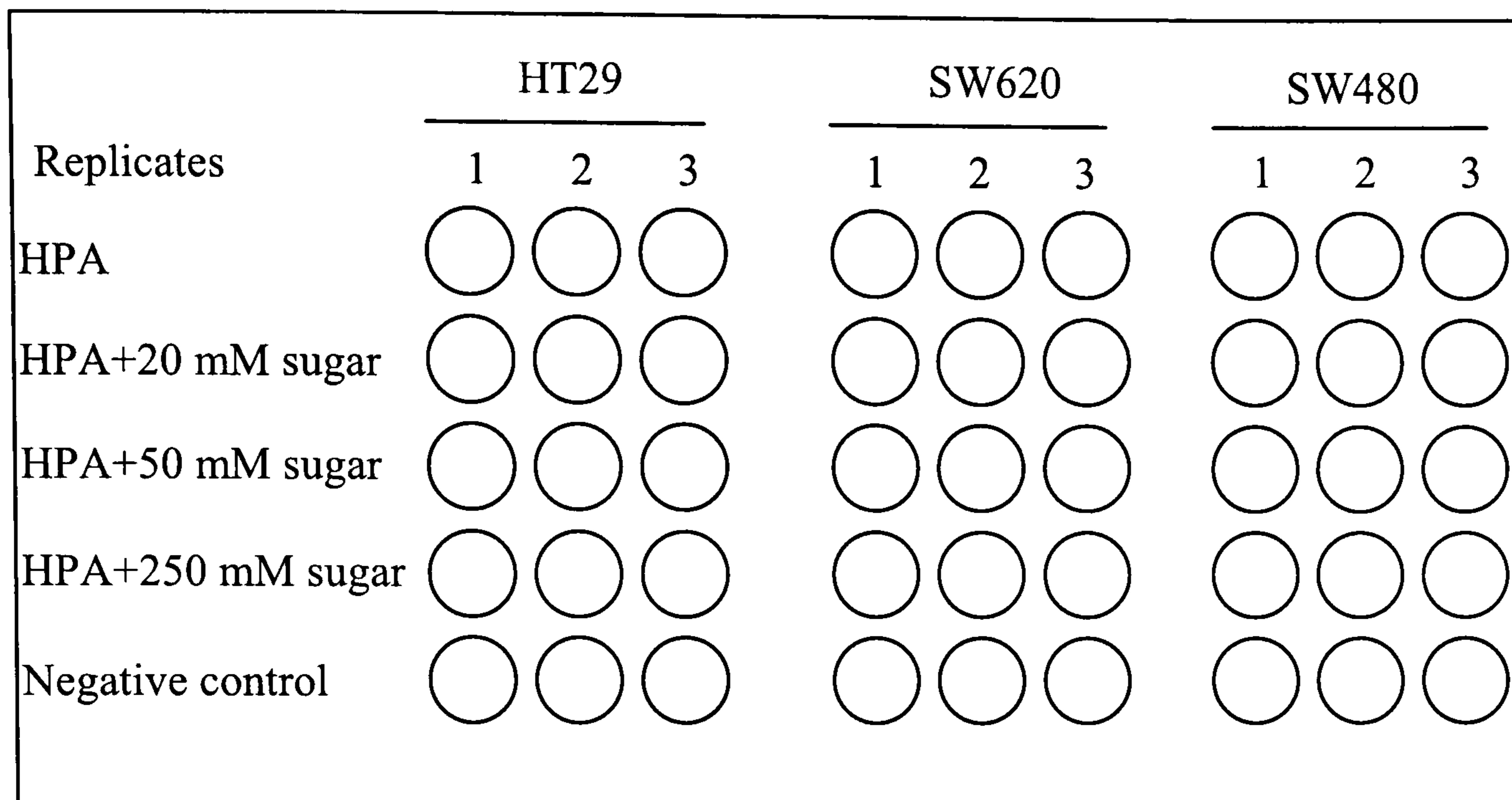
	Replicates					
	1	2	3	4	5	6
HT29 + HPA + Streptavidin	○	○	○	○	○	○
HT29 + Streptavidin (negative control)	○	○	○	○	○	○
SW620 + HPA + Streptavidin	○	○	○	○	○	○
SW620 + Streptavidin (negative control)	○	○	○	○	○	○
SW480 + HPA + Streptavidin	○	○	○	○	○	○
SW480 + Streptavidin (negative control)	○	○	○	○	○	○

**Figure 2.1:** HPA binding to HT29, SW620 and SW480. Experiment layout

## 2.8 Assessing the specificity of HPA binding using the microtitre plate assay

For each of the three cell lines used, the specificity of HPA binding was evaluated using four sugars: GlcNAc, GalNAc, SA and mannose. One separate 96-well microplate was used for each sugar tested. As shown on the experimental layout in Figure 2.2, the cells were incubated with HPA alone or HPA preincubated with 20 mM, 50 mM and 250 mM of appropriate sugar as before and finally with streptavidin alone as a negative control. The cells were incubated with the HPA/sugar mixture for 2 hours followed by streptavidin-FITC (Figure 2.2). A negative control (without lectin) was used to normalise the data.





**Figure 2.2:** Experimental layout for inhibition experiments of HPA binding to HT29, SW620 and SW480.

## 2.9 Cell lysate preparation

Colorectal cancer cells were grown to confluence in 175 cm<sup>3</sup> tissue culture flasks (Falcon) and harvested using a sterile cell scraper (Falcon) into 20 ml PBS as described in section 2.1. Cells in suspension were centrifuged for 5 min at 400 g in a bench top Centaur I MSE centrifuge before being stored as a dry cell pellet at -80°C.

Cell pellets were resuspended and proteins solubilised in 3 ml of lysis solution U7M/T2 containing urea 7M, CHAPS 4% w/v, DTT 1% w/v, ampholytes 2% v/v and thiourea 2M (added after protein assay), by sonication using an ultrasonic probe (MS73 Status 200) at 40% power, 5 times for 10 seconds with intermittent cooling on ice cold water. Cellular debris were removed by centrifuging the cell lysate in a Sorvall Super T21 centrifuge with SL50T rotor for 30 min at 11,000 g before the protein concentration was determined.

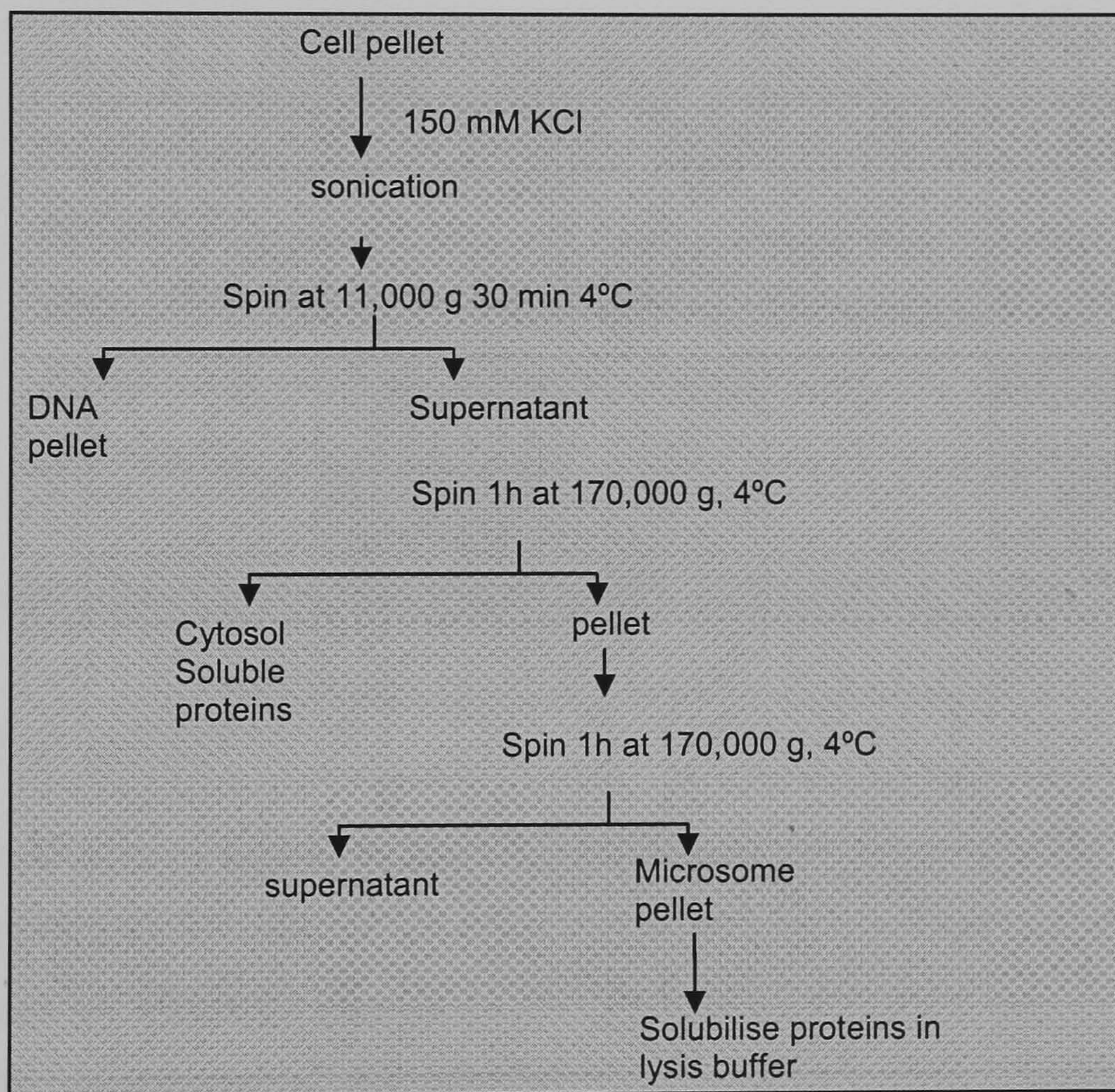
## 2.10 Preparation of a membrane-enriched fraction

Microsomes were prepared from frozen cell pellets by ultracentrifugation using a modified version of the procedure described by Lehner *et al.* (2003). The preparation process is summarised in Figure 2.3.

Briefly, the colorectal cancer cell lines were disrupted using an ultrasonic probe MS73 Status 200 at 40% power, 5 times for 10 seconds in 3 ml of 150 mM KCl. The homogenate was centrifuged at 11,000 g for 30 min at 4°C to pellet the cell debris and



nucleic acids. The supernatant was subsequently centrifuged for 1h at 170,000 g at 4°C (Sorvall Discovery 90SE ultracentrifuge with rotor T-865). At this stage soluble proteins in the supernatant were separated from the pellet containing microsomes. The pellet was re-suspended in 150 mM KCl, and sonicated as before and centrifuged for a further 1h at 170,000 g at 4°C to pellet the microsomes. The membrane-enriched proteins were then solubilised using 1 ml of the lysis buffer U7M/T2 described in section 2.9, prior to protein assay. Several preparations were performed to evaluate the reproducibility of the method and its efficiency to concentrate the HPA-binding protein in the membrane-enriched fraction. No characterisation of the purity of the membrane preparation was carried out and residual soluble proteins may be present in the preparation.



**Figure 2.3:** Schematic diagram showing the preparation of a membrane enriched fraction. Cells were disrupted by sonication in 150 mM KCl. The cell lysate was then centrifuged at 11,000 g in order to remove the cell debris and the nucleic acids from the cellular solution. The supernatant was centrifuged at high speed (170,000 g) in order to pellet the cellular membranes prior to protein extraction.



## 2.11 Protein solubilisation

A total of four solubilisation buffers were investigated for their suitability as 2-DE solubilisation buffers (Görg *et al.*, 1999). The compositions of the complete 2-DE buffers are shown in Table 2.4.

<b>Buffer acronym</b>	<b>Composition</b>
U	Urea 9M, CHAPS 4%, DTT 1%, Ampholytes 1%
T	Urea 7M, Thiourea 2M, CHAPS 4% w/v, DTT 1% w/v, Ampholytes 1% v/v
U7M/T	Urea 7M, Thiourea 2M, CHAPS 4% w/v, DTT 1% w/v, Ampholytes 1% v/v (Thiourea added after protein assay was performed)
U7M/T2	Urea 7M, Thiourea 2M, CHAPS 4% w/v, DTT 1% w/v, Ampholytes 2% v/v (Thiourea added after protein assay was performed)

**Table 2.4:** The solubilisation buffers used in this study and their composition.

## 2.12 Protein assay

Three protein assay kits were tested for compatibility with the chemicals used to make the solubilisation buffers; (i) Bradford reagent (Sigma) based on the original method developed by Bradford (Bradford, 1976), (ii) DC protein assay kit (Bio-Rad) and (iii) RCDC protein assay kit (Bio-Rad), both based on the original method developed by Lowry (Lowry *et al.*, 1951).

Bovine serum albumin, BSA (Sigma) was used as the protein for the generation of standard calibration curves. BSA solutions were prepared in lysis buffers 1 to 4 (Table 2.5) at a concentration ranging from 0.25 mg/ml to 2 mg/ml and were assayed using the Bradford, DC and RCDC protein assay according to the manufacturer's recommendations. The protocols are shown in Appendix 2.



The concentration of protein in the cell lysates, membrane protein preparations and tissue sample lysates were determined using the Bradford reagent (Sigma) which was found to be the most suitable system to allow compatibility with the buffers used.

<b>Buffers</b>	<b>Composition</b>
1	Urea 7M, CHAPS 4%
2	Urea 7M, CHAPS 4%, DTT 1%
3	Urea 7M, CHAPS 4%, Ampholytes 2%
4	Urea 7M, CHAPS 4%, DTT 1%, Ampholytes 2%

**Table 2.5:** Buffer composition used for protein assay

## 2.13 1-D electrophoresis (1-DE)

Whole cell lysate or cell membrane proteins prepared and assayed as described earlier, were separated by SDS-PAGE according to the method developed by Laemmli (1975). Briefly, a suitable volume of cell lysate or enriched cell membrane proteins to obtain between 5 and 20  $\mu\text{g}$  of protein in a maximal volume of 10  $\mu\text{l}$  were mixed with an equal volume of two times Laemmli reducing buffer (125 mM Tris-HCl pH 6.8, 5% glycerol (v/v), 4% SDS (w/v), trace of bromophenol blue, 10%  $\beta$ mercaptoethanol). The samples were boiled for 5 min to achieve complete denaturation of the proteins and were allowed to cool to room temperature before being loaded on the SDS-PAGE gel as detailed below.

## 2.14 SDS-PAGE

Protein samples along with protein molecular weight markers (Bio-Rad or GE Healthcare) were loaded on either 10 % or 12% SDS PAGE gels and electrophoretically separated in running buffer (25 mM Tris, 192 mM Glycine, 0.1% SDS pH 8.3) at 150 V for 1h to 1h15min in the Mini Protean 3 gel system (Bio-Rad). A stacking gel (4%) was poured over the resolving gel in order to ensure better separation by concentrating the



proteins into a thin zone (the stack) prior to separation in the separating gel. All the buffers and recipes are summarised in Appendix 3.

## **2.15 Data analysis**

Digital images of 1DE separations and Western blots were obtained using transillumination with white light and a UVP Biochemi Image Capture System. The Labworks software (UVP) enabled the analysis of electrophoretically separated proteins by calibration of protein gels and Western blot membranes using molecular weight marker proteins. A lane profile and intensity histogram was obtained with relative intensity values for each of the bands detected.

## **2.16 2-D electrophoresis (2-DE): Sample preparation**

The appropriate volume of cell lysate or membrane protein preparation equivalent to between 50 and 100  $\mu\text{g}$  of protein was mixed with rehydration buffer (T buffer) containing 7M Urea, 2M Thiourea, CHAPS 4% w/v, DTT 1% w/v, Ampholytes 2% v/v, to a final volume of 125  $\mu\text{l}$  prior to loading onto an IPG strip followed by in-gel rehydration. DTT was added fresh to the T buffer prior to use in order prevent denaturation of the DTT .

## **2.17 In-gel rehydration**

The protein samples were loaded onto 7 cm Immobiline Drystrips (GE Healthcare) of pH3-10, pH5-8, and pH4-7 depending on the experiment. The protocol used for rehydration was identical regardless of the pH gradient used. Briefly, 125  $\mu\text{l}$  of sample containing 50 to 100  $\mu\text{g}$  of proteins prepared in T buffer was pipetted into a groove of the reswelling tray. The dry IPG strip was inserted into the groove, gel face down, to cover the sample previously loaded. Silicone oil was layered on top of the strip to prevent the strip drying out and rehydration was allowed to proceed overnight at 20°C.



## 2.18 Isoelectric focussing

Rehydrated IPG strips were rinsed in distilled water, placed on the isoelectrofocussing unit (Multiphor, GE Healthcare), gel facing up with wet filter paper between the gel and the electrodes to trap the fast moving ions that may cause an increase in current intensity and unwanted heating.

The isoelectrofocussing was performed under the conditions summarised in Table 2.6. The two parts of the focussing proceeds at low voltage (300 V and 600 V) to enable the removal of fast moving ions, being eventually trapped in the filter paper placed at the electrode. In the second part of the focussing step at 3500 V, isoelectrofocussing of the protein takes place. The charged proteins move toward the anode/cathode depending on their charge. They eventually come to rest at the pH zone equal to their pKa.

---

Gel length: 7 cm

Temperature: 20°C

Current max: 2 mA

Voltage max: 3500 V

<b>IEF</b>	<b>Volts</b>	<b>Time</b>
Initial steps	300 V	30 min
	600 V	30 min
Focussing step	3500 V	2h 30 min
Total Volt hours	9200 Vhour	

**Table 2.6:** The isoelectrofocussing of the proteins loaded onto a 7 cm IPG strips are shown. The IEF consists of three steps. The two first steps at 300V and 600V remove ions. At 3500V the charged proteins move within the pH gradient of the IPG strip until they reach a zone of pH corresponding to their pKa where the proteins precipitate.



## **2.19 Equilibration of IPG strip**

Focussed strips were equilibrated for 15 min in 50 mM Tris-HCl, pH 8.8, 6 M urea, 30% v/v glycerol, 2% w/v sodium dodecyl sulphate (SDS), 1% w/v DTT and then for 15 min in 50 mM Tris-HCl, pH 8.8, 6M urea, 30% glycerol, 2% w/v SDS, 2.5% w/v iodoacetamide. These steps are included with the aim of reducing the disulfide bonds and unfolding the proteins in order to facilitate their exit from the IPG strip as well as to reduce electroendosmotic effects which may result in poor transfer of protein from IPG strip to SDS-PAGE gel.

## **2.20 Second dimension: SDS-PAGE**

Focussed and equilibrated strips were gently applied on top of a vertical 1mm thick, 8x7 cm, 10% SDS-PAGE gel prepared according to the procedure described earlier. Molecular weight standards were loaded on a small piece of filter paper inserted next to the low pH side of the IPG strip. An agarose containing solution (0.5% w/v agarose, 25 mM Tris, 192 mM glycine, 0.1% SDS, trace of bromophenol blue, pH 8.3) was poured over the strip to seal the system in order to prevent the IPG strip from moving during protein separation. Gels were run for 1h15 min at 150 V prior to protein staining or Western blotting.

## **2.21 Protein staining using Coomassie Brilliant Blue**

The solutions used to fix, stain and destain the protein gels, were freshly prepared and are shown in Table 2.7. All three steps of the staining protocol were performed under gentle agitation on a rocking tray. First the gels were fixed for 30 min in methanol/acetic acid; the gels were then stained in Coomassie Brilliant Blue solution for one hour and destained for two hours. Complete destaining was achieved by leaving gels overnight in water until the protein bands were stained deep blue against a transparent background. Gel images were then captured using the UVP Bioimaging Autochemi System as before.



<b>Solutions</b>	<b>Composition</b>	<b>Time</b>
Fixing solution	50% methanol, 10% Acetic acid, dH <sub>2</sub> O	30 min
Staining solution	10% acetic acid, 0.025% Coomassie Brilliant Blue, dH <sub>2</sub> O	1h
Destaining solution	10% Acetic acid, dH <sub>2</sub> O	2h

**Table 2.7:** Coomassie blue staining

The solutions to fix, stain and destain, the proteins separated on SDS-PAGE, are reported in this table along with the time used for each step.

## 2.22 Fluorescent staining of proteins separated by 2-DE

2-DE protein minigels were stained with the fluorescent dye, Sypro Ruby (Molecular Probes) according to the manufacturers recommendations. Sypro Ruby enables the detection of protein at a concentration as low as 0.25 ng hence increasing the sensitivity of detection (up to 10 times more sensitive than Coomassie Brilliant Blue). Briefly, gels were fixed in methanol/acetic acid solution, stained overnight with the undiluted dye and washed with a methanol/acetic acid containing solution. The complete composition of the solutions and protocol are given in Table 2.8.

The Sypro Ruby stained gels were visualised using a laser scanner (Typhoon 8610, GE Healthcare) at an excitation wavelength of 450 nm and emission wavelength of 610 nm.

<b>Solutions</b>	<b>Composition</b>	<b>Time</b>
Fixing solution	50% methanol, 7% Acetic acid, dH <sub>2</sub> O	2x 30 min
Staining solution	Undiluted Sypro Ruby gel stain	Overnight
Destaining solution	10% Methanol, 7% Acetic acid, dH <sub>2</sub> O	30 min

**Table 2.8:** The composition of the solutions and the duration of each step of the fluorescent staining with Sypro Ruby, are shown.



## **2.23 Data analysis from 2-DE experiments**

The 2-DE gel images were obtained using either, for Sypro Ruby stained gels, a dual-laser Typhoon scanner (GE Healthcare) or, for Western blot membranes, transillumination with white light and UVP Biochemi Image Capture System. Images in .TIFF format were imported into two 2D analysis softwares: Progenesis SameSpots (Nonlinear Dynamics) and Image Master 2D Platinum (GE Healthcare) and processed by background subtraction, spot detection, landmarking, overlay analysis and 3D reconstruction of the proteins separated by 2-DE.

## **2.24 Protein transfer by Western blotting**

The electrophoretically (1-DE or 2-DE) separated proteins were transferred onto nitrocellulose membranes (GE Healthcare) by wet transfer using a Mini Trans Blot transfer cell (Bio-Rad). The proteins were electrophoretically transferred from the gel to the nitrocellulose membrane at 100 V for 1h 30 min in transfer buffer (25 mM Tris, 192 mM glycine and 20% v/v methanol pH 8.3). The system was cooled throughout the experiment.

## **2.25 Ponceau S staining of Western blots**

The membranes were stained with the reversible protein dye, Ponceau S (Sigma) to check the efficiency of the transfer and also to localise the lanes and molecular weight markers prior to immunodetection. 0.1% w/v Ponceau S in 5% acetic acid was used for this purpose. The stain was subsequently removed in water.

## **2.26 Immuno/Lectin detection on Western blots**

Prior to immunodetection, the nitrocellulose membrane was blocked with 2% w/v BSA in Tris buffered saline/Tween 0.05% v/v, pH 7.6 (TBS/T) overnight at 4°C and washed 3 times for five minutes in TBS/T. Lectin/antibody steps and washing steps were carried out in TBS/T. The incubation steps with antibodies, lectin or streptavidin were all performed at room temperature under gentle agitation.



## **2.27 Lectin blotting with HPA**

After blocking, the blots were incubated for 2 hours with 5µg/ml biotinylated HPA (Sigma). The protein blots were subsequently incubated with 2µg/ml HRP-conjugated streptavidin (Pierce, UK) for 1h. The blots were washed 3 times for 5 min in TBS/T between each step. The detection was performed by addition of the chromogenic substrate diaminobenzidine (DAB) prepared in TBS/ H<sub>2</sub>O<sub>2</sub> (6mg of DAB prepared in 9ml of TBS and 60 µl of 30 volume H<sub>2</sub>O<sub>2</sub>). The reaction was stopped by addition of excess water.

## **2.28 Probing Western blots with anti-blood group A antibody**

After blocking, the proteins were probed with a murine monoclonal antibody directed against blood group A antigen (anti-blood A) from Ortho-Diagnostics, Johnson and Johnson, kindly provided by Dr P Greenwell (University of Westminster) using the anti-blood A antibody at a dilution of 1/5 for 2 hours. After washing 3 times for 5 min in TBS/T the membrane was incubated for one hour with a goat anti-mouse antibody (Sigma) conjugated with HRP, prepared at a dilution of 1/10000 in TBS/T. The detection was performed using the chemiluminescent reagent, Super Signal West Dura (Pierce), according to manufacturer's instructions. The detection was performed by exposure of an X-ray film for 10 sec to 1 min.



## **2.29 Probing Western blots with anti-integrin $\alpha 6$ (anti-CD49f) antibody**

Proteins separated by SDS-PAGE and transferred to nitrocellulose were blocked in BSA as before and probed with a rat monoclonal biotinylated anti-integrin  $\alpha 6$  antibody (Anti-CD49f, AbD Serotec) prepared in TBS/T to a concentration of 4  $\mu\text{g/ml}$ . The membrane was incubated with the antibody anti-CD49f for 2 hours and washed 3 times for 5 min followed by one hour incubation with 2  $\mu\text{g/ml}$  streptavidin-HRP. The detection was performed using the chemiluminescent reagent Super Signal West Dura (Pierce) as before. Visualisation of the results was achieved by exposure of a X-ray film to the blot for 10 sec to 1 min.

## **2.30 Inhibition of HPA binding to HT29 proteins**

HT29 membrane proteins separated by 1-DE or 2-DE and transferred to nitrocellulose membranes were blocked with BSA and incubated for 2 h with 5  $\mu\text{g/ml}$  biotinylated HPA in TBS/T preincubated for 30 min with either 250 mM GlcNAc, GalNAc or SA. The membrane was washed in TBS/T and incubated with 2  $\mu\text{g/ml}$  of HRP-conjugated streptavidin before detection with DAB as before.

## **2.31 HPA affinity chromatography**

A 1ml lectin affinity chromatography column (0.7 cm diameter x 2.5 cm length) was prepared and used to purify the HPA binding membrane proteins from HT29, SW480 and SW620. 1 mg HPA (Sigma) was coupled to a HiTrap NHS-activated Sepharose prepacked column (GE Healthcare) according to the manufacturer's recommendations. The packing matrix contains activated N-hydroxysuccinimide (NHS) functional groups capable of forming covalent bonds with the primary amino groups of proteins. During the preparation of the column the flow rate was maintained below 1ml/min. The column was first washed with 6ml ice cold 1mM HCl. 1 ml of 1mg/ml HPA was prepared in standard coupling buffer (0.2M  $\text{NaHCO}_3$ , 0.5M NaCl, pH8.3), injected onto the column and allowed to interact at room temperature to enable the formation of covalent bonds between the matrix and the lectin. The unbound ligand was



washed away and unbound active NHS groups were inactivated with 18 ml of 0.5M ethanolamine, 0.5M NaCl, pH8.3 and 18 ml of 0.1M acetate, 0.5M NaCl, pH 4.

The column was fitted to an AKTA prime semi-automated system with in-line UV detector and fraction collector (GE Healthcare). The column was washed and equilibrated with 10 column volumes of binding buffer (20 mM Tris, 0.5M NaCl, pH 7.4) at a flow rate of 0.5 ml/min. Cell pellets from HT29, SW480, and SW620 were prepared in the binding buffer, solubilised using an ultrasonic probe as before and centrifuged at 11,000g. 0.5mg of each cell line protein preparation was injected onto the column using a 500µl injection loop. The column was washed with 15 column volumes of binding buffer to remove any unbound proteins. The bound proteins were eluted with a gradient of binding buffer supplemented with 1M GlcNAc. GlcNAc has been shown previously to bind to HPA (Vretblad *et al.*, 1979). Finally the column was extensively washed and re-equilibrated with 10 column volumes of binding buffer. Fractions of 1ml were collected and the eluted material pooled, dialysed against distilled water overnight at 4°C and freeze dried (Super Modulyo D, Thermo Electron). The bound fractions were resuspended in buffer before separation by 1-DE or 2-DE.

## **2.32 Improved method for the purification of the HPA binding partners from HT29**

The method described above was further refined using proteins from the cell line HT29. A 1 ml HiTrap NHS-activated Sepharose column (GE Healthcare) was used to prepare the HPA affinity column as before according to the manufacturer's recommendations with the exception that 5 mg of lectin was coupled to the NHS-Sepharose matrix rather than 1mg. The column was first washed and equilibrated with 10 column volumes of lectin buffer containing 0.1% w/v CHAPS. 0.5 mg of membrane proteins were loaded onto the column in the lectin CHAPS buffer using a 500 µl injection loop as before, the column was washed with 5 column volumes of lectin buffer containing 0.1% CHAPS to remove unbound proteins and bound proteins were eluted with freshly prepared lectin buffer containing 0.1% CHAPS and 250mM GlcNAc in a step gradient of 10 column volumes. The flow rate was maintained at 0.5 ml/min throughout the experiment.



Protein peaks detected at 280 nm were integrated using the Prime Evaluation software (GE Healthcare) and this enabled evaluation of the relative amount of unbound and bound proteins.

### **2.33 Spot picking from 2-DE separated HPA binding proteins**

The HPA binding proteins of HT29 above were dialysed, freeze dried and resuspended in T buffer prior to 2-DE. Approximately 100µg of HPA binding proteins were loaded onto an IPG strip of pH 3-10, the proteins were focussed, separated on a 10% SDS-PAGE and stained with Coomassie Brilliant Blue as before. Proteins spots were excised from the gel using a clean scalpel and stored at -80°C in 0.5 ml sterile Eppendorf tubes.

### **2.34 Protein identification by MALDI-TOF Mass spectrometry**

Protein identification was carried out by commercial arrangement with Dr Jerry Thomas at the University of York. A MALDI-TOF/TOF 4700 analyser from Applied Biosystems was used. The 2-DE protein spots stained with Coomassie Brilliant Blue excised from the gel were provided to the Thomas laboratory. The proteins were reduced using DTT and S-carbamidomethylation was performed using iodoacetamide prior to the tryptic digest. The gels pieces were washed three times in 50% v/v acetonitrile / 25mM ammonium bicarbonate and air dried before rehydration in 10 µl of 0.02µg/µl sequencing-grade, modified porcine trypsin (Promega). Digestion was performed over night at 37°C. A 0.5 µl aliquot of each tryptic digest and 0.5 µl of a solution of 4-hydroxy- $\alpha$ -cyano-cinnamic acid (Sigma, Poole, UK) in 50% aqueous (v/v) acetonitrile containing 0.1% TFA (v/v), was applied to the MALDI target plate. Mass spectra were obtained in reflection mode with an accelerating voltage of 20kV. The peptide mass fingerprint generated was compared to the masses of all theoretical tryptic peptides generated *in silico* by the MASCOT search program. MASCOT produces a statistically based Mowse score that evaluates the significance of the matches. A



significant event would be expected to occur at random with a frequency less than 5% (Pappin *et al.*, 1993). The Mowse score generated by the search depends on parameters such as the sequence database used, the protein studied and hence there is no absolute threshold value for MASCOT (Perkins *et al.*, 1999). Collision induced dissociation (CID) MS/MS was also performed to corroborate the significant matches from the MALDI/MS. The results are shown as mass spectra as well as a list of peptides detected and the sequence coverage.

## **2.35 Selection of colorectal tissue samples for evaluation of HPA binding glycoproteins**

Tissue samples were used to determine whether the differential expression of HPA binding proteins observed between the metastatic cell line HT29 and the non-metastatic cell line are also found in human colorectal cancer.

The human colorectal tissue samples were collected following informed consent and subject to local ethics committee approval at the University Hospital of Martinique and were provided by Dr Smith-Ravin (University of Guadeloupe). We selected eight samples which were categorised into two cohorts. Four samples (1-4) showed no regional lymph node involvement at the time of tumour resection and were described as the “early stage” group termed C1. Four other tissue samples (5-8) with more advanced tumour stage and lymph node involvement were selected and were described as the “advanced” group termed C2. For each patient a piece of healthy tissue was also surgically removed. The healthy tissue from patients 1 to 4 and 5 to 8 were used to prepare the healthy counterpart of C1 and C2 termed N1 and N2. The characteristics of the samples are shown in Table 2.9. The samples were evaluated by cutting frozen sections and H+E staining prior to use.

Samples were pooled into two groups “early stage” and “advanced” cancers in order to facilitate the analysis and the correlation with the findings made *in vitro* in the metastatic and non-metastatic cell lines. The use of pooled samples of human tissues does not provide information on the HPA binding glycoproteins of a given individual, rather, it provides a quick overview of HPA binding to the glycoproteins in an entire pool. Proteomic studies based on pooling samples are a relatively quick method for identifying fold-changes in protein levels in individuals from different clinical groups.



Such changes may be subject to bias as, in the extreme situation; the altered protein levels may simply represent a change in protein level from a single individual of the group. It is widely accepted that proteomic studies with pooled samples require further verification and validation with proteins from the individuals in the group run separately.

<b>Patients</b>	<b>Age</b>	<b>Gender</b>	<b>TNM status</b>
1	44	Female	T2N0
2	62	Female	T2N0
3	54	Female	T2N0
4	70	Male	T3N0
5	64	Female	T4N1
6	70	Male	T4N2
7	49	Female	T3N2
8	57	Female	T3N2

**Table 2.9:** Characteristics of the patient samples used in this study.

## 2.36 Protein preparation

2 mm<sup>2</sup> tissue pieces from each patient were individually disrupted in U7M/T2 buffer using a “rotor stator type” hand-held homogeniser. The protein mixture was then centrifuged at 11,000g for 10 min in order to remove cellular debris and nucleic acids and the protein solution was eventually assayed.

100 µg of the protein mixture extracted from cancerous and healthy tissue of patient 1 to 4 were pooled to prepare respectively the “early stage” cancer protein mixture (C1) and its healthy counterpart (N1). The “advanced” cancer protein mixture (C2) and its healthy counterpart (N2) were prepared identically.



## **2.37 Protein separation**

90  $\mu\text{g}$  of proteins from C1, C2, N1 and N2 were individually separated by 2-DE. IPG strips of pH3-10 were used to separate the proteins in the first dimension, and 10% SDS-PAGE was used for the second dimension. The running conditions were the same as those described earlier. The gels were stained by Coomassie Brilliant Blue and images acquired via the Autochemi Bioimaging system.

## **2.38 Analysis of HPA binding proteins separated by 1-DE**

20  $\mu\text{g}$  of the two cancerous and corresponding healthy protein mixtures (C1, C2, N1 and N2) were separated by 10% SDS-PAGE and transferred onto nitrocellulose membrane before being probed with biotinylated lectin (HPA) as before

## **2.39 Analysis of HPA binding proteins separated by 2-DE**

90  $\mu\text{g}$  of proteins from C1, C2, N1 and N2 were separated by 2-DE and transferred onto nitrocellulose membranes and probed with HPA as before.

## **2.40 Probing Western blots of CRC samples with antibody to integrin $\alpha 6$**

The presence of integrin  $\alpha 6$  in C1, C2, N1 and N2 protein mixtures was evaluated by separating 20 $\mu\text{g}$  of proteins on 10% SDS-PAGE and transferring to nitrocellulose membrane. The membrane was then probed with the monoclonal rat anti-integrin  $\alpha 6$  antibody (anti-CD49f) using conditions identical to those described in section 2.29, detection was performed using chemiluminescence and exposure of X-ray film as described earlier.



## **CHAPTER THREE**

**Lectin binding to colorectal  
cancer cells: A focus on *Helix  
pomatia* agglutinin (HPA)**



### 3.1 Introduction

The carbohydrate moieties of glycoconjugates on the outside of cells are known to play an important role in maintaining cellular integrity by controlling cell-cell, cell-matrix interactions and correlate with cell differentiation. Over the past two decades a large range of glycoconjugates including glycoproteins and glycolipids have been shown to be modified during differentiation and malignant transformation (Aoki *et al.*, 1993; Iwakawa *et al.*, 1996; Remani *et al.*, 2000). Observations have strongly suggested that the post-translational modification of proteins, particularly glycosylation is altered in relation to cancer progression to a metastatic phenotype (Smets and Van Beek, 1984; Altevogt *et al.*, 1983).

The study of the modified carbohydrate structures occurring during malignant transformation has often been achieved using lectins. Carbohydrate binding proteins including lectins have attracted interest in cancer research applications. In the 1960's researchers discovered that some lectins bound specifically to particular cancer cells (Aub *et al.* 1963, 1965; Burger 1969). Over the past thirty years lectins have been used to study changes in glycoconjugates involved in pathophysiological events including cancer development as well as cancer prognosis. A study by Rhodes *et al.* (1986) investigated the binding properties of various peroxidase-labelled lectins including PNA, WGA, SBA, DBA and UEA-1 to nineteen colorectal carcinoma (CRC) and twenty normal mucosa specimens. They found an association between PNA/UEA-1 binding and poor patient prognosis. Iwakawa *et al.* (1996) studied the binding of two lectins, UEA-1 and DBA, on colorectal tissues and highlighted changes in the N-acetyl-galactosamine, L-fucose and O-acetylated-sialic acid expression in cancer. These authors also unravelled the differential binding of these lectins to normal tissue, adenoma and adenocarcinoma. A study by Sams and co-workers reported a decreased reactivity of FITC- labelled WGA towards colorectal cancer tissue specimens (Sams *et al.*, 1990) whereas a recent study by Garcia De Albuquerque Garcia Redondo *et al.* (2004) reported increased binding of WGA on invasive colorectal cancer cell lines. These two studies contradict the findings of Rhodes *et al.* (1986) discussed earlier who failed to identify any significant difference in binding of peroxidase-labelled WGA in normal colon and carcinoma tissue samples. This non-exhaustive list of studies



highlights the usefulness of lectins as tools for detecting changes in the carbohydrates found on the surfaces of malignant cells but illustrates the problem of reproducibility amongst the systems and methodologies used. The lectin HPA, which binds to N-acetylgalactosamine residues, has been studied for over twenty years. Retrospective studies have shown HPA to bind to tumours and correlate with poor patient prognosis in breast (Leathem, 1983; Leathem and Brooks, 1987; Thomas *et al.*, 1993; Brooks and Leathem, 1991), gastric (Kakeji *et al.*, 1991), oesophageal (Yoshida *et al.*, 1993), prostate (Shiraishi *et al.*, 1992) and colorectal cancer (Ikeda *et al.*, 1994; Schumacher *et al.*, 1994; Mitchell and Schumacher, 1999; Brooks, 2000). A correlation between HPA staining and poor prognosis colorectal cancer was first established by Ikeda *et al.* in 1994 and has since been reported by several research groups and found to correlate with a metastatic phenotype. In later studies the Schumacher group established an animal model to study the role of HPA binding cancer cells in the metastatic process. Colorectal cancer cell lines (HT29, SW480) were implanted subcutaneously in SCID mice and the development of lung metastases showed a positive correlation with the HPA staining pattern of the cell lines (Schumacher *et al.* 1994b, 1996; Schumacher and Adams 1997). The use of appropriate methodology is important when studying lectin binding as argued by Brooks *et al.* in 1996. Earlier studies of HPA binding to colorectal tumours that were contradictory were probably due to differences in the lectin binding methods used (Kim and Isaacs, 1975; Hakamori, 1989; Kemmer *et al.*, 1992).

The choice of cellular model and methodology is an important consideration when using lectins as a tool to study the glycoconjugates potentially involved in the metastatic process. For this study we have chosen a well-characterised metastatic and non-metastatic colorectal cancer cell line model. HT29 and SW480 are two cell lines derived from human colorectal adenocarcinomas. SW480 was established from a Dukes type B colorectal adenocarcinoma of a 50 year old Caucasian male; HT29 was established from a colorectal adenocarcinoma of a 44 year old female. The cell lines have been extensively used to study the binding of HPA directly on cells or in tumours grown in immuno-deficient mice and HT29 has been shown to be HPA positive whilst SW480 has been shown to be HPA negative (Schumacher and Adam, 1997). Each of the cell lines has been studied in SCID mice in an attempt to evaluate their metastatic potential and it appears that their HPA-binding properties correlate with their ability to



metastasise. SW480 did not form metastases after growing a tumour when implanted in SCID mice, whereas HT29 did grow a tumour and form lung metastases (Schumacher *et al.*, 1994a, 1996; Schumacher and Adam, 1997).

Earlier studies of HPA binding to breast and CRC cancer tissues and/or cells have mainly used light microscopy-based methods of detection. These systems do not allow absolute quantification of HPA binding; they are rather subjective qualitative assessments potentially subject to variation. In a study by Brooks *et al.* 2001, fluorescent HPA (FITC) was used in conjunction with confocal microscopy and a range of breast cancer cell lines. This approach allowed localisation of the binding (perinuclear or surface/cytoplasm) but the quantification used a scale ranging from “-” for low binding to “+++” for intense binding, in a similar approach to the scoring system used in light microscopy. Although the results gathered in their study were of interest in terms of their qualitative observations, there remained an opportunity to improve the methods to allow semi-quantification. De Albuquerque Garcia Redondo *et al.* (2004) used gold-labelled lectin in an attempt to quantify binding of HPA to CRC cells but most of the studies with HPA over the past 15 years relied on colorimetric detection systems with light microscopy or, alternatively, fluorescence-based methods.

In this study an effort was made to standardise the analysis of HPA binding and minimise reporter variation particularly to improve the scoring systems. For this we used a confocal microscopy-based and a microtitre plate-based approach. We also used simple sugars (mannose, GlcNAc, GalNAc, SA) to inhibit the binding of HPA to determine the specificity of binding. A combination of 2D reconstruction and 3D views generated from the confocal microscopy-based method enabled visualisation of the binding of HPA to HT29 and SW480. This approach offered an insight into localisation of HPA binding. This method also enabled us to visualise the inhibition of HPA binding to HT29 cells by GalNAc. The confocal microscopy was a useful tool to evaluate the extent of HPA binding but used a relatively small number of cells.

The development of a microplate-based assay allowed evaluation of HPA binding on a larger population of cells and enabled inhibition experiments using a variety of simple sugars and provided an insight into the binding partners of HPA.



## 3.2 Lectin binding to HT29 and SW480

In the first part of the evaluation of lectin binding to the CRC cell lines, we used several fluorescently-labelled lectins, including HPA and confocal microscopy. We sought to confirm using the HT29 and SW480 cell lines, previous findings in which HPA was reported to bind more strongly to the cells with metastatic capability (HT29) compared to other lectins.

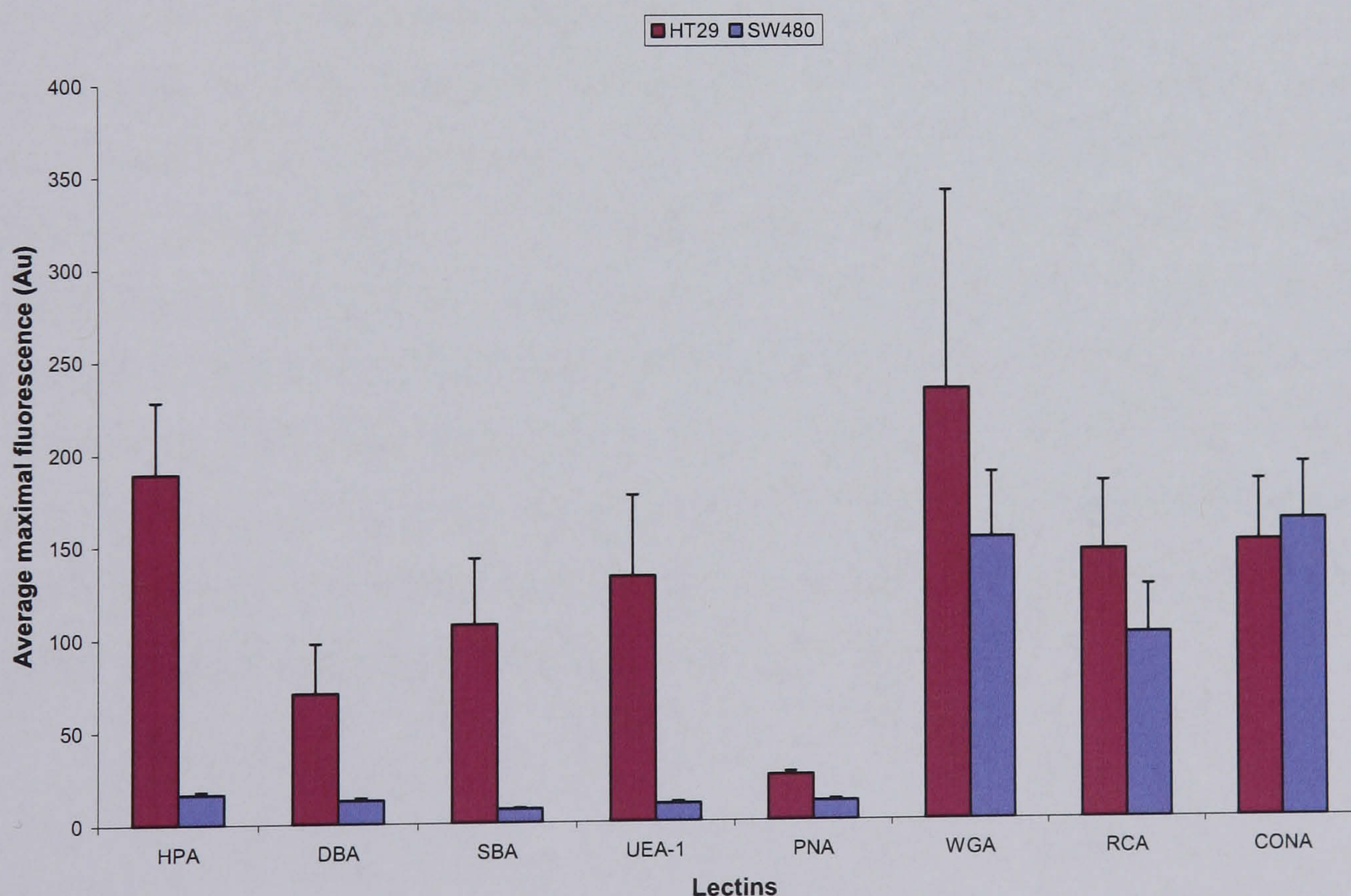
HT29 and SW480 cells were grown to near confluence in Petri dishes, the cells were fixed in formalin to mimic the tissue processing methods used in Histopathology Departments and the binding of eight fluorescently-labelled lectins was evaluated.

The total fluorescence intensity of the 8 lectins mentioned above was measured for each cell line and the results are shown in Figure 3.1. In this system, no significant differences in binding to HT29 and SW480 cells were observed for RCA, ConA and PNA, however the binding pattern were not identical for the three lectins. PNA bound weakly (<25 Au) to both HT29 and SW480 suggesting that low expression of T antigen whereas the binding of RCA and Con A was about 6 times more intense than PNA but was not significantly different between HT29 and SW480. All three lectins failed to bind differentially to our cell line model and therefore were not considered relevant for the study glycoconjugates changes associated with metastatic tumour cells. On the contrary, HPA, UEA-1, DBA and SBA all bound more intensely to HT29 than to SW480. UEA-1 and DBA were previously reported to detect changes in glycosylation occurring during development from adenoma to adenocarcinoma and behaved similarly in this system (Iwakawa *et al.* 1996), Here UEA-1 bound 7 times and DBA bound 4 times more intensely to the metastatic cell line, HT29 as compared to the non-metastatic cell line, SW480. Similarly, SBA bound 5 times more intensely to HT29 than SW480. In our system HPA bound 9 times more intensely to the metastatic cell line HT29 than to the non-metastatic cell line SW480 and was the lectin that showed the most differential binding to glycoconjugates of HT29 compared to SW480, confirming previous studies that suggest HPA has the ability to detect changes in glycosylation associated with a metastatic colorectal cancer phenotype. The lectin WGA bound in a very intense manner (233 Au) to HT29 but bound also intensely to the non metastatic cell line SW480 (152 Au), the difference of binding was not found to be significant and



hence we failed to confirm the observation by De Albuquerque Garcia Redondo *et al.* (2004) that WGA staining increases in malignancy (IEC-6, CaCo-2 and HCT-116). This apparent dichotomy may be due to variations in the methodology and the cell line used. CaCo-2 used by De Albuquerque Garcia Redondo *et al.* is a cell line with low metastatic potential and may be one of the causes of the ambiguous results. The CaCo-2 cell line has been studied by many other groups including Mitchell *et al.* (1998) who reported that it metastasised in only 25% of the cases when implanted in SCID mice. In the study by De Albuquerque Garcia Redondo *et al.* using gold labelled HPA, the lectin was found to bind similarly to the CRC cell lines irrespective of their invasive potential whereas several studies, including our own, concluded that HPA is a useful tool for identifying cancer cells with a metastatic phenotype (Ikeda *et al.* 1994; Schumacher *et al.* 1994; Mitchell *et al.* 1998).

This experiment confirmed the validity of HPA as a tool for detecting colorectal cancer cells with metastatic potential.



**Figure 3.1:** Lectin binding to HT29 (blue) and SW480 (red) cell lines observed and evaluated using a confocal microscope. The mean values of the global binding intensity evaluated for 22 cells +/- SE are presented.



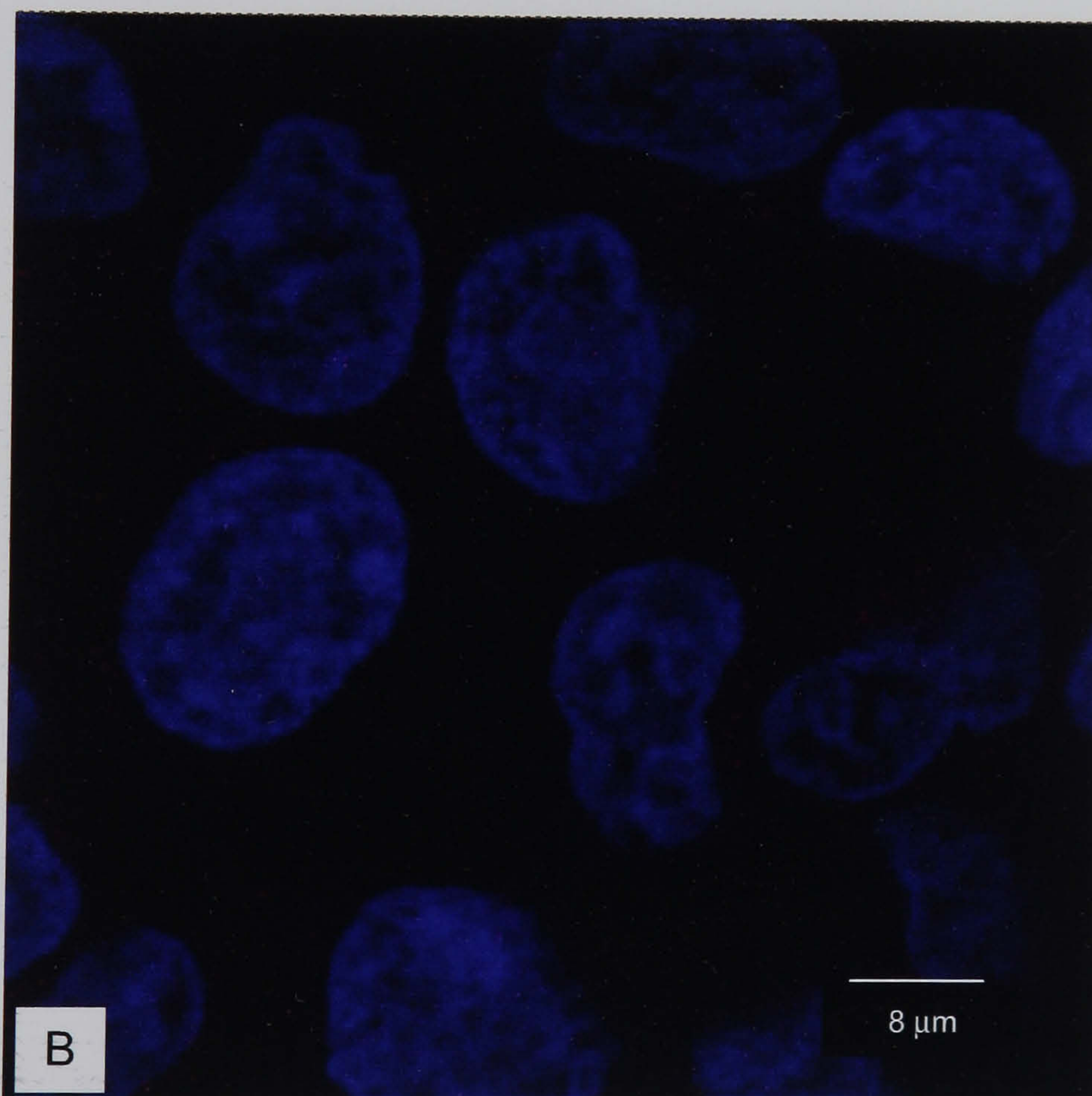
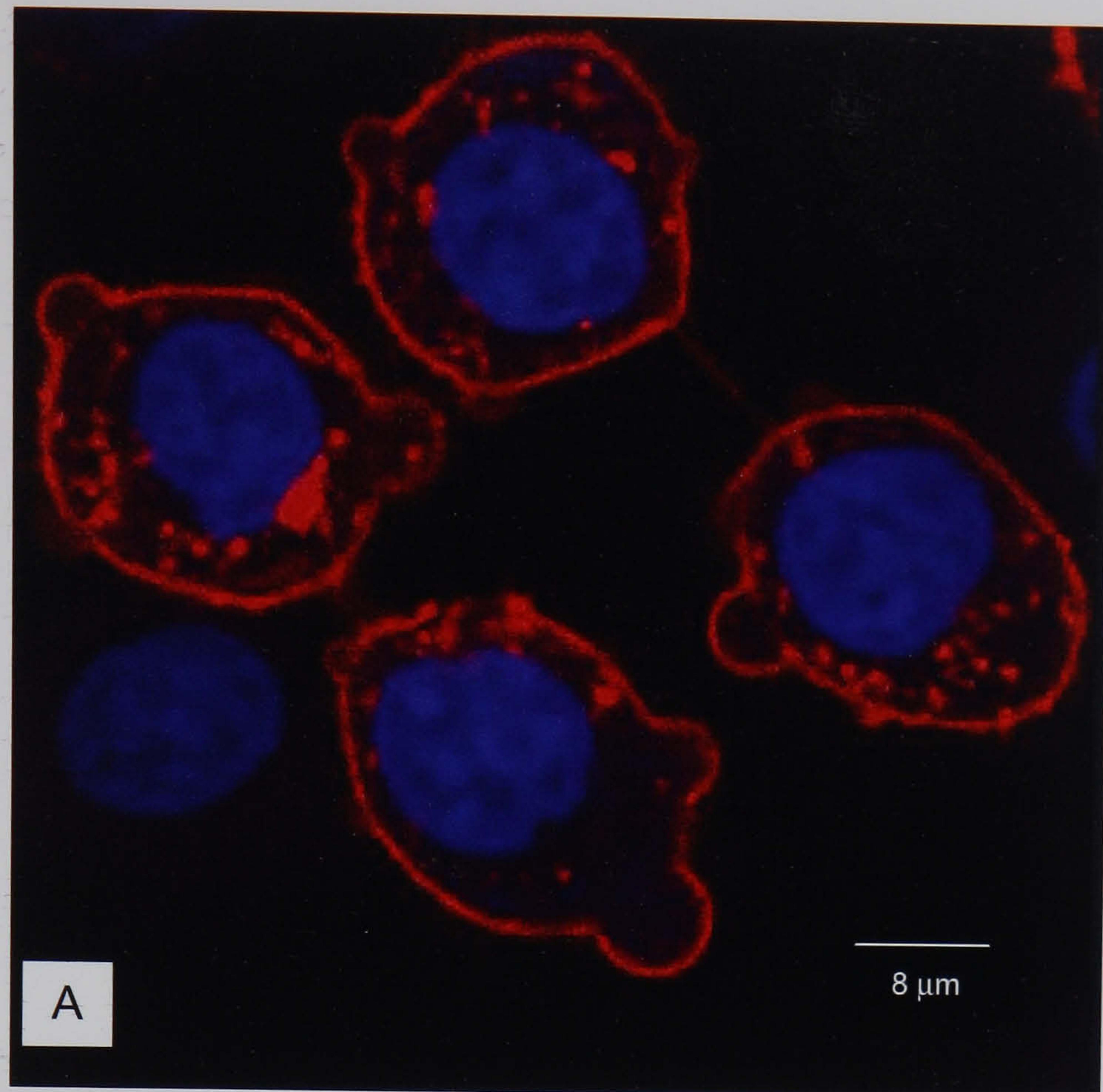
### 3.3 Localisation of HPA binding in HT29 and SW480

In order to obtain a clear overview of the localisation of HPA binding in metastatic (HT29) and non-metastatic (SW480) cell lines HPA-TRITC was used and the staining analysed by confocal microscopy. In this way we confirmed the observations of Schumacher *et al.* (1994b) who used an indirect colorimetric method and found HT29 to be HPA positive whereas SW480 was HPA negative. We performed at least five reproducible experiments and Figure 3.2 shows a typical result.

HPA bound intensely to the HT29 cell membrane with granular staining in the cytoplasm and in the perinuclear region. On the other hand, HPA showed very faint binding to SW480 in both membrane and cytoplasmic compartments. Brooks *et al.* (2001) used FITC-labelled HPA with confocal microscopy and breast cancer cell lines and reported perinuclear and membrane binding to the metastatic breast cancer cell lines.

Although a direct detection system was used in these confocal experiments (TRITC-labelled HPA) the results were consistent with those obtained using an indirect method (Schumacher *et al.*, 1994). The use of peroxidase-labelled HPA as a direct detection method may not be appropriate (Leathem, 1983) as the horseradish peroxidase molecule (MW: 40,000 Da) used to label HPA (MW: 76,000 Da) is relatively large and may interfere with HPA binding to cancer cell glycans. The indirect methods which use either native HPA (Brooks *et al.*, 1996) or biotinylated HPA (Brooks and Wilkinson, 2003) (biotin MW: 244 Da) are comparable to our fluorescent system. The TRITC fluorophore (MW: 443.5 Da) used to label HPA and used in our study is about 100 fold smaller than HRP (MW: 40,000 Da) used in colorimetric direct detection methods and our results suggest that it does not hinder HPA binding even though it is slightly larger than both biotin and FITC. In summary, this experiment validated the use of HPA-TRITC to study lectin interaction with HT29 and SW480 cells.



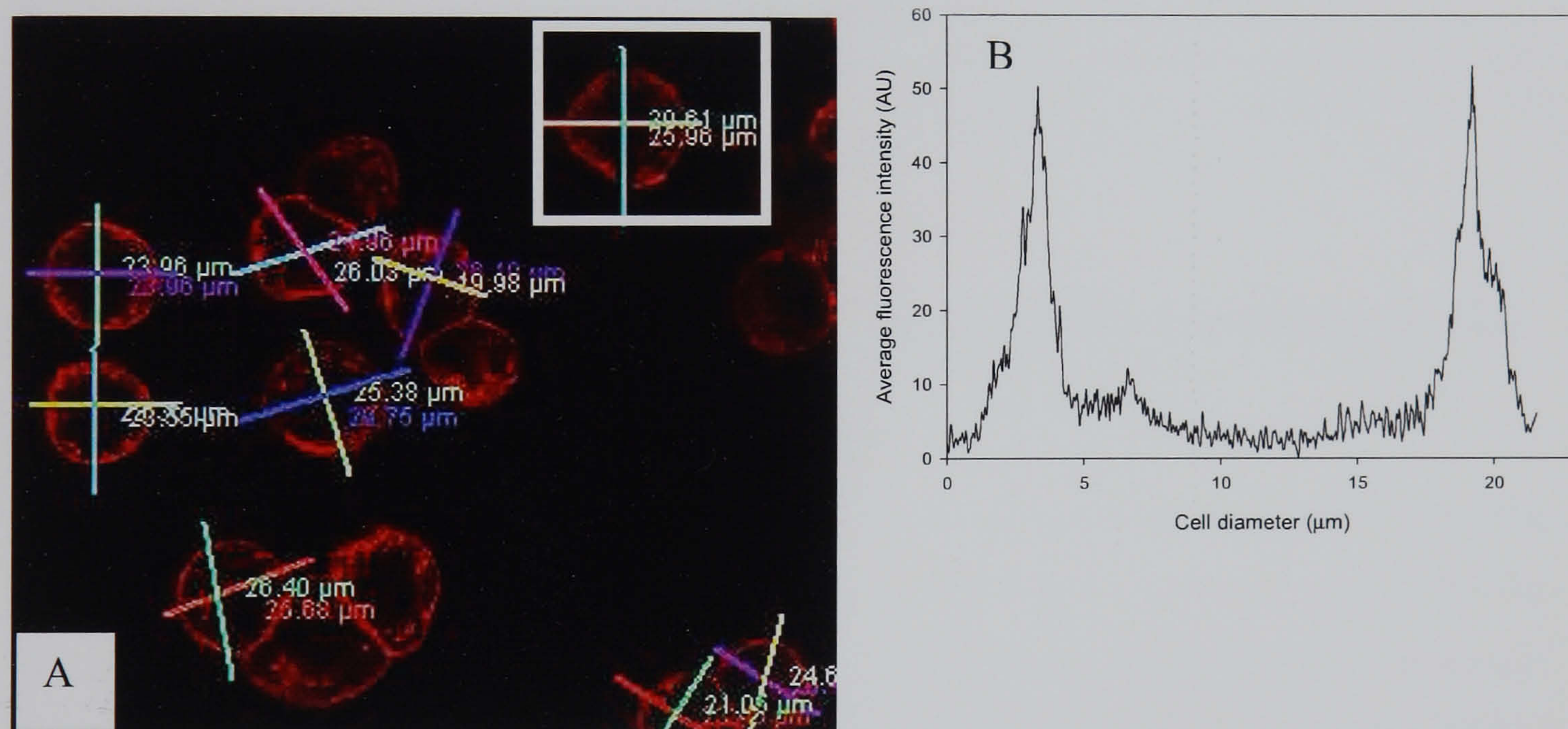


**Figure 3.2:** The binding of HPA -TRITC to HT29 and SW480 cells  
Confocal images showed the binding of TRITC-labelled HPA (red) to HT29 (panel A) and SW480 (panel B). The nuclei were counterstained with ToPRO-3 (blue). Image A shows intense lectin binding on the surface of HT29 with some granular staining inside the cell. There is almost no HPA binding to SW480 on the surface or inside the cell. *Scale bars 8 μm*



### 3.1 Quantification and localisation of HPA binding:

In an attempt to localise the binding of the lectin HPA and obtain relative quantification of binding, the confocal images were used with data analysis using the Leica software analysis package. Briefly, two lines were drawn across either HT29 or SW480 cells. On the field shown in Figure 3.3, nine cells were evaluated and the intensity of fluorescence was calculated along the two lines drawn on each of the cells. An example of a linear profile taken across one of the cells (framed in Figure 3.3 panel A) is also presented (Figure 3.3, panel B). This approach enabled relative quantification values to be obtained and generated digitised data showing localisation of the binding of HPA via the measurement of the fluorescence intensity along the lines drawn across the cells. This method confirmed that HPA binding was focussed to the cell membrane of HT29.



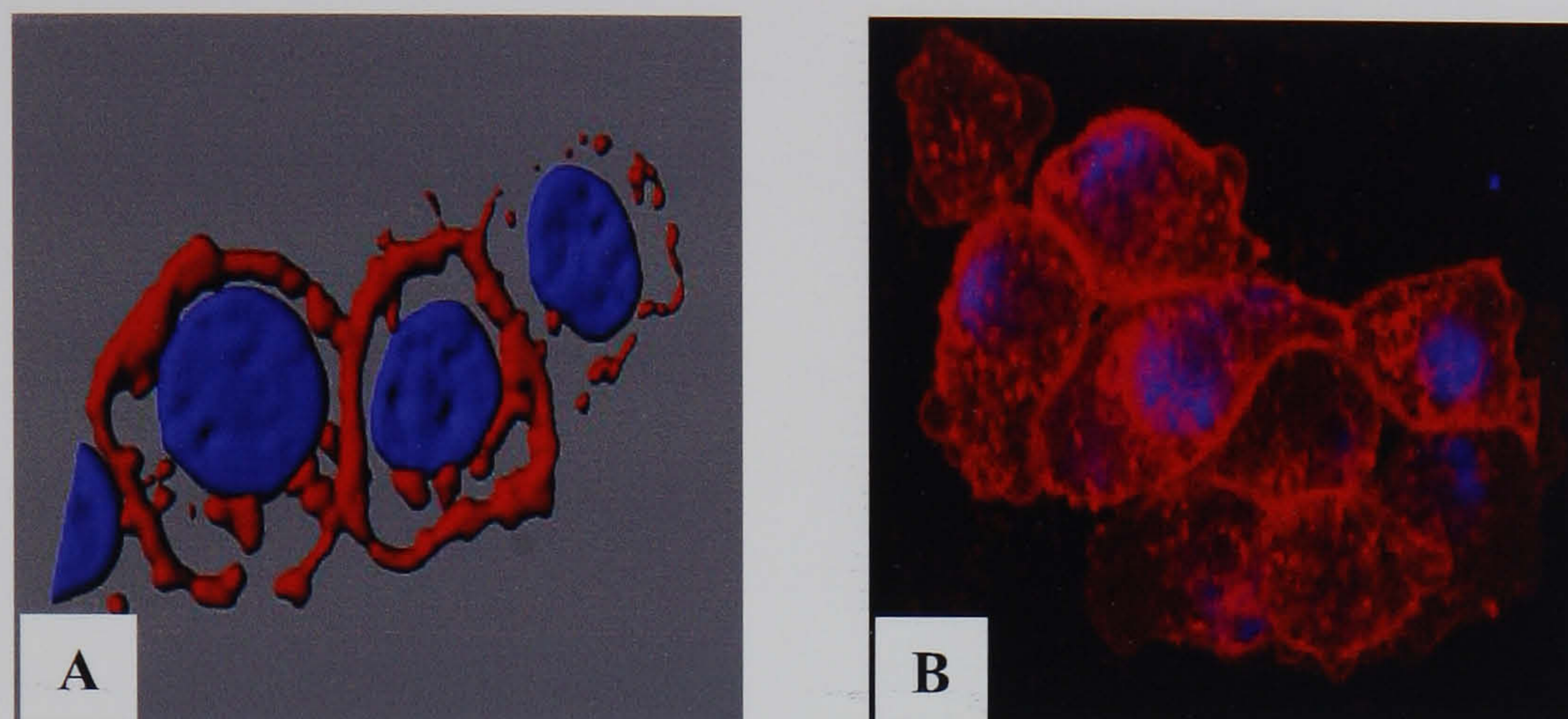
**Figure 3.3:** An example of linear profiling of HPA binding to HT29 using the Leica analysis software. Two lines were drawn across each of the nine HT29 cells chosen in this field in order to evaluate the intensity of the HPA binding in the different compartments of the cells (panel A). An example of the linear profile generated with this technique is shown in the framed region (panel B).

### 3.2 2D and 3D reconstruction of confocal images

The Leica software package was used to produce 2D and 3D models of HPA binding (Figure 3.4). Sections were taken of HPA-stained cells at increments of 1 μm. The sections were compiled to generate a 3D representation of HPA binding. Figure 3.4



shows nine HT29 cells stained with TRITC-labelled HPA and counterstained with the nuclei dye ToPRO-3. The 3D view offers an insight into the location of HPA binding and confirmed the observations made in the 2D mode.



**Figure 3.4:** 2D and 3D representation of HPA binding to HT29 cells. The Leica software in “Surpass view” shows a 2D representation of HPA binding to HT29 cells. The nuclei are displayed in blue and the lectin in red. As observed under the microscope the 2D reconstruction showed binding mainly located on the membrane with some staining within the cells at perinuclear sites consistent with the endoplasmic reticulum and Golgi apparatus (panel A). The Leica software Imaris 4.0 was used to generate a 3D view of nine HT29 cells. The 3D image was reconstructed from Z stacks taken at 1 $\mu$ m increments. Blue nuclei are displayed and the HPA binding is shown in red (panel B).

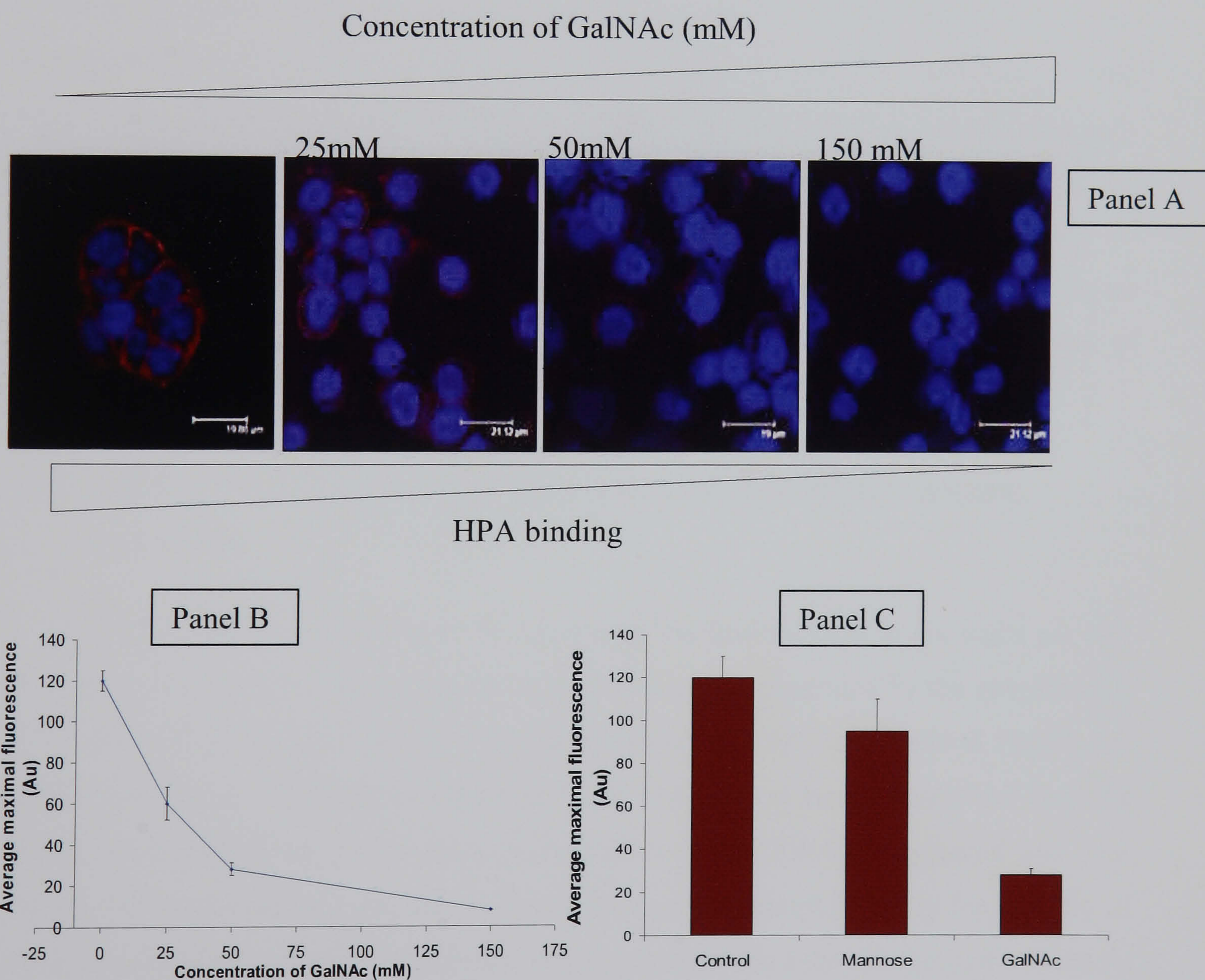
### 3.3 Evaluation of the specificity of HPA binding at the cellular level

The experiments presented in this section were designed to evaluate the binding of HPA to HT29 and SW480 at the cellular level. HPA has a nominal specificity for glycans bearing GalNAc and GlcNAc residues (Vretlab *et al.* 1979). In this experiment GalNAc was used to competitively inhibit the binding of HPA. HT29 cells were used in binding experiments with HPA, pre-incubated with different concentrations of GalNAc (20 mM - 150 mM). Three experiments were performed and provided reproducible results, Figure 3.5 shows the typical inhibition observed across the experiments. Specific inhibition of HPA-binding by GalNAc was observed (Figure 3.5 panel A) and the detection of HPA-TRITC binding decreased with the addition of GalNAc, in a dose dependent manner. The intensity of fluorescence, as a measure of the HPA binding, was evaluated as described earlier and is displayed graphically in Figure 3.5 panel B. A decrease in fluorescence intensity with increasing concentration of inhibiting sugar was



detected and the presence of GalNAc at 25 mM and 50 mM resulted in inhibition of the binding by 50 % and 75 % respectively. Almost complete inhibition of binding was obtained when the lectin was preincubated with 150 mM GalNAc. This experiment confirmed that the lectin specifically binds to glycoconjugates via GalNAc residues on the surface of the malignant colorectal cancer cell line, HT29.

By way of a negative control an experiment was performed in which HT29 cells were incubated either with HPA alone or with HPA preincubated with 50 mM of GalNAc or 50 mM of mannose. An inhibition of 75 % of binding was observed when HPA was preincubated with 50 mM GalNAc whereas only 17% inhibition was observed when HPA was preincubated with 50 mM mannose. These results support the observations of Hammerstrom and Kabat (1971) that the primary specificity of HPA is towards GalNAc-containing glycoconjugates.



**Figure 3.5:** Specificity of the binding of HPA to HT29 cells. The cells were incubated with 10  $\mu\text{g/ml}$  HPA-TRITC alone or after preincubation with increasing concentrations of GalNAc at 25 mM, 50 mM or 150 mM GalNAc (panel A). Substantial inhibition of HPA binding was observed at 50 mM GalNAc. Quantification of membrane binding was achieved by linear profiling using the Leica software (panel B). Error bars represent the Standard Error of the Mean (SEM) of 10 cells (single inhibition experiment). A control experiment was performed and is presented in panel C.



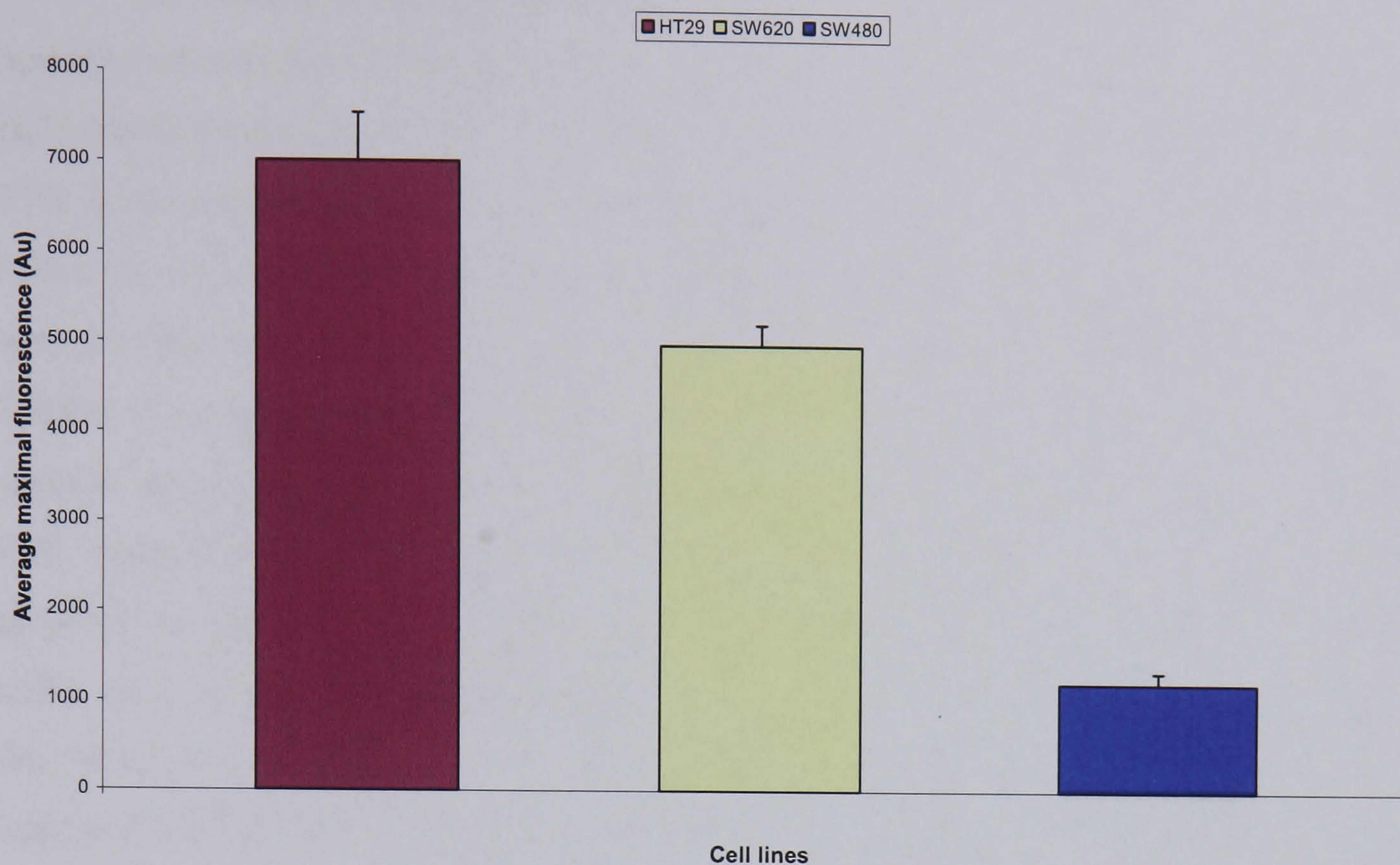
### **3.7 The development of a microtitre plate assay to evaluate HPA binding to SW480, SW620 and HT29 cells**

A microtitre plate assay was designed to quantify the binding of HPA using a large population of cells in each well. This served as a higher throughput system for assessing the HPA binding than the confocal microscopy based system. It also allowed further evaluation of the monosaccharides that inhibit HPA interaction. For this assay 100,000 cells were seeded into individual wells in a 96 well plate and fixed in formalin. The assay used an indirect method of detection, with biotinylated-HPA and a FITC-labelled streptavidin detection system thereby modelling the tissue based histochemistry methods of other researchers. The second step with streptavidin increased the background signal but this was normalised using a negative control of cells incubated with streptavidin-FITC alone. Brooks and co-workers (2001) used FITC-HPA to study binding to breast cancer cell lines but the methodology was different and, importantly, they used cells grown on a coverslip which hinders the study of a large population of cells. Five experiments were performed for the evaluation of the binding and the sugars specificities and reproducible results were observed. In this section one typical result of each experiment is presented.

### **3.8 HPA binding using the microtitre plate assay system**

HPA binding was assessed by measuring the fluorescence in six wells per cell line and normalised by subtracting the background signal generated by the streptavidin-FITC alone. The results of the six wells for HT29 and SW480 are shown in Figure 3.6. In this experiment we included a third cell line. SW620 has been established from the lymph node metastases of the same patient from whom SW480 originated one year earlier. We evaluated this cell line in this system in an attempt to verify the binding of HPA to SW620 which may be regarded as an intermediate between the non metastatic SW480 and the highly metastatic HT29.





**Figure 3.6:** An assessment of the binding HPA using a microtitre plate assay. For each cell line, HT29, SW620 and SW480 the mean fluorescence from six wells and standard deviation are shown. This chart clearly showed the difference of HPA binding between HT29, SW620 and SW480. The average fluorescence for HT29, SW620 and SW480 was 7014, 4985 and 1216 respectively.

A significant difference in HPA binding was observed between HT29 and SW480 and this data concurs with the results obtained previously with confocal microscopy. Whilst this system does not allow detailed localisation of the lectin binding it provided a reliable overview of binding of HPA using a larger population of cells. SW620 was described as HPA negative by Mitchell *et al.* (1998) but was consistently 'HPA positive' in this system.

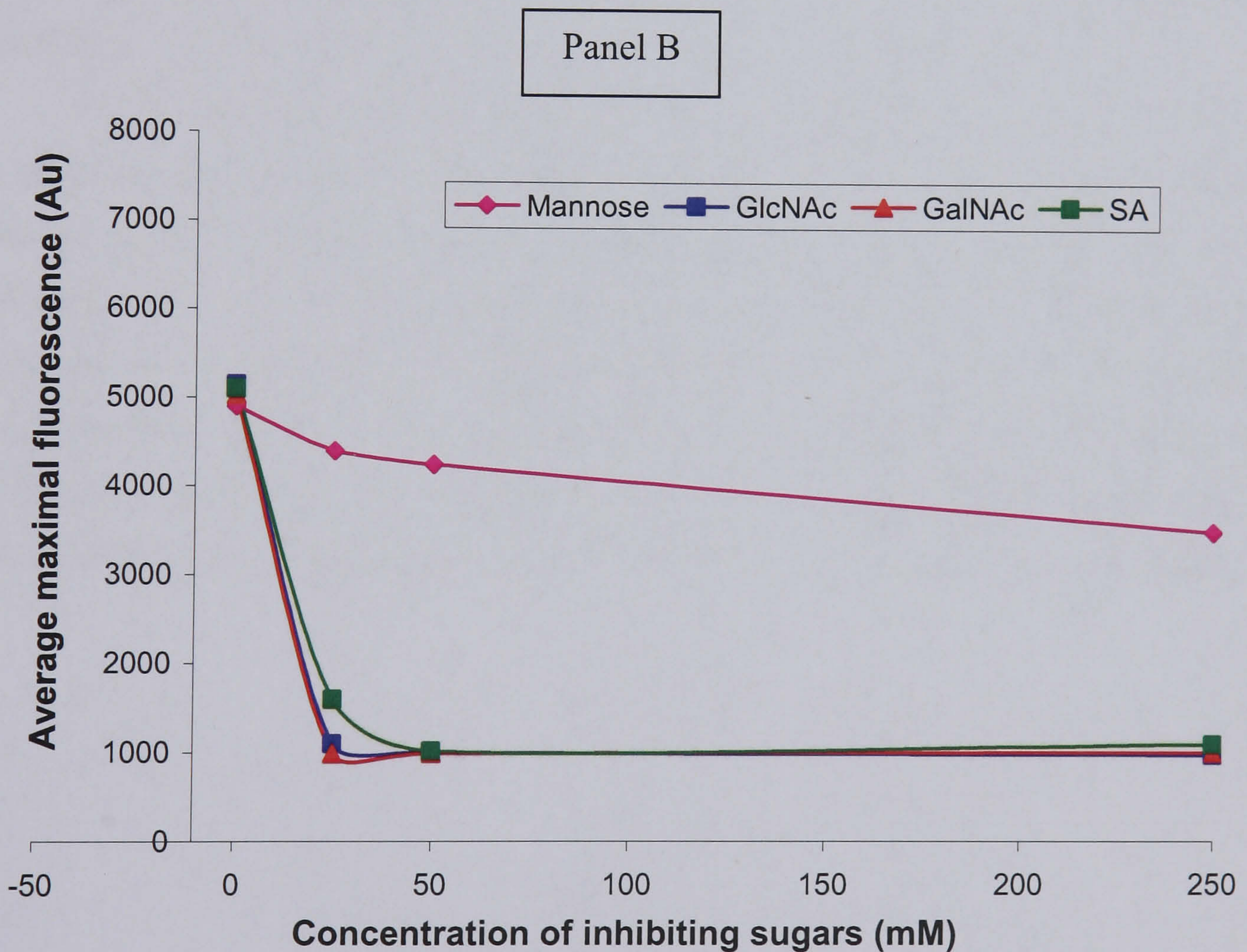
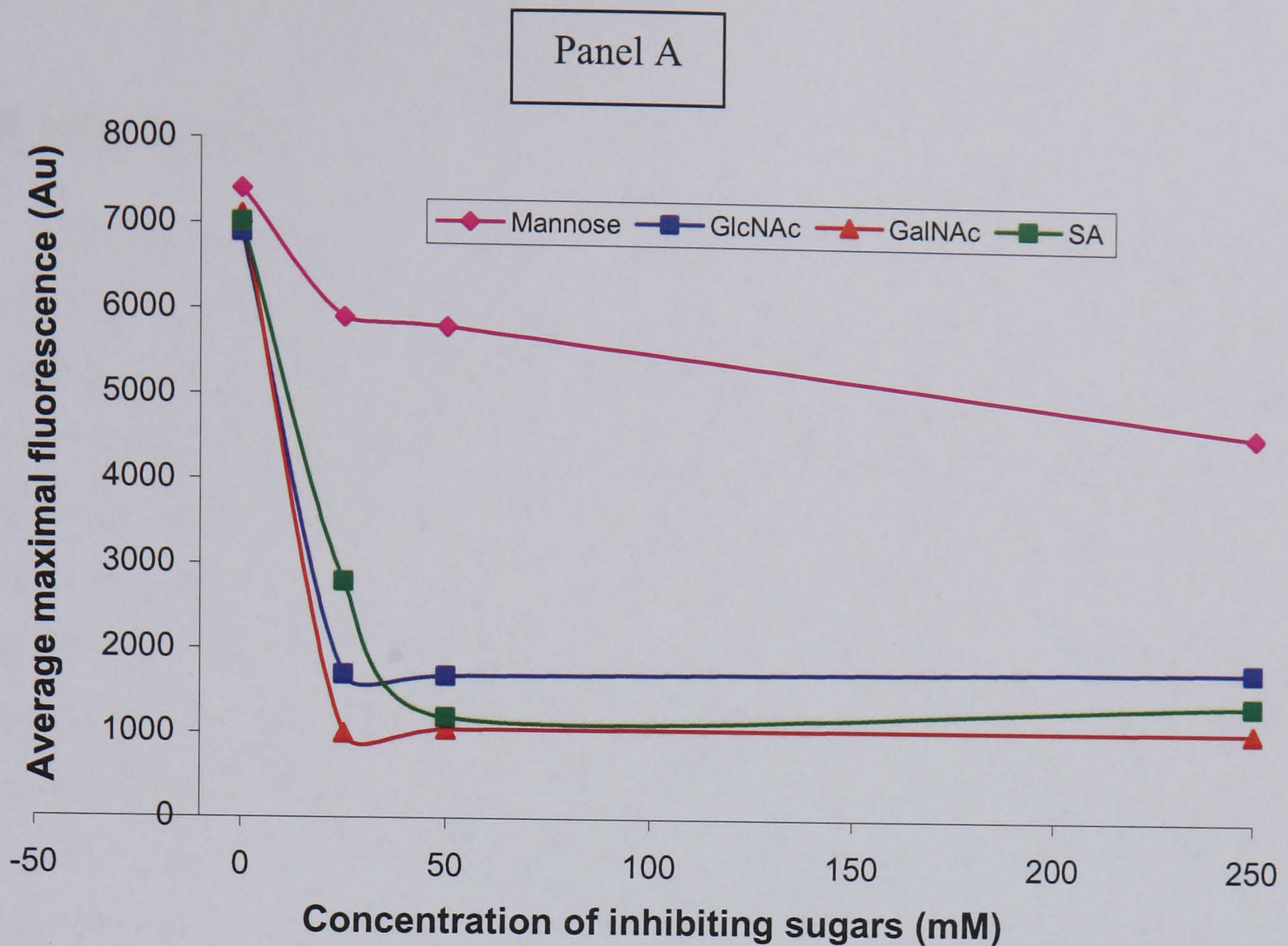
### 3.9 Inhibition of the binding of HPA in the microtitre plate assay

This experiment aimed to test a wider variety of inhibitory monosaccharides and to better characterise the binding properties of HPA to the colorectal cancer cell lines. Cells were prepared as before and were incubated with HPA alone or HPA preincubated with 20 mM, 50 mM or 250 mM competing sugar.



The binding of HPA to HT29 glycoproteins was inhibited in a dose dependent manner with GlcNAc, GalNAc and SA, as shown in Figure 3.7, panel A. Inhibition of HPA binding was achieved using 50 mM GalNAc, GlcNAc and SA with 77%, 87% and 79% inhibition respectively. When 50 mM mannose was used in this system only 23% inhibition was achieved. The inhibition reached with GalNAc at 50 mM (87%) concurs with the observations made using the confocal microscopy-based approach (75%). Similar observations were made with the cell line SW620 (Figure 3.7 panel B) with specific inhibition observed with 50 mM of GlcNAc, GalNAc and SA whereas in the HPA negative cell line, SW480, the binding of HPA remained low and unchanged in presence or absence of sugars (not shown). Intriguingly, this experiment showed that sialic acid (SA) could inhibit HPA binding to HT29. SA has not previously been described as a specific sugar involved in HPA binding, although in this system it inhibited 79% and 80% of the HPA binding in HT29 and SW620 respectively. A study by Dwek *et al.* (2001) reported that HPA binding glycans were sialylated, and Fenlon *et al.* (1987) reported a loss of the predictive power for HPA when tissue sections were pretreated with neuraminidase. The results shown here reinforced these findings and suggest a role for sialic acid as part of the glycan epitopes recognised by HPA. Findings by A Markiv (unpublished data) suggest that one of the proteins in the HPA preparation (NCBI entry gi:93209532) has 55% sequence similarity to a sialic acid binding protein from *Cepaea hortensis* and this data supports the idea that the HPA preparation comprises both GalNAc and SA binding proteins.





**Figure 3.7:** Inhibition of the binding of HPA to HT29, SW620 and SW480 colorectal cancer cells. Competitive inhibition of HPA using GalNAc, GlcNAc, SA and mannose was assessed using a microtitre plate assay. The monosaccharides were added to the HPA solution prior to incubation with the cells and the intensity of the binding was evaluated by reading the fluorescence emitted at 520 nm. Each sugar was used in an independent experiment including a positive control (biotinylated-HPA and Streptavidin-FITC). In HT29 (panel A) a high signal was detected with HPA alone and competitive inhibition was observed with the maximum inhibition reached at 50 mM of GlcNAc, GalNAc and SialAc and no significant inhibition was observed with mannose at 50 mM. The experiment using SW620 showed similar results although the binding of HPA alone was less intense than HT29 (panel B).



### 3.10 Discussion

The development of a microtitre plate assay confirmed, using a large population of cells, that the lectin HPA binds to the metastatic cell lines HT29 and SW620 via carbohydrate-mediated interactions, in contrast, HPA did not bind to the non-metastatic cell line SW480. Immunohistochemistry using TRITC-labelled HPA combined with confocal microscopy enabled localisation of binding of HPA to HT29 both via classical images of the cells as well as using reconstructed 2D and 3D images. This method confirmed observations that had previously been made that HPA binds intensely to the metastatic cell line HT29 and weakly to the non-metastatic cell line SW480. HPA binding was located mainly to the surface of HT29 but also some granular intracellular staining was observed. This is consistent with binding of HPA to the Golgi apparatus that has been reported elsewhere (Virtanen, 1990) and is attributed to binding of glycoproteins in transit through the secretory pathways or to glycosyltransferase / glycosidase enzymes resident in this organelle as described in chapter 1.

The development of the microtitre plate assay conferred a greater confidence in the inhibition experiments. The two methods developed in this study provided a reliable analysis of HPA binding to the two cell lines HT29 and SW480 and showed that HPA binding to these cells is via a lectin-glycan mediated interaction that can be inhibited using GalNAc, GlcNAc and SA. The experiments also showed that the HPA binding site encompasses glycoconjugates containing N-acetylgalactosamine as well as charged residues (SA) and N-acetylglucosaminylated structures. The development of this system also allowed us to establish the cell line SW620 as a model of moderate HPA binding.



## **CHAPTER FOUR:**

**Method development for  
the separation of  
membrane-enriched  
proteins via a proteomics-  
based approach**



## 4.1 Introduction

In the previous chapter, we observed that HPA has the ability to detect changes in the glycoprotein composition on the cell membrane of two cell lines with different metastatic potential. Studying glycoproteins from cells which differ in their biological behaviour may provide new insights into the role of glycosylation of proteins in the metastatic spread of primary tumours. In this new aspect of the work we aimed to analyse HPA-binding glycoproteins of HT29 and SW480 cells using a proteomic approach. 2-DE was selected for this as it is capable of resolving thousands of proteins in a single experiment (O'Farrell, 1975).

The solubilisation of proteins remains a critical step for high performance 2-DE and a wide range of protein solubilisation cocktails have been reported with the composition adapted to each analysis. Such a mixture needs to contain, as a minimum requirement, chaotropes to unfold proteins, non ionic detergents, reducing agent(s) and ampholytes (O'Farrell, 1975). However, it is also important to note that the accurate determination of protein concentration of the sample of interest is a prerequisite towards a comparative study of the protein expression in any two cell lines and the use of complex solubilisation mixtures may be incompatible with protein assay reagents and hence a source of misinterpretation of results.

In this chapter we evaluated the compatibility of the chemicals contained in the solubilisation buffers using three commercially available protein assay kits. We aimed to determine the most suitable method to evaluate the protein concentration of our sample prior to analysis by 2-DE. Secondly we used a method developed by Lehner *et al.* (2003) to isolate cell membranes by ultracentrifugation in an attempt to prepare a protein fraction enriched in HPA-binding proteins. We then evaluated the efficiency of four home-made solubilisation buffers by comparing the quality of the protein separation on 2-D gels. Finally we analysed the robustness of our system by assessing the reproducibility of protein migration of two replicates of the same sample. We also evaluated the suitability of our 2D system to compare the protein composition of HT29 and SW480.



## 4.2 Compatibility of solubilisation buffers with protein assay reagents

HPA mainly binds to membrane proteins of the metastatic cell line HT29. These membrane proteins are present in relatively small quantities and possess hydrophobic domains and they therefore fall into the category of “biochemically challenging proteins”. The method developed by Laemmli in 1970, based on the use of an SDS-containing buffer, has been successfully used as a standard method to solubilise a wide range of proteins, including hydrophobic proteins. The use of 2-DE does not, however, allow the use of the anionic detergent SDS (or only if followed with excess dilution in classical 2D buffer, Harder *et al.*, 1999) since SDS imparts a negative charge to all the proteins in solution thereby causing problems in the isoelectric focussing step.

To take advantage of the high resolution of 2-DE, proteins have to be denatured, disaggregated, reduced and solubilised to achieve complete disruption of molecular interactions and ensure that each spot represents, ideally, an individual polypeptide. In order to solubilise proteins prior to separation by 2-DE, high concentrations of chaotropes such as urea and thiourea are commonly used to unfold hydrophobic proteins (O’Farrell, 1975; Rabilloud *et al.*, 1997) and are combined with zwitterionic detergents such as CHAPS or sulfobetaines (Santoni *et al.*, 2000; Molloy, 2000). As a standard procedure, reducing agents such as DTT are used in lysis buffers to break disulfide bonds, along with carrier ampholytes to facilitate solubilisation with the aim that each spot contains a single polypeptide. The lysis buffer is a complex mixture of chemicals and compatibility with commercially available protein assay kits had to be tested in order to ensure an accurate evaluation of the concentration of solubilised proteins. Thiourea is known to not be compatible with commercially protein assays and can lead to inaccurate evaluation of the protein content of a solution. An accurate evaluation of the protein concentration of the cell lysate or membrane protein mixture is an essential step prior to separation by 2-DE or any other proteomics-based methodologies.

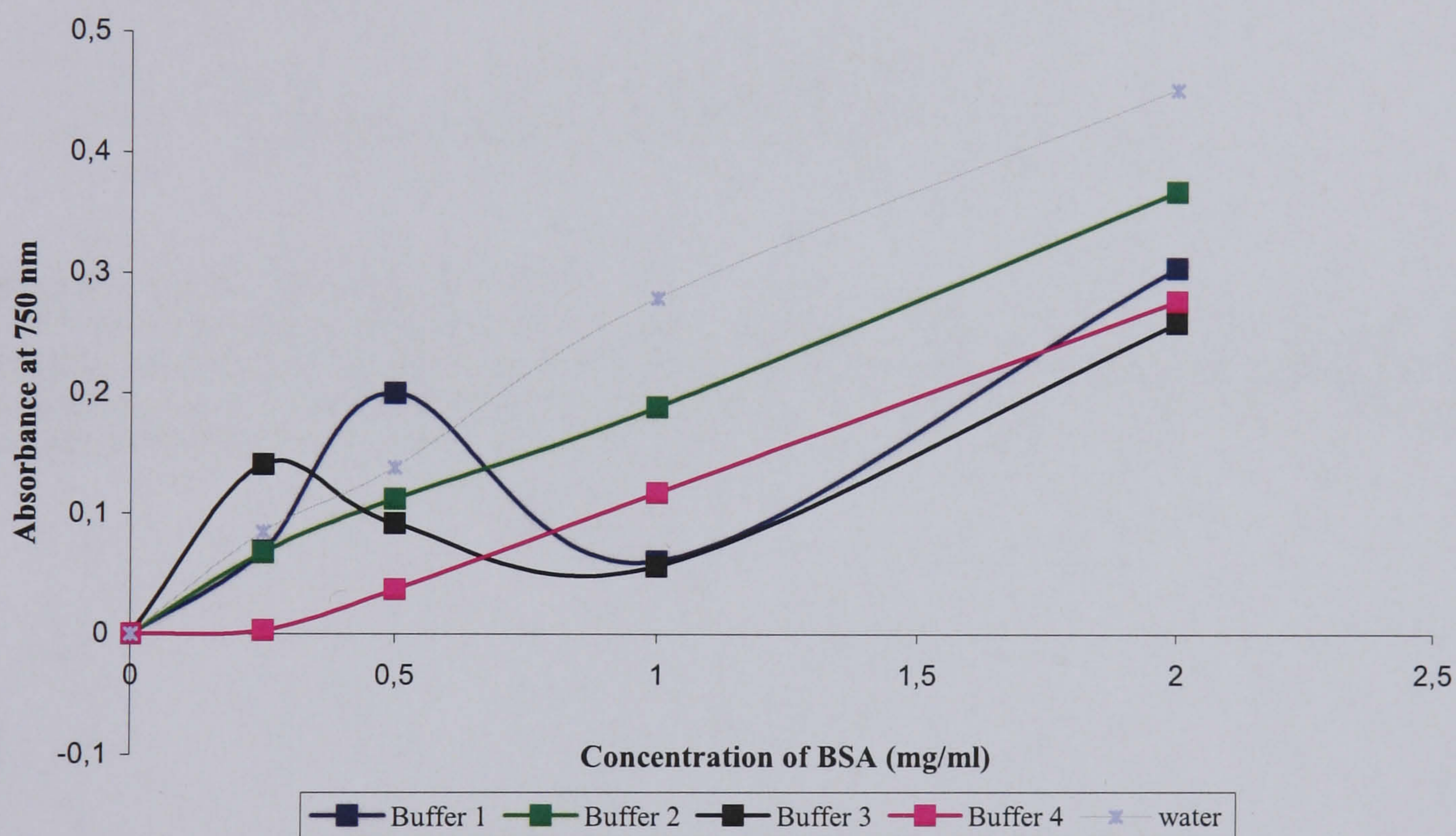
We tested the effect of the classical components of a 2D buffer Urea, CHAPS, DTT and ampholytes (O’Farrell, 1975) on three protein assay kits, (i) DC protein assay, (ii) RC-DC protein assay and (iii) Bradford assay, in an attempt to select the most reliable method for evaluating the protein content of our cell line protein extracts. The



results obtained for the standard curve using BSA prepared in a range of buffers (Table 4.1) and assay systems are shown in Figures 4.1, 4.2, and 4.3.

Buffer	Composition
1	Urea 7M, CHAPS 4%
2	Urea 7M, CHAPS 4%, DTT 1%
3	Urea 7M, CHAPS 4%, Ampholytes 2%
4	Urea 7M, CHAPS 4%, DTT 1%, Ampholytes 2%

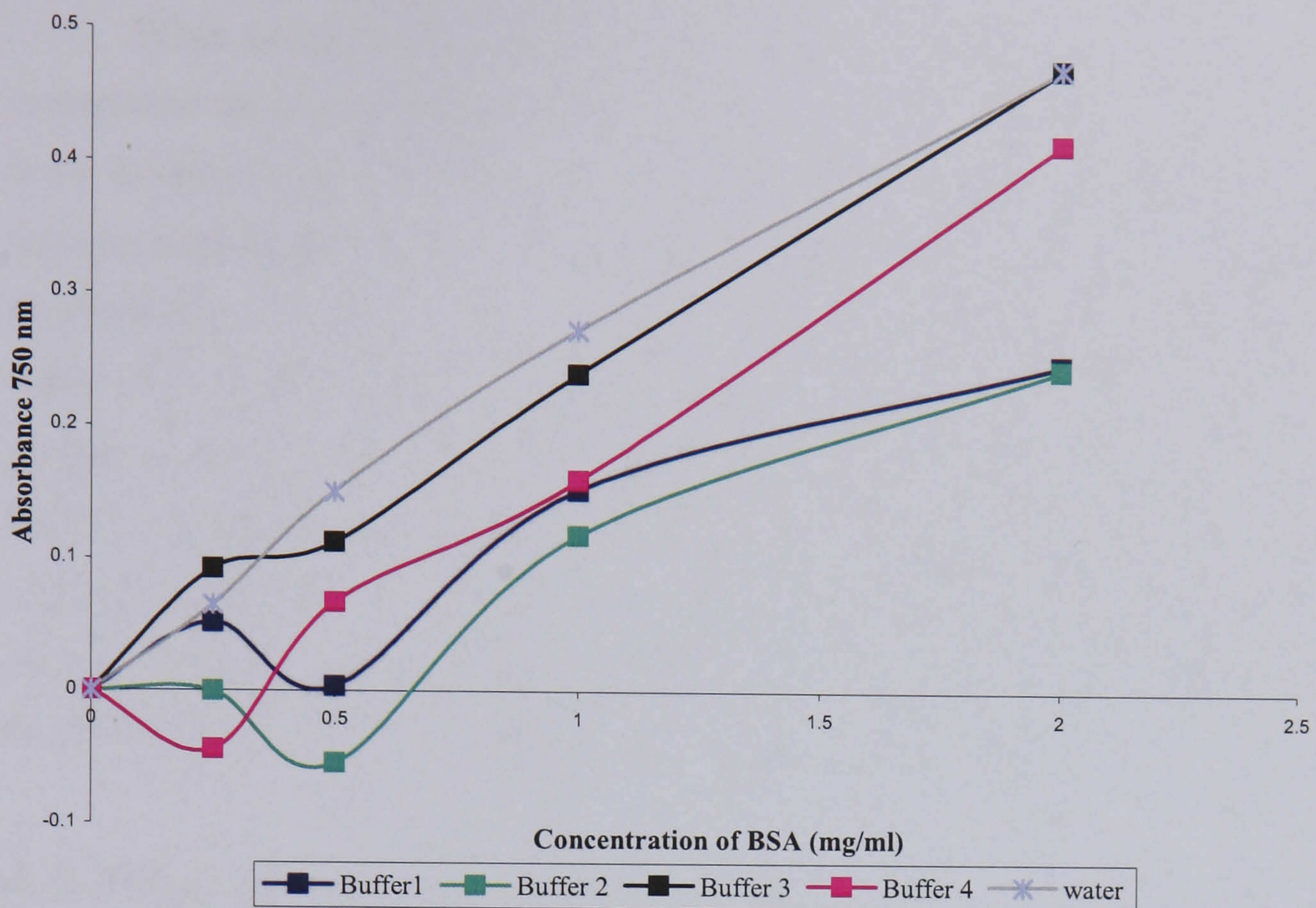
**Table 4.1:** Buffer composition used for the preparation of the BSA solution used as the standard protein for the protein assay.



**Figure 4.1:** Bio-Rad DC protein assay.

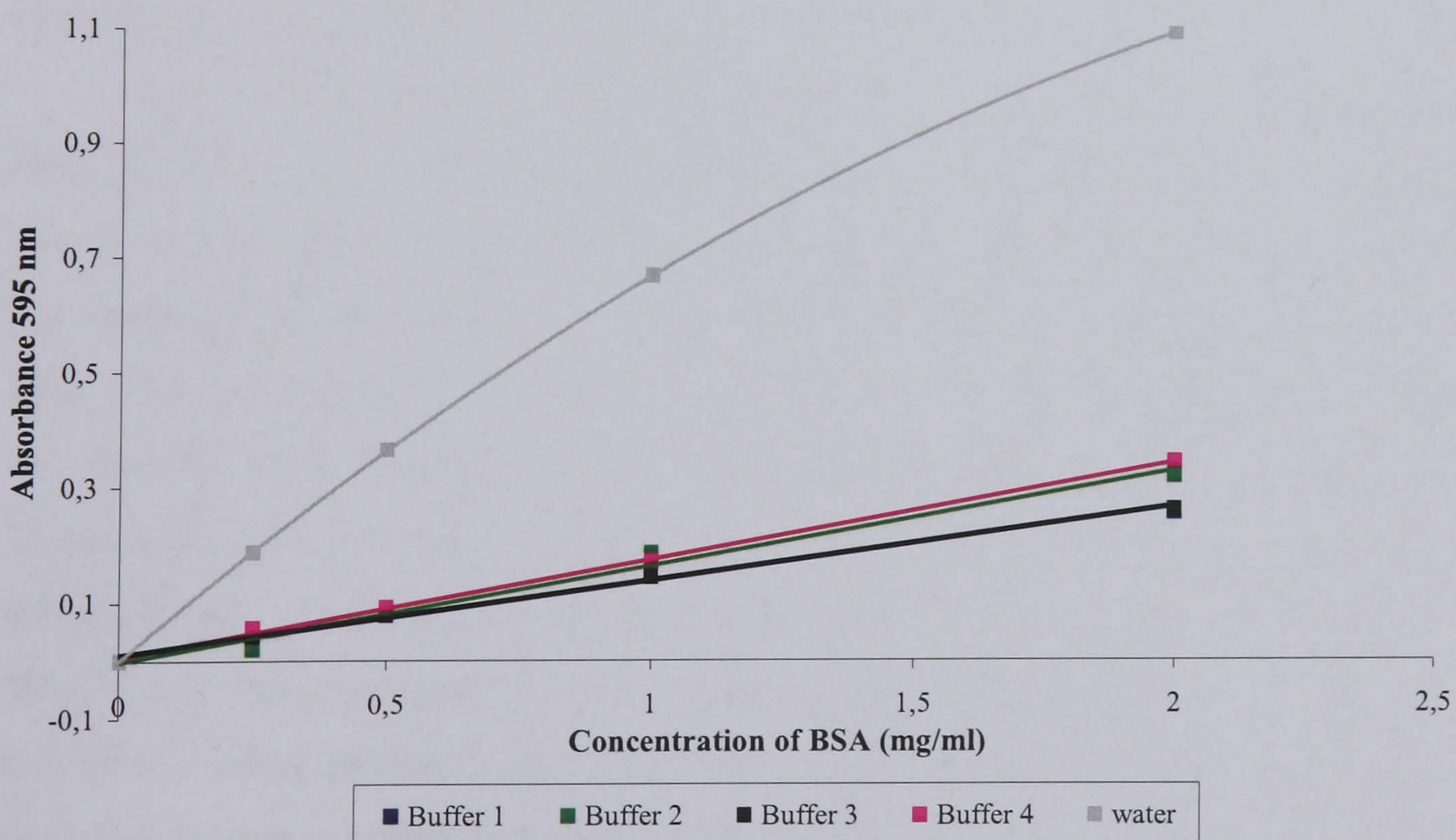
An increasing concentration of BSA ranging from 0.25 to 2 mg/ml was prepared in four buffers. The protein assay was performed according to manufacturer recommendations. Buffers 1,2,3,4 appear in blue, green, black and pink respectively, a positive control consisting of BSA prepared in water (grey) was added to the experiment and the absorbance was read at 655nm.





**Figure 4.2:** Bio-Rad RC/DC protein assay.

A BSA solution ranging from 0.25 to 2 mg/ml was prepared in four buffers. A protein assay was performed according to manufacturer recommendations. Buffers 1,2,3,4 appear in blue, green, black and pink respectively. A control consisting of BSA prepared in water (grey) was included and the absorbance was read at 750 nm.



**Figure 4.3:** Bio-Rad Bradford protein assay. Calibration curves using BSA prepared in buffers 1, 2, 3, 4 respectively in blue, green, black and pink, were drawn. A control consisting of BSA prepared in water (grey) was included and the absorbance was read at 595 nm.



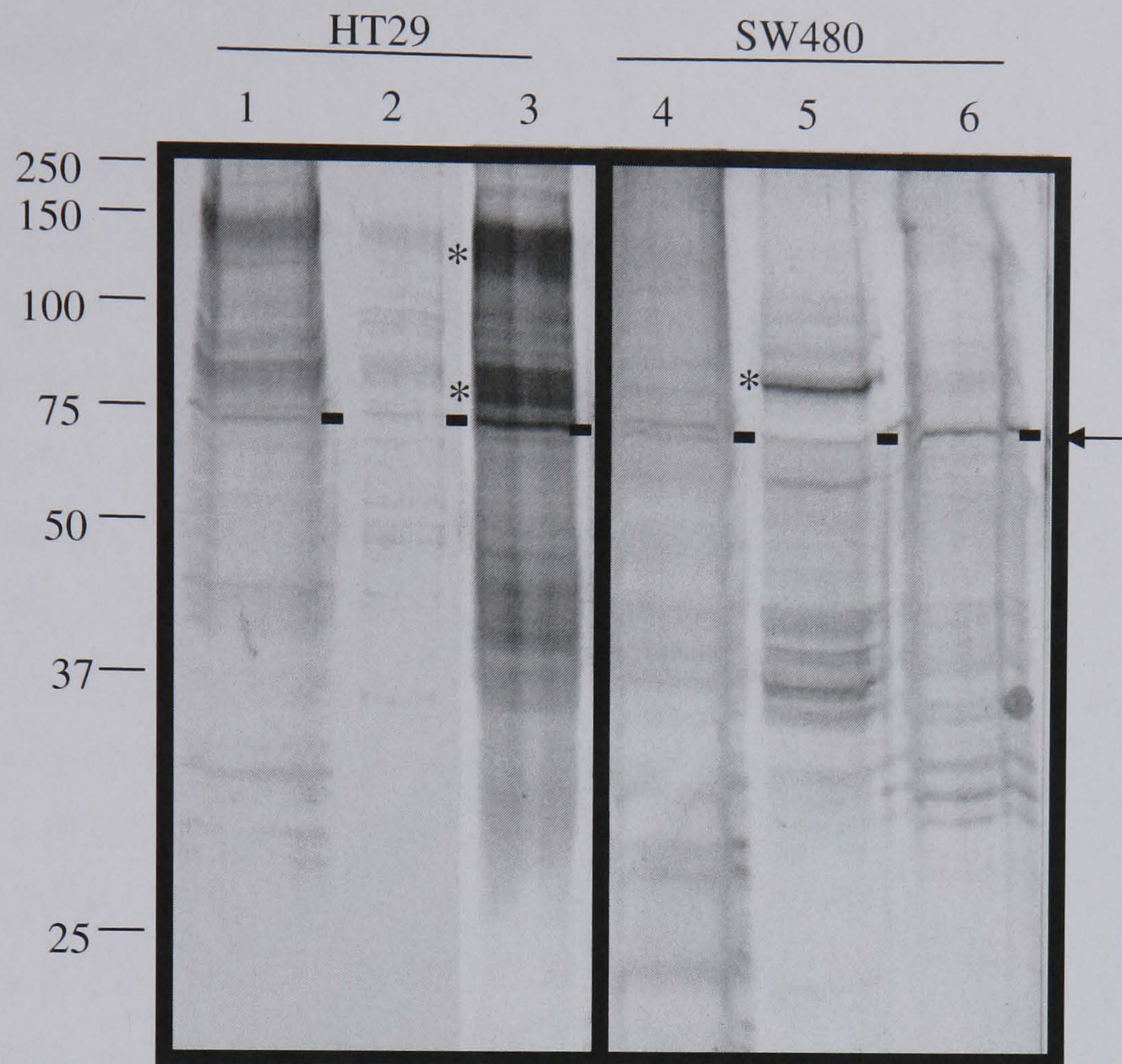
When using the DC protein assay the absorbance read at 655 nm was rather inconsistent throughout the experiment and hence the DC protein assay does not appear to be suitable to accurately assess the protein content of protein mixtures prepared in buffers containing any of the following chemicals, urea, CHAPS, DTT, and ampholytes. In experiments using the RC/DC protein assay the absorbance read at 750 nm was also rather inconsistent and was not sufficiently reliable to allow accurate assessment of the protein content of the protein mixtures prepared in buffers containing urea, CHAPS, DTT, and ampholytes. However, when the Bradford assay was used the results showed a linear association between absorbance and protein concentration for BSA prepared in the buffers above. The consistency of the readings throughout the experiment showed that the Bradford reagent is a reliable assay to use in this system.

### **4.3 Membrane preparation**

In chapter 3, studies using confocal microscopy demonstrated the predominant cell membrane binding of HPA to the metastatic cell line HT29 and hence the preparation of a cell membrane-enriched fraction was a focus of this work. Five cell membrane-enriched preparations were reproducibly carried out and the result a typical experiment is presented in this section.

In this experiment the two cell lines SW480 and HT29 were used. The proteins from the three fractions generated during the membrane preparation, (i) cell debris and nuclei, (ii) soluble proteins and (iii) membrane-enriched proteins were extracted in Laemmli buffer and analysed to evaluate the HPA-binding proteins. Proteins (20 µg) from the three fractions were separated by SDS-PAGE, transferred onto nitrocellulose and probed with biotinylated-HPA. This experiment shows that most of the HPA binding protein were recovered in the membrane fraction of the metastatic cell line HT29 (including the two most intense protein bands shown by asterixes in Figure 4.4, lane 3) confirming our previous observations and suggesting a successful enrichment of the HPA binding proteins. Amongst the cytoplasmic proteins of SW480 (Figure 4.4, lane 5) a protein is intensely bound by HPA and shown with an asterix. A protein of 75 kDa is consistently bound in the 6 protein fractions (indicated with a black arrow in Figure 4.4) and represent the non specific signal due to the streptavidin binding (Banks *et al.*, 2003).





**Figure 4.4:** Evaluation of the HPA binding glycoproteins content of the three fractions from the membrane preparation. 20  $\mu$ g of proteins extracted from the debris, cytosol and membrane of HT29 and SW480 were separated on a 12% SDS-PAGE and probed with the lectin HPA. The proteins extracted from the cell debris, the cytosol and the membrane-enriched fraction are shown in lane 1, 2 and 3 for HT29 and 4, 5 and 6 for SW480. The two main protein bands recognised by HPA in the membrane-enriched fraction of HT29 are shown by asterixes in lane 3. A protein band is intensely detected in the cytosolic fraction of SW480 and shown by an asterix in lane 5. A non specific protein band is detected in each lane and is indicated with an arrow.

The results the Western blot data for the HPA binding proteins are shown in a illustrate the successful enrichment of HPA-binding proteins in the membrane fraction of HT29 cells.

The binding of HPA to each of the three fractions from SW480 confirmed the observations of the confocal microscopy that HPA only binds weakly to this cell line. In HT29 the HPA binding varied between the fractions from low binding in the debris and soluble fractions to very intense binding in the membrane fraction. In HT29, most of the HPA binding was recovered in the membrane-enriched fraction.



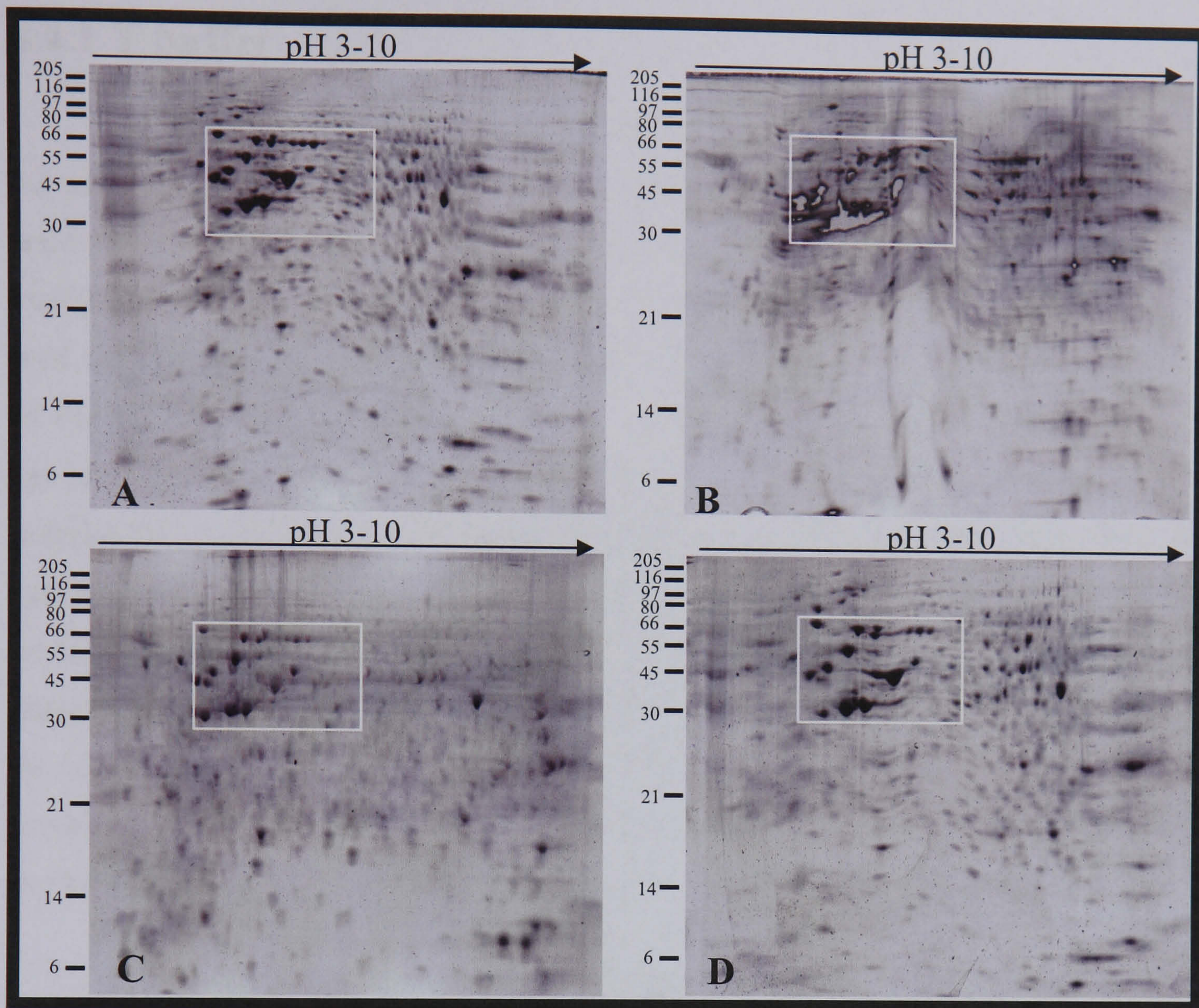
## 4.4 Solubilisation of membrane-enriched proteins for separation by 2-DE

The analysis of membrane proteins appeared crucial in our study because they comprise most of the HPA-binding epitopes which we intend identify. 2-DE offers one of the highest resolution available and enable the separation of post-translationally modified proteins. However the use non ionic/switterionic detergents do not guaranty a total recovery of membrane proteins, hence the solubilisation of membrane-enriched proteins and the choice of the solubilisation buffer remains a key element for proteomic analysis by 2-DE. The limitations of the use of 2-DE to study membrane-enriched proteins and possible improvements will be discussed in section 4.6. Four buffers were used to solubilise the proteins of the membrane-enriched fraction of HT29. U buffer (Urea 7M, CHAPS 4% w/v, DTT 1% w/v, Ampholytes 1% v/v), T buffer (Urea 7M, Thiourea 2M, CHAPS 4% w/v, DTT 1% w/v, Ampholytes 1% v/v), U7M/T buffer (Urea 7M, CHAPS 4% w/v, DTT 1% w/v, Ampholytes 1% v/v with 2M Thiourea added after protein assay is performed) and U7M/T2 (Urea 7M, CHAPS 4%, DTT 1%, Ampholytes 2% with 2M Thiourea added after protein assay is performed).

HT29 membrane-enriched protein samples were prepared in each buffer and reproducibly separated by 2-DE in duplicate. The gels stained with Sypro Ruby and one typical gel per condition is shown in Figure 4.6.

The HT29 membrane-enriched proteins solubilised in T buffer (Figure 4.6, panel A), U buffer (Figure 4.6 panel B), U7M/T buffer (Figure 4.6 panel C) and U7M/T2 buffer (Figure 4.6 panel D) presented a similar pattern of protein migration however proteins in U buffer showed distortion and poor resolution. The remaining three gels showed protein migration across the pI and MW range with slight variation in the quality of separation due to the yield of solubilisation achieved in each buffers. The T buffer (Thiourea 2M, Urea 7M, CHAPS 4% w/v, DTT 1% w/v, Ampholytes 1% v/v) seemed to offer the best solubilisation in this system.





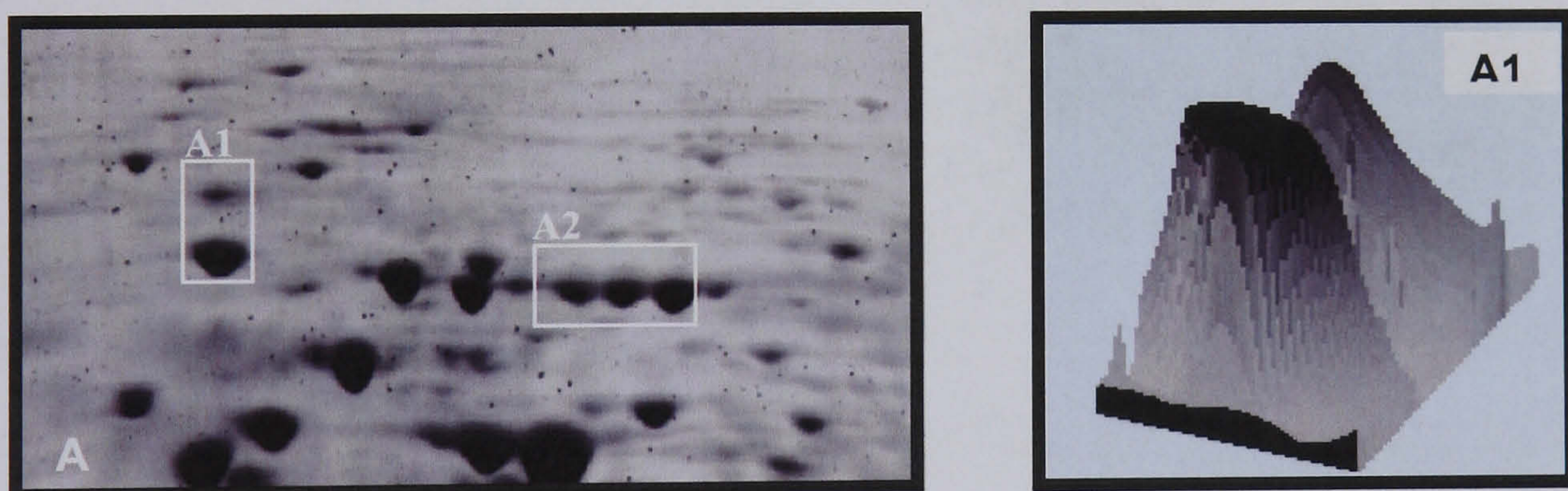
**Figure 4.5:** Profile of HT29 membrane-enriched proteins solubilised in four different buffers and separated by 2-DE. HT29 membrane proteins were prepared and solubilised in T buffer (panel A), U buffer (panel B), U7MT buffer (panel C) and U7MT2 buffer (panel D). 70  $\mu$ g of proteins were then loaded on a pH 3-10 IPG strip and subsequently on a 12% SDS-PAGE in order to achieve a bidimensional separation of the proteins. Similarities in the general pattern are observed in the four gels

We have selected a region MW 30-70 kDa and pI 4.5- 6.5 (Figure 4.5, white box) subject to variation in resolution depending on the buffer used to solubilise the proteins. The framed region contains a large selection of proteins and was used to further compare the quality of the protein separation of the four gels using the 2-D analysis software Melanie Image Master 2D Platinum (GE Healthcare).



### 4.4.1 T buffer

The framed region in Figure 4.5, panel A is presented in a zoom view (Figure 4.6 panel A). In this system the proteins were all well separated. Two specific regions were investigated further (Figure 4.6, panel A, boxes A1, A2) as they contain challenging proteins that seemed to vary across the four gels. Zone A1 contained two well separated protein spots, when presented in the 3D view this allows an assessment of the quality of the separation. The two peaks representing the two protein spots appeared sharp and well separated suggesting a good resolution, with one of the proteins being extracted in higher quantity and showing overloading in this detection system. The three proteins highlighted in box A2 were also well separated and again were somewhat overloaded as shown by the 3D view (Figure 4.6, panel A2). This detailed view confirmed that T buffer offers good extraction and separation of proteins including those with similar isoelectric points but unfortunately this buffer is not compatible with the protein assay and alternative buffers were tested. This gel was used as the reference gel for subsequent analyses.

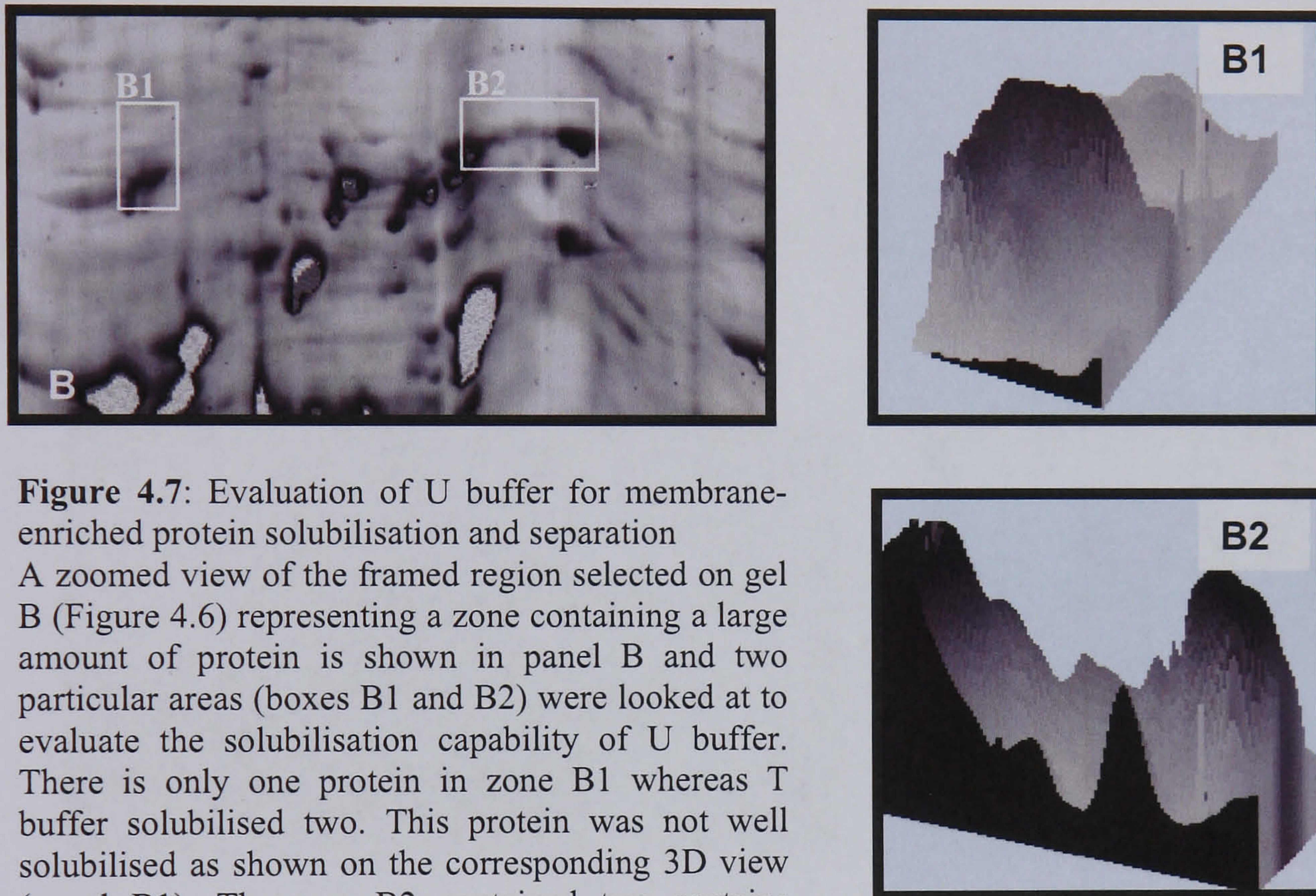


**Figure 4.6:** Evaluation of T buffer for membrane-enriched protein solubilisation and separation  
The zoom view of the framed area selected on the gel A (Figure 4.5) is shown in panel A and two particular areas (boxes A1 and A2) were looked at in order to evaluate the solubilisation capability of T buffer. The proteins in region 1 appeared well separated as illustrated by the two sharp and well separated peaks in the 3D view (panel A1). The area A2 contained three proteins of similar MW and very close pI; these proteins were well separated as shown by the three distinct peaks observed on the 3D view (panel A2).



#### 4.4.2 U buffer

The framed region from Figure 4.6 is shown in a zoom view as for the T buffer (Figure 4.7), in this view it is clear that U buffer does not enable protein solubilisation and resolution at the level observed with the T buffer (Figure 4.6).



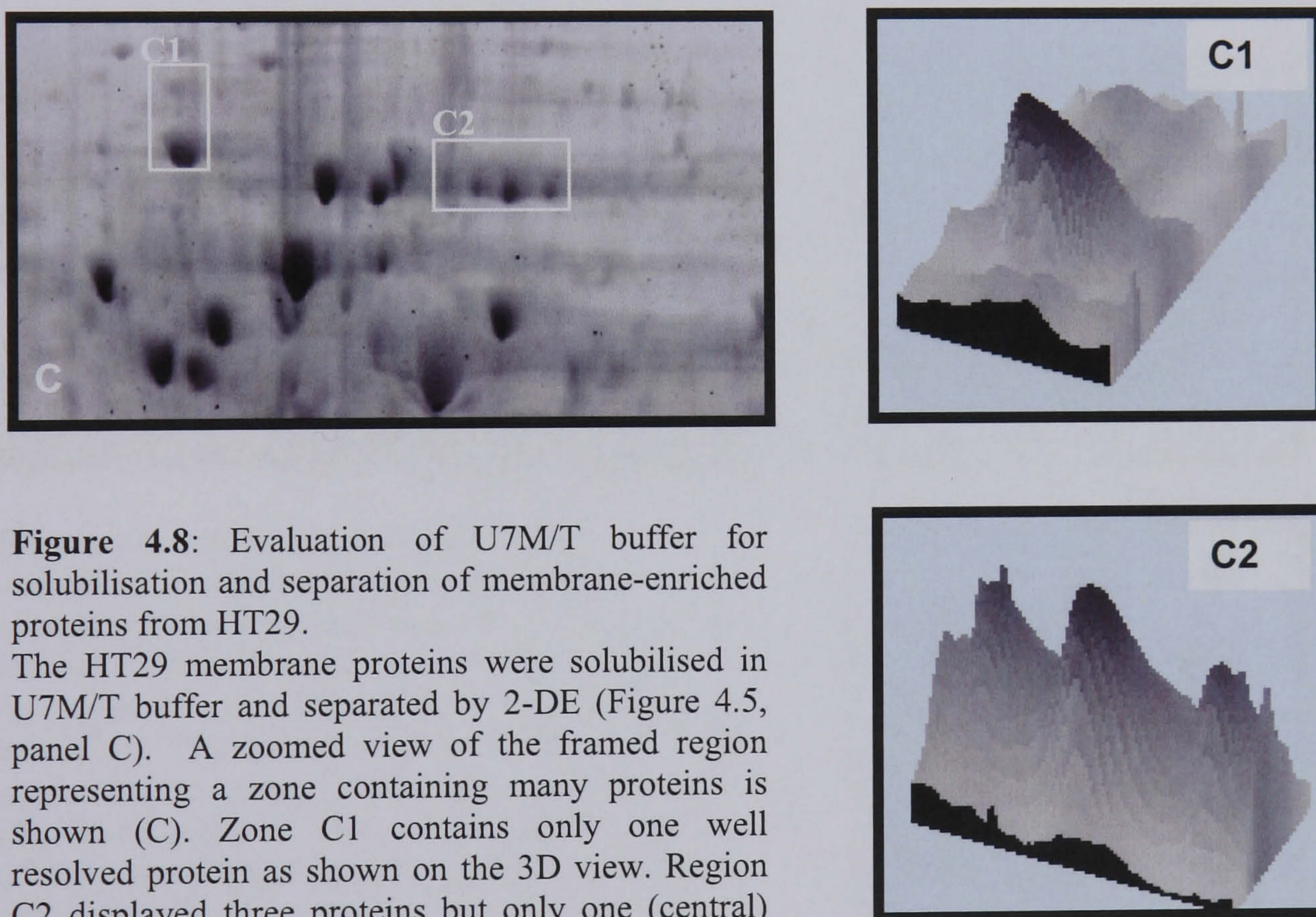
**Figure 4.7:** Evaluation of U buffer for membrane-enriched protein solubilisation and separation

A zoomed view of the framed region selected on gel B (Figure 4.6) representing a zone containing a large amount of protein is shown in panel B and two particular areas (boxes B1 and B2) were looked at to evaluate the solubilisation capability of U buffer. There is only one protein in zone B1 whereas T buffer solubilised two. This protein was not well solubilised as shown on the corresponding 3D view (panel B1). The area B2 contained two proteins instead of three with T buffer and those proteins were not well separated (panel B2).



### 4.4.3 U7M/T buffer

The U7M/T buffer contains the same components as T buffer with the difference that thiourea was added after protein assay. Thiourea in conjunction with urea has been shown to allow the solubilisation of hydrophobic proteins (Rabilloud, 1998). The framed region of gel C (Figure 4.5) was investigated with a particular interest in area C1 and C2 (Figure 4.8). The area C1 contained only one protein rather than the two observed with T buffer. In region C2 three proteins were recovered with a good resolution but in lower quantity compared to the T buffer. Therefore it can be surmised that the addition of thiourea after the protein assay improved the solubilisation of proteins although improvements would be needed to achieve the solubilisation and separation that was obtained with the T buffer.



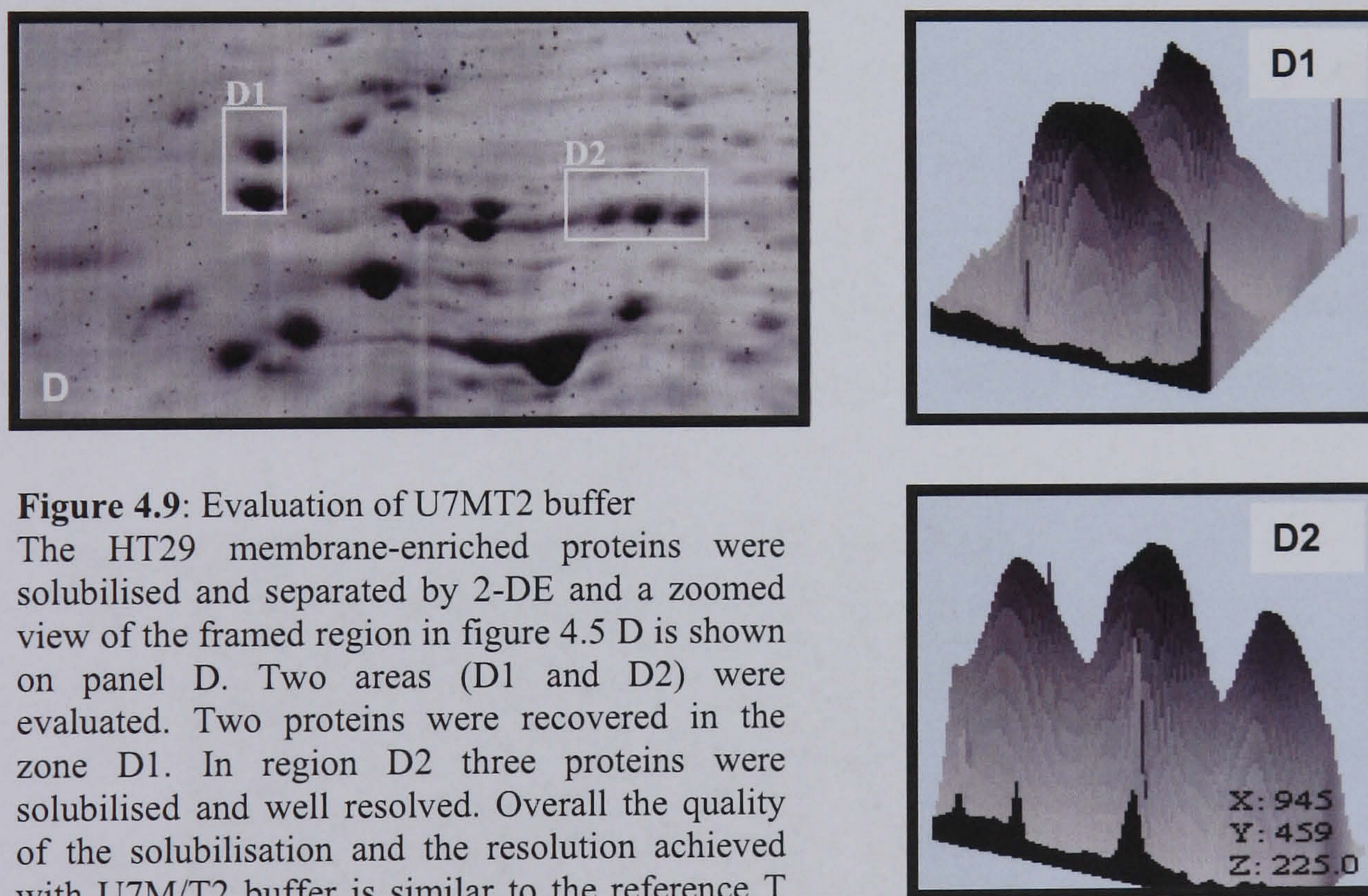
**Figure 4.8:** Evaluation of U7M/T buffer for solubilisation and separation of membrane-enriched proteins from HT29.

The HT29 membrane proteins were solubilised in U7M/T buffer and separated by 2-DE (Figure 4.5, panel C). A zoomed view of the framed region representing a zone containing many proteins is shown (C). Zone C1 contains only one well resolved protein as shown on the 3D view. Region C2 displayed three proteins but only one (central) showed good resolution.



#### 4.4.4 U7M/T2 buffer

To try to obtain the separation of proteins observed with buffer “T” and retain compatibility with the protein assay we used a buffer similar to the previous buffer U7M/T but the concentration of ampholytes was increased from 1% to 2% as the addition of carrier ampholytes enhances the solubility of individual proteins as they approach their isoelectric points. Ampholytes also produce an approximately uniform conductivity across the pH gradient. The quality of resolution was assessed as before. Figure 4.9 shows the proteins were solubilised and well separated and the quality of the separation obtained with U7M/T2 was similar to that achieved with T buffer and in addition it offered the possibility to evaluate the protein concentration of the sample prior to loading onto IPG strip and therefore this buffer was used in subsequent experiments.



**Figure 4.9:** Evaluation of U7MT2 buffer

The HT29 membrane-enriched proteins were solubilised and separated by 2-DE and a zoomed view of the framed region in figure 4.5 D is shown on panel D. Two areas (D1 and D2) were evaluated. Two proteins were recovered in the zone D1. In region D2 three proteins were solubilised and well resolved. Overall the quality of the solubilisation and the resolution achieved with U7M/T2 buffer is similar to the reference T buffer.



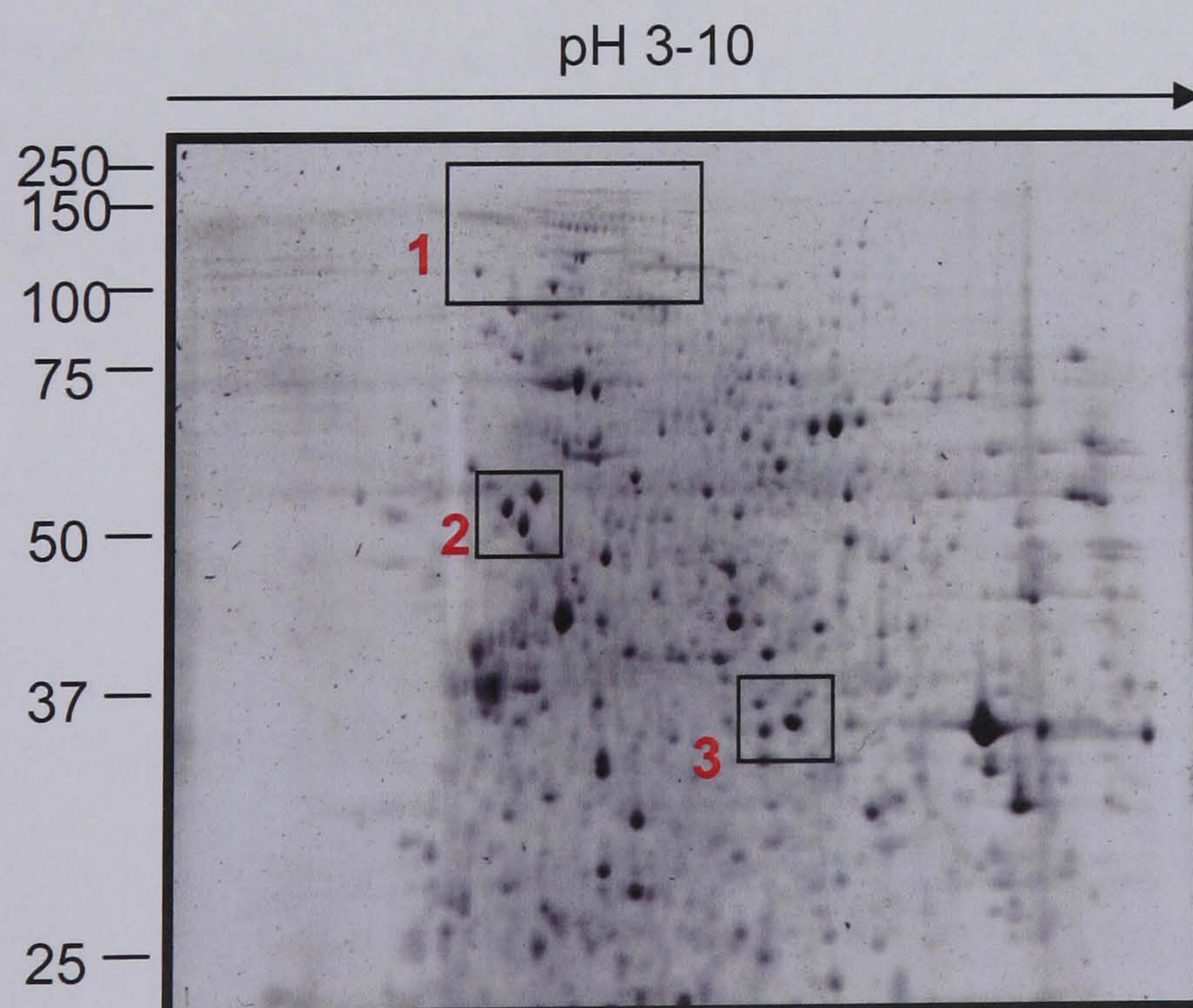
## 4.5 Reproducibility of the 2-DE system

In this section the reproducibility of the 2-DE separation was assessed using two analytical replicates of the same sample (HT29 membrane-enriched proteins). Next two gels from different samples were compared, one from HT29 and one from SW480.

Overall we aimed to determine whether the 2-DE system was suitable for the study of changes in protein expression in two cancer cell lines originating from the same organ and hence having organ/cell type specific protein composition but also having different malignant potential.

### 4.5.1 Analytical reproducibility using two replicates of HT29 membrane-enriched proteins

HT29 membrane-enriched proteins from the same membrane preparation were run on two separate gels in parallel and the reproducibility was assessed. An overview of the separation is shown in Figure 4.10. To evaluate the reproducibility between the two gels, three regions were chosen, these contained proteins which were low to high abundance and from low to high molecular mass. The three regions were numbered 1, 2, 3 on the gel shown in Figure 4.10 and are shown in more detail in Figure 4.11.



**Figure 4.10:** HT29 membrane-enriched proteins separated by 2-DE on a pH 3-10 IPG strip. 90  $\mu$ g of protein were loaded and proteins were stained with Sypro Ruby. Three regions numbered 1, 2, 3 were analysed in two replicates of the same gel.



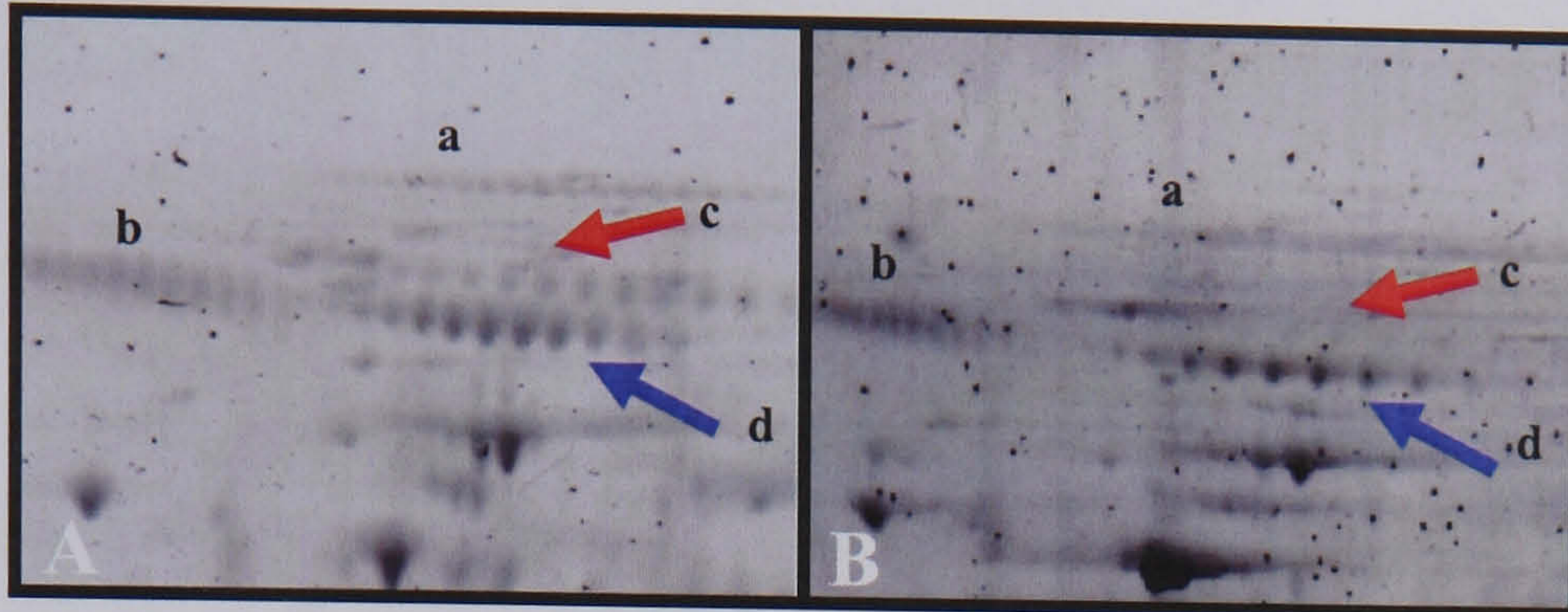
For region 1 we observed similar protein separation in both gels with some slight differences due to gel-to-gel variation. The zone is located from pH 5.5 to 7 and MW from 100 to 250 kDa and contained 4 groups of proteins (Figure 4.11, “a”, “b”, “c”, “d”) each containing several isoforms of very low abundance proteins as suggested by their faint Sypro Ruby staining (Figure 4.11, area 1). Some differences were observed in terms of intensity, for example protein “c” was less intense in replicate B as compared to replicate A. On the contrary protein “b” was less intense in replicate A. Overall, the pattern of migration was reproducible. Protein “d” was composed of 8 well separated isoforms in both gels A and B and these were of similar relative intensity. The reproducibility between these replicates confirmed the efficiency of the solubilisation buffer and showed the capacity of the 2D system to reproducibly separate low abundance protein isoforms with high resolution.

Area 2 (Figure 4.11) is located at pH 5.5-6, MW 45-60 kDa and represented three high abundance proteins between 50 and 60 kDa. Figure 4.11 shows a zoom view of area 2 from the two replicates A and B, the three proteins were reproducibly separated, the intensity of the staining was also very similar between A and B although the lower MW protein showed a slight decrease in intensity in replicate B. These three proteins are high abundance proteins and their migration pattern on 2-DE was reproducible across both replicates.

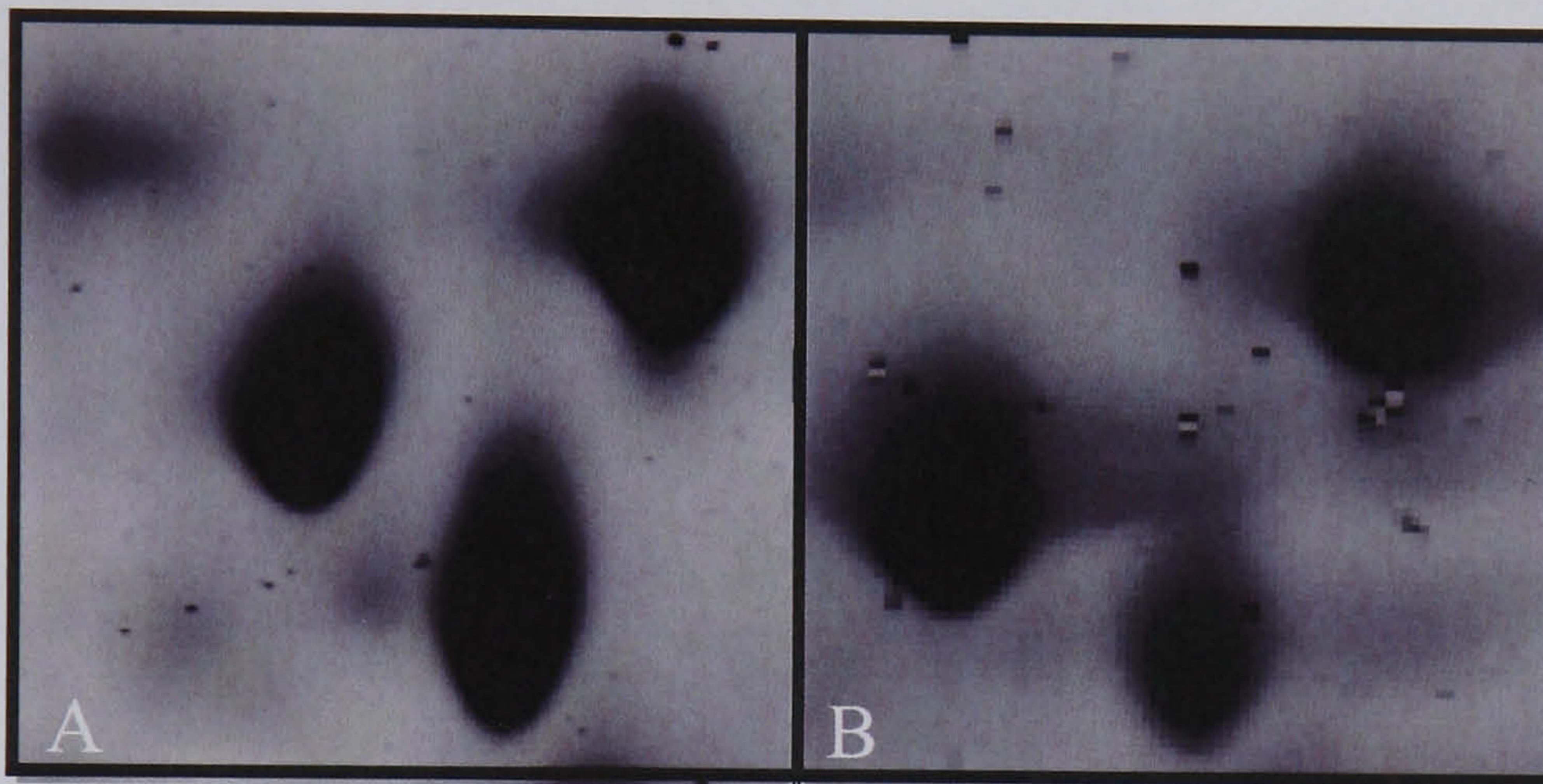
Area 3 (Figure 4.11) is located at pH 7.5-8, MW 30-35 kDa and contained four medium abundance proteins of size less than 37 kDa, the protein spots were found in both replicates with a similar relative intensity.



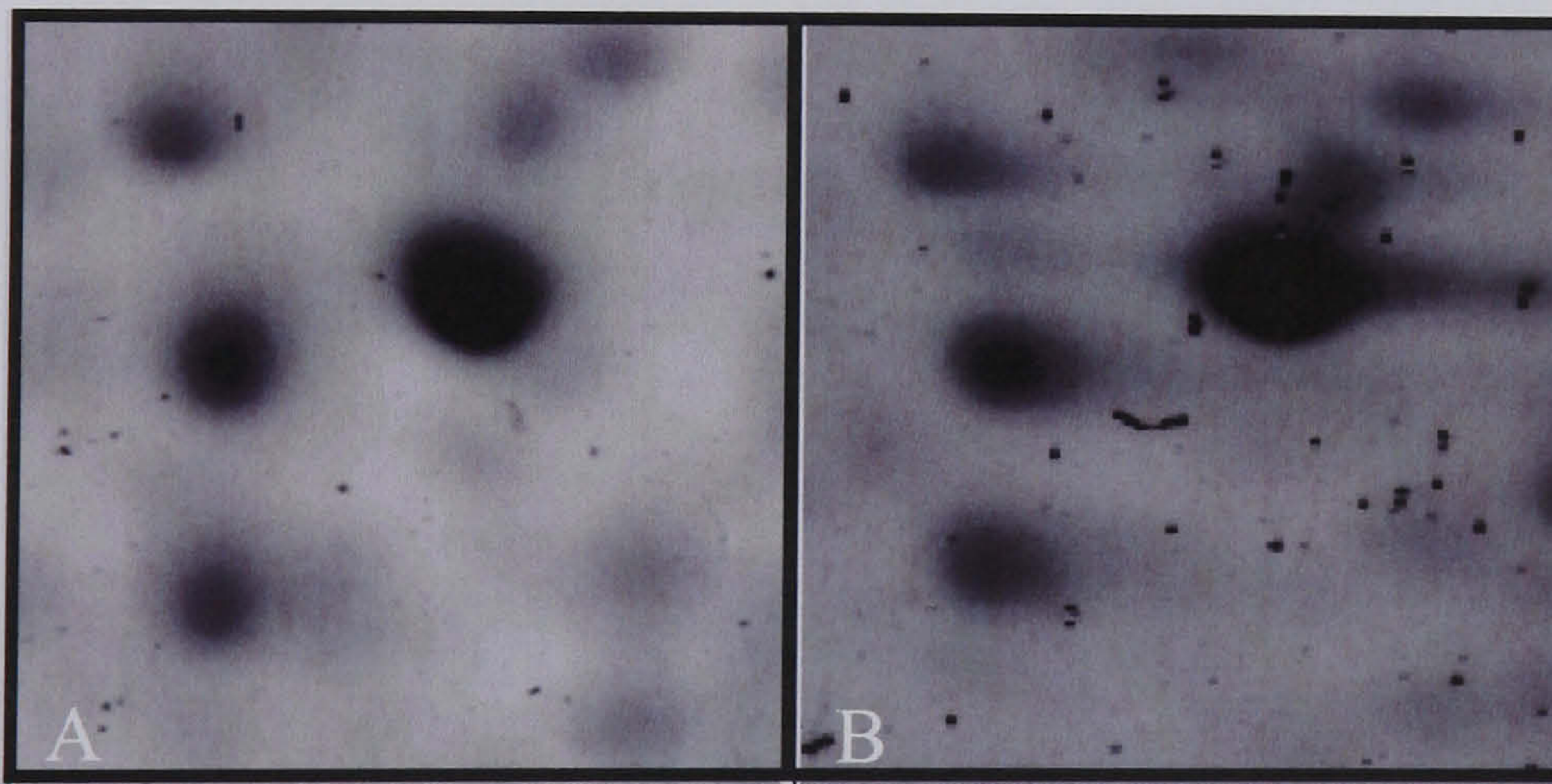
1- AREA 1 (MW 100-250 kDa; pH 5.5-7)



2- AREA 2 (MW 45-60 kDa; pH 5.5-6)



3- AREA 3 (MW 35-40 kDa; pH 7.5-8)



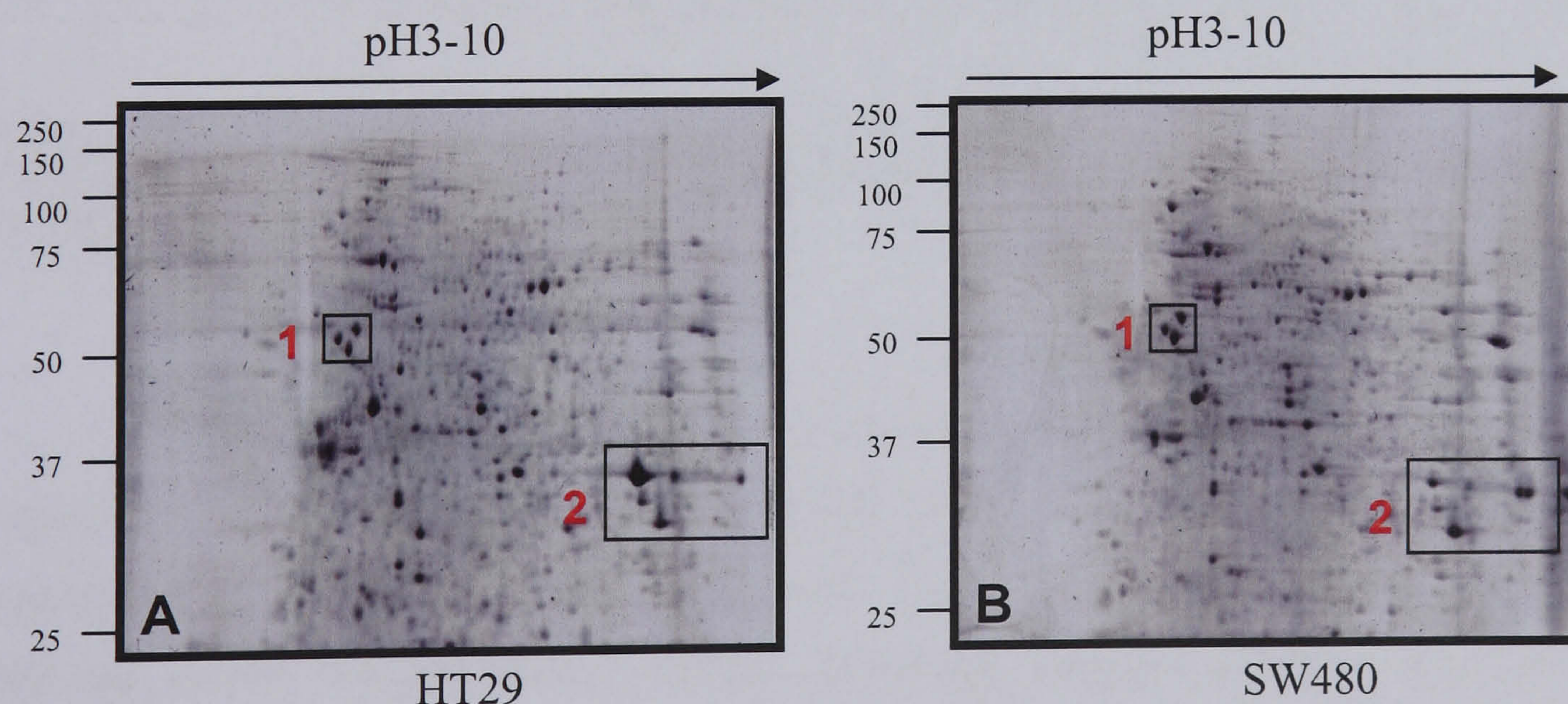
**Figure 4.11:** Zoom view of area 1, 2 and 3 in replicates A and B of HT29 membrane-enriched proteins. Region 1 is located from pH 5.5 to 7 and MW from 100 to 250 kDa. Four trails of protein are highlighted and named “a”, “b”, “c” and “d”. Those four low abundance proteins comprised many isoforms and were reproducible in both replicates A and B. Protein “c” seemed to sustain a significant variation in quantity indicated with the red arrow. The eight isoforms of the protein d were reproducibly found in both replicates (blue arrow). Region 2 is located at pH 5.5-6, MW 45-60 kDa and contained three high abundance proteins. The 2-DE migration pattern of these three proteins is similar in the two replicates A and B with a slight decrease in abundance of the lower MW protein in gel B. The four proteins of the region 3 are located at pH 7.5-8, MW 30-35 kDa and show similar pattern of migration in replicates A and B.



The analysis of the three characteristic regions of the two replicates of the same sample showed that we have developed a sensitive, reproducible and robust methodology to separate CRC membrane-enriched proteins by 2-DE.

#### 4.5.2 Comparison of HT29/SW480 membrane-enriched proteins using a 2-DE system

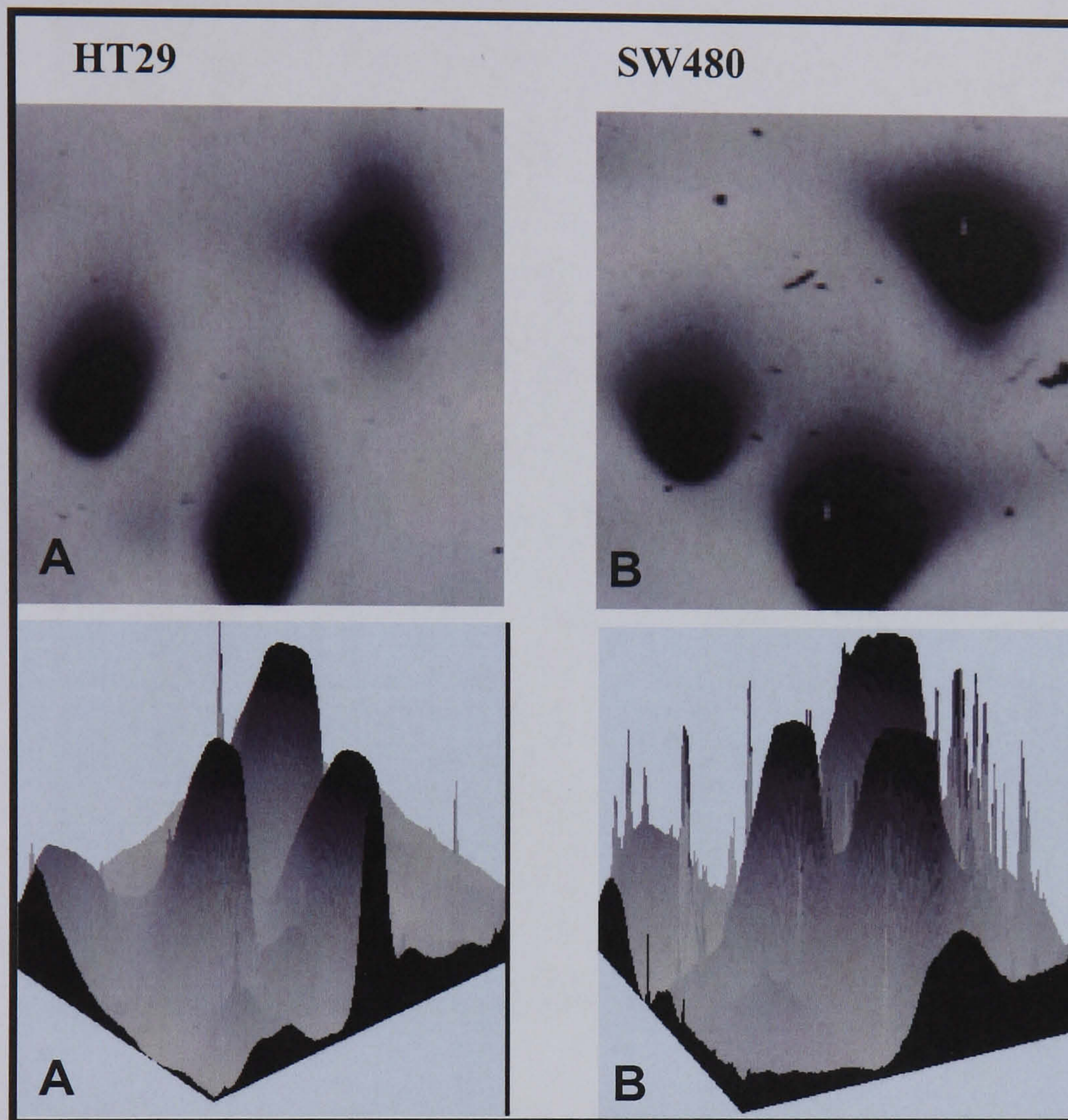
HT29 and SW480 both originate from colorectal adenocarcinomas and would therefore be expected to contain similar structural proteins, these might be used as reference proteins. We analysed the protein profile of the two cell lines separated by 2-DE. In this experiment 90  $\mu$ g of membrane-enriched proteins from either HT29 or SW480 were loaded on two gels, separated and stained with Sypro Ruby. The general pattern of migration of the proteins from HT29 and SW480 appeared very similar with some variations of protein expression (Figure 4.12). Two regions were evaluated in more detail. The first region contained structural proteins (tubulin  $\alpha$  and  $\beta$ ) that were expressed in near same proportions in both of the cell types, the second region was composed of proteins which showed differences in intensity in HT29 and SW480.



**Figure 4.12:** Comparison of HT29 and SW480 membrane-enriched proteins separated by 2-DE. 90  $\mu$ g of proteins from HT29 (A) and SW480 (B) were separated on a pH3-10 IPG strip, then on a 12% SDS-PAGE before fluorescent staining with Sypro Ruby. The two regions that were investigated further are framed and numbered 1 and 2 in both gels.



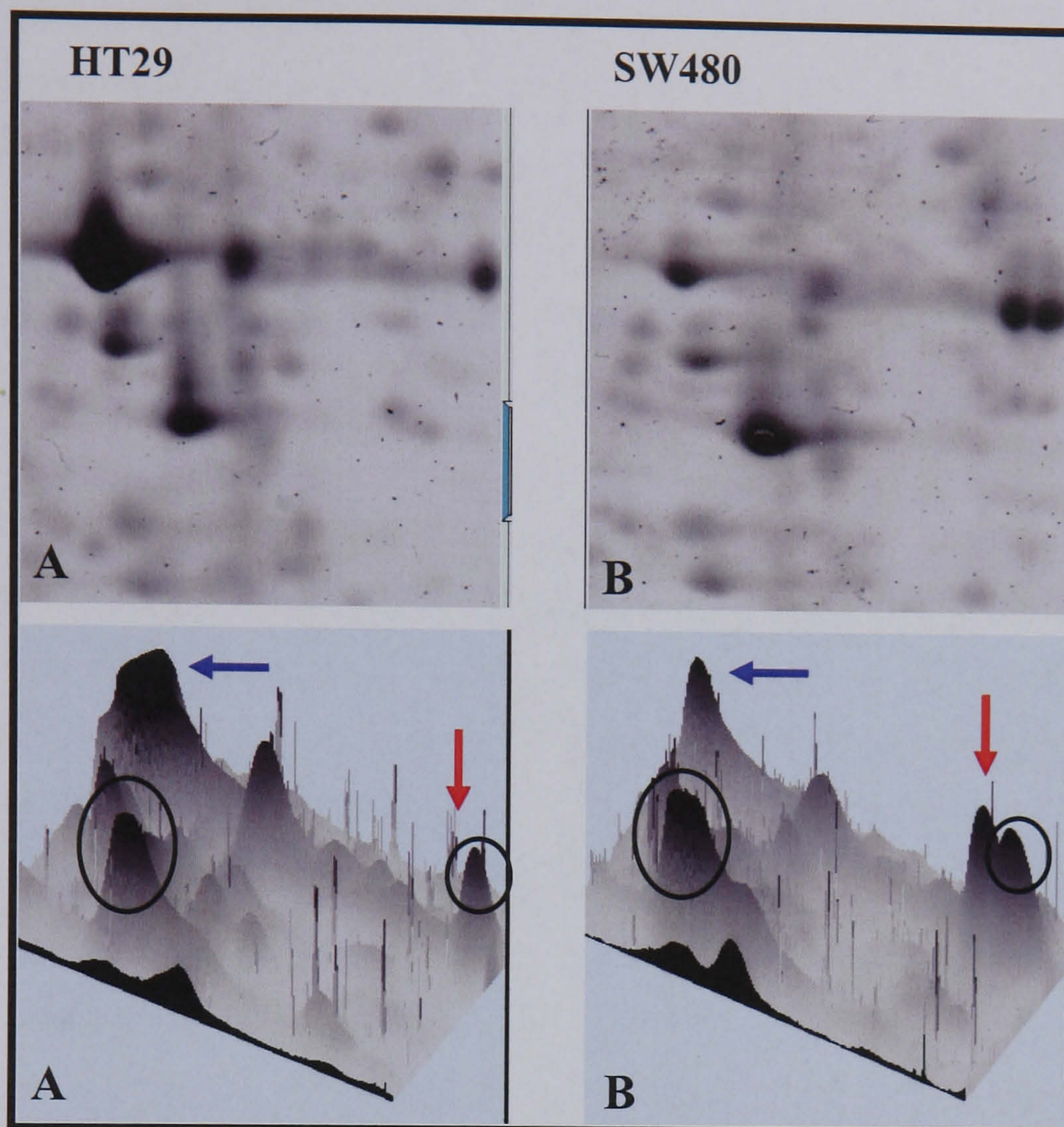
A zoom view and a mountain plot from region 1 of Figure 4.12 contained three proteins uniformly expressed in both cell lines (Figure 4.13).



**Figure 4.13:** Zoom and 3D view of area 1 (MW 45-60 kDa, pH 5.5-6) from Figure 4.12. The three proteins contained in this box are identically separated on HT29 (panel A) and SW480 (panel B). Their patterns of migration are also very similar.

The framed-zone 2 from Figure 4.12 was similarly analysed and an enlarged view is shown in Figure 4.14. This showed similar protein patterns with discrete changes in the amounts of some of the proteins. In the 3D view of Figure 4.14 the blue and red arrows indicate proteins present in greater amounts in HT29 and SW480 respectively. Two proteins (circled in Figure 4.14) have similar intensities in both of the two cell lines, this taken with the observation regarding similar quantity of structural proteins rules out the possibility of a variation due to protein loading. The reproducibility of the 2-DE system therefore offered potential to study the level of expression of HPA-binding proteins in these two cell lines.





**Figure 4.14:** Zoomed and 3D view of area 2 (MW 30-35 kDa, pH 9-10) from Figure 4.12. The proteins of this area are identically separated but variations in their expression are noted. The two circled proteins are expressed in similar quantity but the arrows show protein spots sustaining increase in protein expression in HT29 (blue) and in SW480 (red).

## 4.6 Discussion

In this chapter we first evaluated the compatibility of the chemicals used in solubilisation buffers with several protein assay methods and we established that the Bradford assay was the most suitable and reliable to determine the protein content when a complex mixture of chemicals containing up to 7M urea, 4% CHAPS, 1% DTT and 2% ampholytes was used.

There have only been a few studies investigating cancer-derived HPA binding proteins and even fewer investigating the HPA-binding partner of membrane-enriched proteins from CRC cells. It seems that high speed centrifugation is the preferred approach to study such proteins (De Albuquerque Garcia Redondo *et al.*, 2004)



although detergent-based approaches are also available to prepare membrane-enriched proteins. De Albuquerque Garcia Redondo *et al.* (2004) were the first group to investigate the membrane proteins recognised by lectins such as HPA and WGA of the surface of CRC cell lines (HCT116 and CaCo2). The methods to prepare membrane proteins in our study are slightly different with respect to the buffers used but the key step is the use of high speed centrifugation and this was a common feature in both studies. A comparison of the membrane-enriched protein content of HT29 (our study) and HCT116 (De Albuquerque Garcia Redondo's group) showed that we solubilised, in higher quantity, the same 8 proteins bands ranging from 20 kDa to 97 kDa that they identified in the membrane-enriched fractions of HCT116 but we also identified several other proteins. In addition, we also showed that the membrane-enriched fraction of HT29 contained the majority of the total HPA-binding proteins of HT29. We concluded that our approach enabled us to prepare a protein fraction enriched in membrane components and that this constitutes an ideal model for studying HPA-binding proteins in CRC.

The analysis of membrane proteins remains a major challenge for 2-DE based proteomics. For a long time membrane proteins could not be solubilised under the condition required for IEF (Wikins *et al.*, 1998). However the use of thiourea, urea and the introduction of zwitterionic detergents has enabled the analysis of some membrane proteins by 2-DE (Rabilloud *et al.*, 1997; Chevallet *et al.*, 1998; Rabilloud *et al.*, 1999). We evaluated the efficiency of several solubilisation buffers by comparing the separation of the proteins by 2-DE and found that the U7M/T2 buffer allowed efficient solubilisation of membrane-enriched proteins and compatibility with the Bradford assay and that this system was the solubilisation buffer of choice. However there are not ideal solutions to the problem of solubilisation of hydrophobic proteins and it is well accepted that various membrane proteins will not be solubilised with a single non ionic/zwitterionic detergent such as CHAPS. This is detrimental as some important HPA-binding proteins may be lost when studied by 2-DE. The solubilisation of membrane proteins has been studied by several research groups contributing to a great improvement of their analysis by 2-DE or by other methods. In particular the use of non-ionic and/or zwitterionic detergents with higher "solubilising power" such as dodecyl maltoside, decaethylene glycol monohexadecyl ether, triton X100 or ASB14



has been shown to improve the solubilisation of integral membrane proteins (Luche *et al.*, 2003). The composition of the solubilisation buffers has to be empirically adapted to reach the best solubilisation rate for the proteins of interest. New techniques have also been developed to study the highly hydrophobic proteins such as proteolytic digestion prior to chromatographic separation (RP LC) and identification by MS (Rabilloud, 2003; Wu *et al.*, 2003). Also the labelling of cell surface proteins (e.g. with biotin) followed by affinity purification and identification of tryptic peptides by MS greatly improve the analysis of membrane proteins (Sabarth *et al.*, 2002; Clifton *et al.*, 2007; Zhang *et al.*, 2003). Although in our case the 2-DE with U7M/T2 buffer appeared well suited to analyse the HPA-binding proteins of HT29, the use of another detergent or other techniques could enhance our analysis.

Finally we evaluated the robustness of the separation method, 2-DE. We assessed the reproducibility between two analytical replicates and we established that the method can be used to detect changes in protein expression in HT29 and SW480 cells. The protein map of the membrane-enriched proteins from HT29 separated using 2-DE with pH3-10 IPG strips was comparable to the 2D map generated for the same cell line by Tan *et al.* (2002). Several high abundance proteins of MW ranging from 40 kDa to 70 kDa were found to be present in both experiments although different samples were used, total lysate (Tan *et al.*) and membrane preparation (our group). For example the three high abundance proteins that we analysed (Figure 4.12, Area 2, MW 45-60 kDa, pH 5.5-6) were common to both our system and that of Tan *et al.*. We also observed high MW proteins (Figure 4.12, Area 1, MW 100-250 kDa; pH 5.5-7) that were not identified in the 2D map of Tan *et al.* This may be because these proteins are membrane proteins which were enriched in the high speed ultracentrifugation step. More over the resolution obtained in our experiment using 7 cm strips was as good as the 2D map obtained using 17 cm strips by Tan *et al.* This suggests that our method for solubilisation and separation generates high quality 2D maps. From this work we concluded that the 2-D system is ideally suited to allow the analysis of protein levels and the HPA binding proteins with a high probability that many of these are found on the surface of HT29 cells.



## **CHAPTER FIVE:**

### **Identification of HPA binding glycoproteins in HT29 and SW480 cells**



## 5.1 Introduction

Many studies have described the ability of the lectin HPA to bind poor prognosis cancer cells and cell lines with metastatic potential such as HT29. We confirmed those findings using the cell lines HT29, SW480 and SW620 in conjunction with fluorescently labelled lectin and using both confocal microscopy and microtitre assay.

Our previous experiments with HT29 showed mainly membrane localisation of the binding (Figure 4.4). The utility of HPA as a prognostic tool would be expected to come from its ability to bind to membrane glycoproteins as HPA binding ligands from cancer cells have been linked to the metastatic spread of colorectal cancer cells *in vivo* (Schumacher *et al.*, 1994) and has also been shown to be implicated in adhesion to endothelial cells (reviewed by Dwek and Brooks, 2004). The investigation of the HPA binding proteins of the cell surface of a metastatic cell line HT29 was therefore used as an approach to assist in the discovery of glycoproteins involved in the metastatic process. De Albuquerque Garcia Redondo *et al.* (2004) was the first group to study the HPA binding proteins of colorectal cancer cell lines using isolated cell membranes and they described glycoproteins that are common to all the cell lines as well as differences in the composition of the HPA binding proteins, for this HCT116, Caco-2 and IEC-6 cell lines were used. Observations were made using 1-DE and lectin blotting but in this study De Albuquerque Garcia Redondo *et al.* did not identify the HPA binding glycoproteins. In 2001, Brooks *et al.* studied the HPA binding proteins in various breast cancer cell lines and highlighted 11 major protein bands ranging from 20 to 200 kDa but the proteins were simply evaluated with reference to their molecular weight. Previously Schumacher *et al.* (1995) and Mitchell *et al.* (1995) reported 4 and 7 HPA binding bands ranging from 20 to 90 kDa including bands at 69 kDa and 90 kDa identified as an N-acetylgalactosaminylated form of albumin and the transferrin receptor.

We used HT29, SW480 and SW620 as cell lines models of metastasis. HT29 and SW480 are HPA positive and HPA negative as described earlier. A proteomic approach was undertaken this was based on 1DE and 2DE separation of membrane-enriched proteins followed by lectin binding. HPA affinity chromatography combined with 2-dimensional electrophoresis (2-DE) and mass spectrometry allowed us to discern

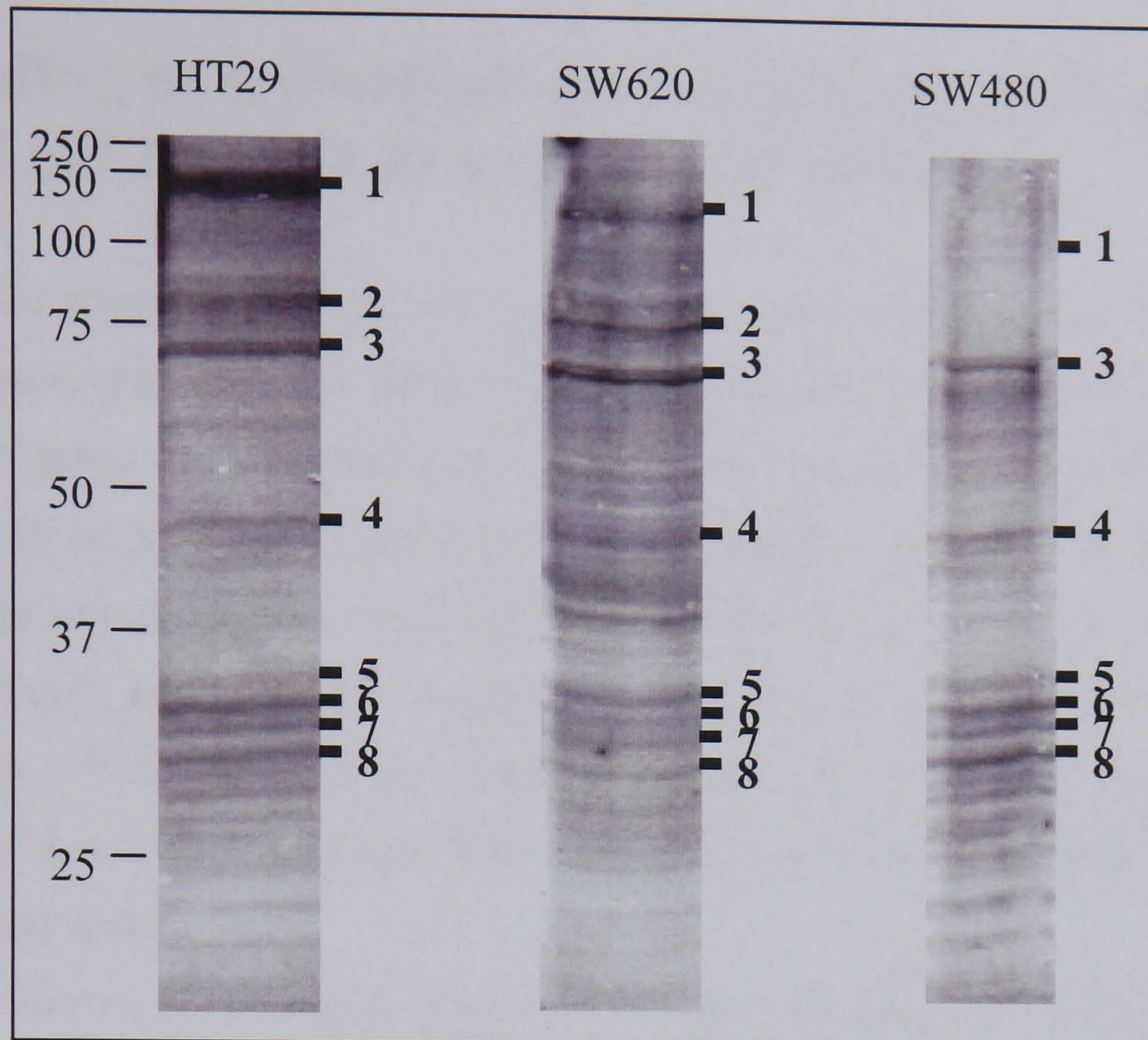


and identify the HPA binding proteins in the cell line bearing the metastatic phenotype (HT29).

## **5.2 Overview of the HPA binding proteins of HT29, SW620, SW480 by SDS-PAGE and lectin blotting.**

SDS-PAGE analysis followed by HPA-western blotting of the enriched plasma membrane fractions from HT29, SW620, and SW480 was performed in three separate experiments. A typical lectin blotting is shown in this sections (Figure 5.1) and highlighted similarities in HPA-binding but also allowed identification of differences between the cell lines. HT29 contained 17 glycoproteins recognised by HPA ranging from 24 kDa to 135 kDa. SW480 and SW620 both contained 18 proteins bands ranging from ~ 24 to 130 kDa and ~25 to 130 kDa respectively, the exhaustive list of proteins bands detected in each cell line as well as their relative abundance and molecular weights are reported in the Appendix 5. Figure 5.1 shows the results of the western blot analysis, the bands which showed varied expression across the cell lines (bands 1, 2) and also the major protein bands common to the three cell lines (bands 3, 4, 6, 7, 8 and 9). The MW of proteins 1-8 for each cell line are shown in Table 5.1. In the HPA positive cell lines HT29 and SW620, two proteins of high intensity (bands 1 and 2 were the predominant HPA binding sites. Protein bands 1 and 2 were subject to variations in intensity across the 3 cell lines. Band 1 (GP130) was a very intense HPA-binding polypeptide in HT29, less intense in SW620 and almost non-detectable in SW480. Similarly band 2, (GP80) showed decreased intensity of HPA-binding across the cell lines from intense in HT29 to moderate in SW620 and non-detectable in SW480. When comparing all the three cell lines at the 1-DE level it became obvious that most of the proteins recognised by HPA in SW480 (3-8) were also recognised in HT29 and SW620, therefore we classified these glycoproteins recognised by HPA as “non specific” binding. Two major glycoproteins GP130 and GP80 attracted our interest as they were the most intense HPA binding species in the HPA positive HT29 and SW620 whereas they were almost non-detectable in SW480.





**Figure 5.1:** HPA lectin blotting after separation by 1DE. 20  $\mu$ g of proteins of the three cell lines were separated on a 12% SDS-PAGE, probed with HPA and detected with DAB/H<sub>2</sub>O<sub>2</sub>. The proteins 1 and 2 are recognised by HPA in HT29 and SW620 and constitute the main HPA binding sites. The protein 3 is consistently found in the three cell lines and constitute the background of signal due to streptavidin binding. The proteins 4-8 are recognised by HPA in the Three cell lines.

Band number	Molecular Weight (kDa)		
	HT29	SW620	SW480
1	135	130	130
2	87	85	N/D
3	74	71	73
4	45	44	44
5	33	32	32
6	31	31	31
7	30	30	30
8	29	29	29

**Table 5.1:** Proteins recognised by HPA in HT29, SW620 and SW480 and their relative molecular weight. N/D=not detectable



### 5.3 Binding of a blood group A antibody to Western blots of HT29, SW620 and SW480 proteins

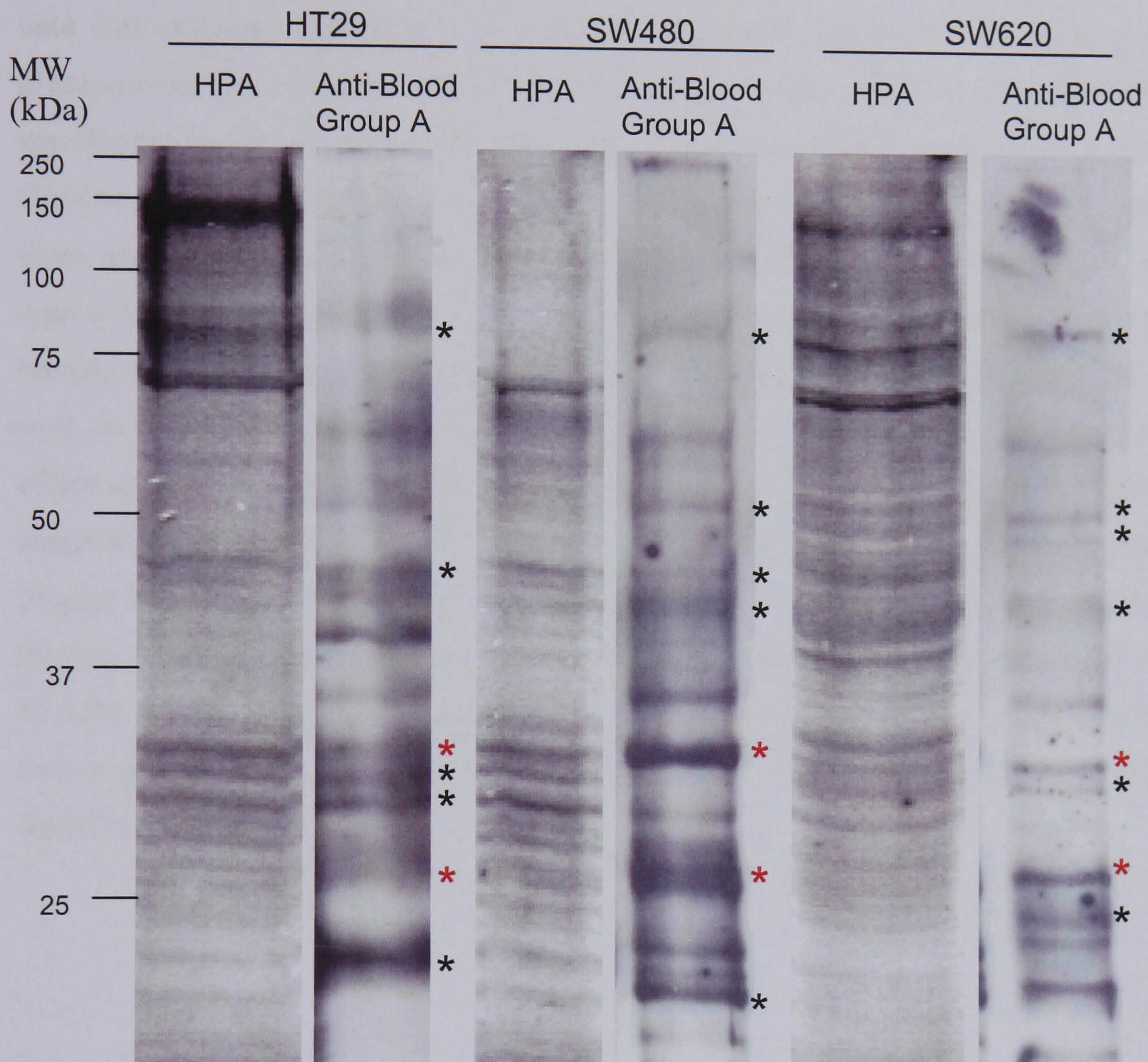
In this experiment we aimed to determine whether HPA simply recognises the blood group A epitope in the three cell lines, HT29, SW480 and SW620 (all derived from blood group A individuals). We used a murine monoclonal antibody directed against the blood group A antigen to probe the proteins of the three cell lines separated by 1-DE, the aim of this step was to determine whether the glycoproteins recognised by HPA are blood group antigen expression or if they are glycans with a different composition. HT29, SW480 and SW620 showed 12, 11 and 13 protein species respectively that were recognised by the blood group A antibody, these ranged from ~20-80 kDa (Figure 5.2)

Similarities in the overall pattern of proteins carrying blood group epitopes are observed between the three cell lines of our model however interesting variations are observed in particular between SW480 and SW620, the two cell lines with different metastatic capabilities derived from the same blood group A patient. Indeed changes in the composition of proteins as well as the intensity of the signal suggest that the cells with a metastatic potential (SW620) undergo modification in their glycan repertoire. In particular the reduction of the blood group A epitopes as detected by the blood group A monoclonal antibody seem to be part of the changes leading to a metastatic phenotype as previously suggested by Ichikawa *et al.* (1998).

The blood group A antibody bound most intensely to proteins between 20 kDa and 85 kDa (shown by asterisks on Figure 5.2) these bands were also recognised by HPA in HT29, SW480 and SW620. Two of these proteins appeared to be common to all of the cell lines (shown by red asterisks on Figure 5.2). The proteins described in section 5.2 as “non specific” with respect to HPA binding also appeared to be the major bands that bound the anti-blood group A antibody although GP 80 seems to be composed of two HPA-binding bands and only one of these carried the blood group A antigen. This suggests that some of the epitopes recognised by HPA contain glycans bearing the blood group A antigen. However the blood group A epitope was not detected on all of the HPA binding glycoproteins, for example, GP130 and several other proteins that bound HPA in a less intense manner. This suggested that the binding of



HPA to the metastatic cell lines was by recognition of epitopes bearing glycosylation motifs distinct from the blood group A antigen potentially encompassing part of the blood group A antigen.



**Figure 5.2:** HT29, SW480 and SW620 membrane-enriched proteins separated by 1-DE and Western blotting.

Detection of blood group A antigen using a monoclonal antibody directed against human blood group A epitope. The equivalent Western blot probed with HPA is shown as a reference blot.

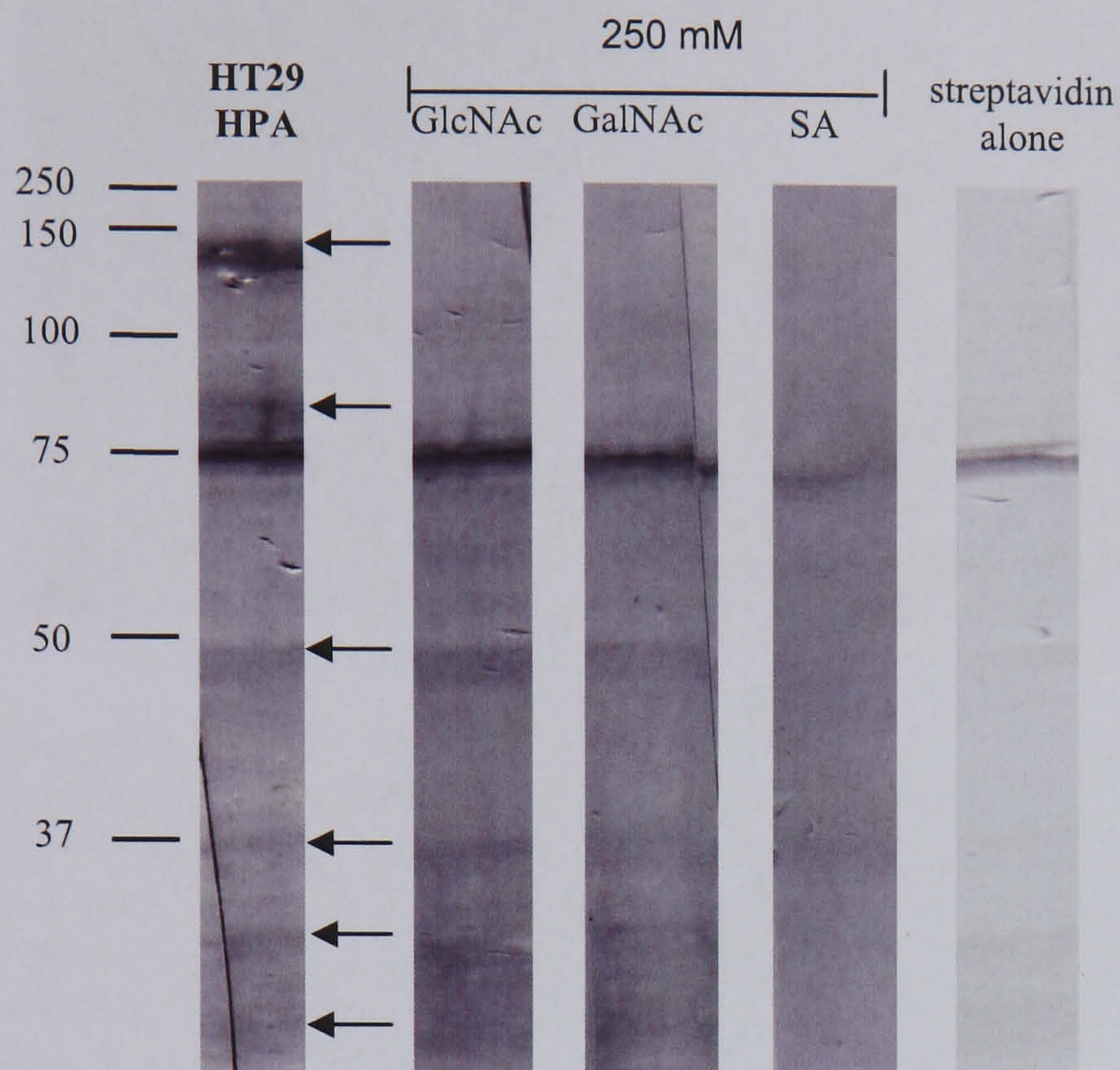
The anti-blood group A antibody detected 12, 11 and 13 protein bands respectively in HT29, SW480 and SW620 in a range of sizes from ~20 to 80 kDa. 7, 7 and 8 proteins (shown by asterisks) were detected by HPA and the anti-blood group A antibody in HT29, SW480 and SW620 respectively. Amongst these, 2 protein bands were commonly found in every cell line and are shown by red asterisks. GP80 was recognised by HPA and the anti-blood group A antibody suggesting that its HPA binding sites contain the blood group A residues. In contrast, GP130 was only detected by HPA and did not bind the anti-blood group A antibody.



## **5.4 Inhibition study of HPA binding to membrane-enriched proteins from HT29 at the protein level**

Membrane-enriched proteins from HT29 were separated by 1-DE and blotted onto nitrocellulose membranes. In order to determine that HPA was binding the glycoproteins via a glycan-lectin interaction and also to determine the monosaccharide specificities we pre-incubated the lectin for 30 min in a buffer containing GalNAc, GlcNAc or SA before probing the Western blot. These experiments were repeated three times and were undertaken using monosaccharides in the range 50 to 250 mM, and the typical data for 250 mM solutions are shown. GlcNAc, GalNAc and SA all inhibited the binding of HPA to the predominant HPA binding polypeptide of 130 kDa (GP130) as well as to the 80 kDa species (GP80) (Figure 5.3). GalNAc was a slightly more effective inhibitor of HPA binding to the lower molecular weight proteins than GlcNAc supporting the data that showed these proteins contain the blood group A epitope (Figure 5.2). Incubation with SA at this concentration abrogated the majority of HPA-binding to HT29 proteins, including the lower molecular weight species of 25, 31 and 45 kDa. The results illustrate that the interaction between biotinylated HPA and the cancer glycoproteins is via a lectin-mediated interaction that may be inhibited using GalNAc, GlcNAc and/or SA.



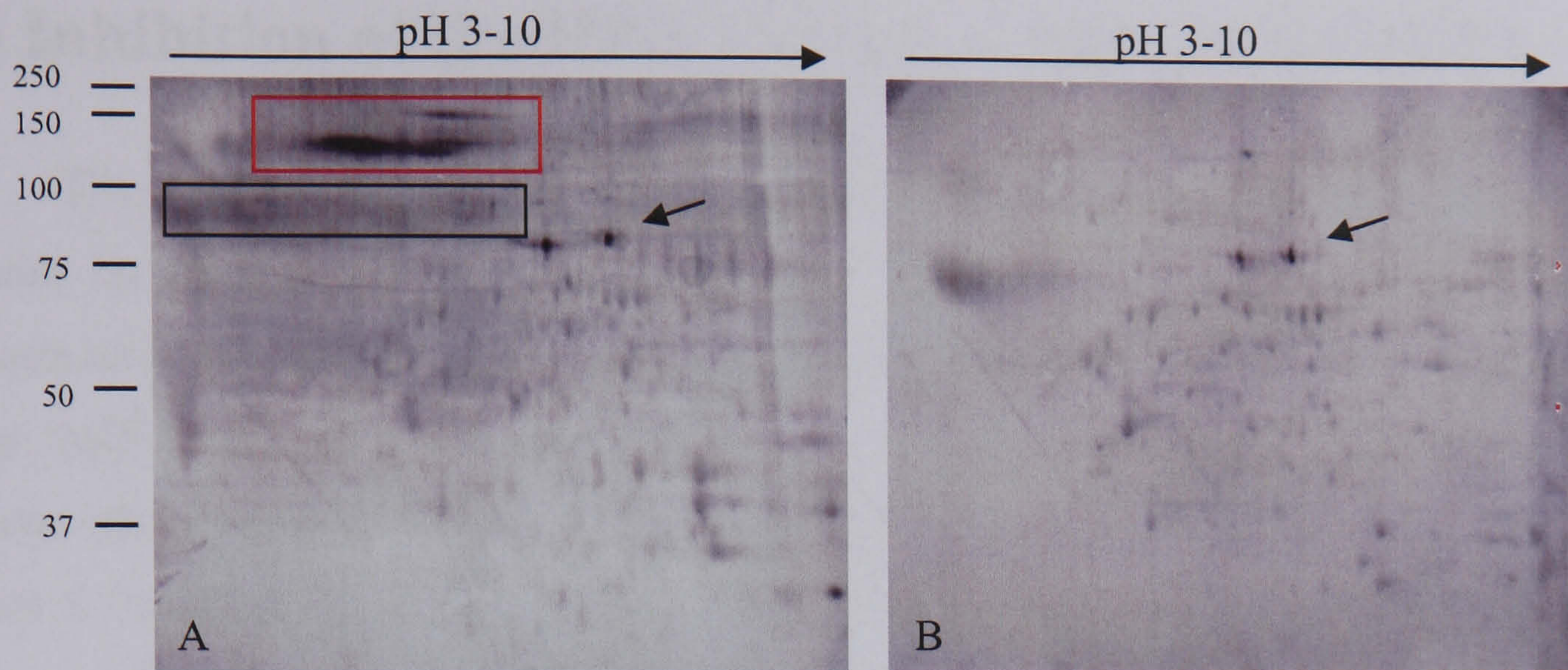


**Figure 5.3:** Inhibition of the HPA binding to HT29 proteins. 20  $\mu$ g of proteins from HT29 were separated on a 12% SDS-PAGE and incubated with HPA-biotin; pre-incubated for 30 min with 250 mM GalNAc, GlcNAc or SA. Inhibition of GP130 and GP80 was observed with all three sugars. The negative control where HPA was omitted showed a non-specific band of 75 kDa present in every experiment.

## 5.5 HPA binding: 2-DE and lectin blotting analysis

The membrane-enriched proteins from HT29 and SW480, were separated by 2-DE, transferred to nitrocellulose and probed with HPA. We used the resolving power of 2-DE to investigate in detail the individual polypeptides recognised by the lectin HPA in three separate occasions and typical results are shown in this section. The observations were similar to those made on 1-DE with several common proteins of MW <80 kDa weakly binding to HPA (Figure 5.4).





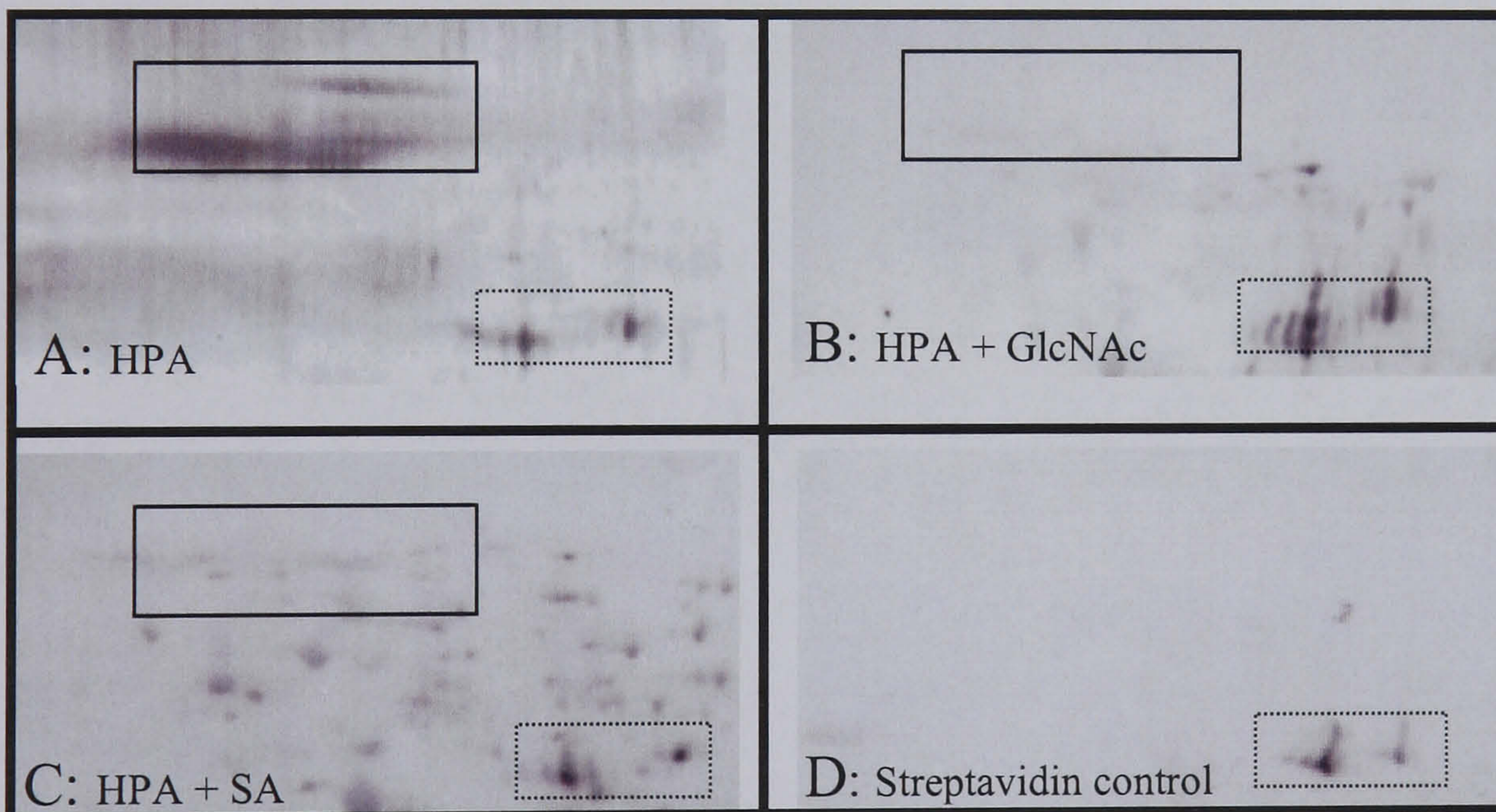
**Figure 5.4:** Comparison of HPA binding proteins of HT29 (panel A) and SW480 (panel B) by 2-DE and lectin blotting with HPA. 90  $\mu$ g of HT29 and SW480 membrane-enriched proteins were separated by 2-DE, transferred to nitrocellulose by Western blotting and probed with HPA. A wide range of proteins were recognised below 80 kDa including the streptavidin binding protein (shown with an arrow) and a large number of proteins weakly bound by HPA in both cell lines. In the HPA positive cell line HT29, HPA bound strongly to GP130 (framed in red) and a group of several glycoproteins badly separated of 80 kDa (black box), whereas HPA did not bind to proteins in the same region in SW480.

In this experiment some of the “non specific” binding was seen to be due to endogenous biotin as represented by the streptavidin binding protein of MW 80 kDa (Figure 5.4, shown by the black arrow). GP80 was detected only in HT29 as a wide, diffuse trail of proteins (black box, Figure 5.4). The intensity of the HPA-binding to GP80 was intense but its protein components were not individually separated. GP130 was separated and the intensity of HPA binding confirmed that it was one of the principal HPA binding sites in the metastatic cell line HT29. This experiment confirmed that higher molecular weight acidic proteins comprising GP130 are the major proteins recognised by HPA in HT29 (red box, Figure 5.4), these proteins were not recognised in SW480. Moreover 2-DE enabled the separation of individual polypeptides hidden within, for example, the GP130 protein band perhaps representing differentially glycosylated forms of the same polypeptide sequence.



## 5.6 Inhibition of the HPA binding to GP130 in HT29

The monosaccharide specificity of HPA was tested after separation of the proteins by 2-DE. We used the previous results obtained in the 1-DE to design this experiment as we have shown previously that HPA binding to GP130 could be inhibited using GalNAc, GlcNAc and SA. We chose the region of the gel containing GP130 proteins and evaluated the inhibition of binding of HPA using GlcNAc and SA. In Figure 5.5 panel A, HPA alone was used and the various components of GP130 (black box) as well as the non specific streptavidin binding (dotted box) were observed. The streptavidin binding signal was used as a landmark to locate GP130 in each subsequent experiment. Preincubation of HPA with GlcNAc at 250 mM (Figure 5.5, panel B) or SA at 250 mM (Figure 5.5, panel C) inhibited the binding of HPA to all of the components of GP130, confirming that HPA binds GP130 via glycan epitopes containing GlcNAc or SA.



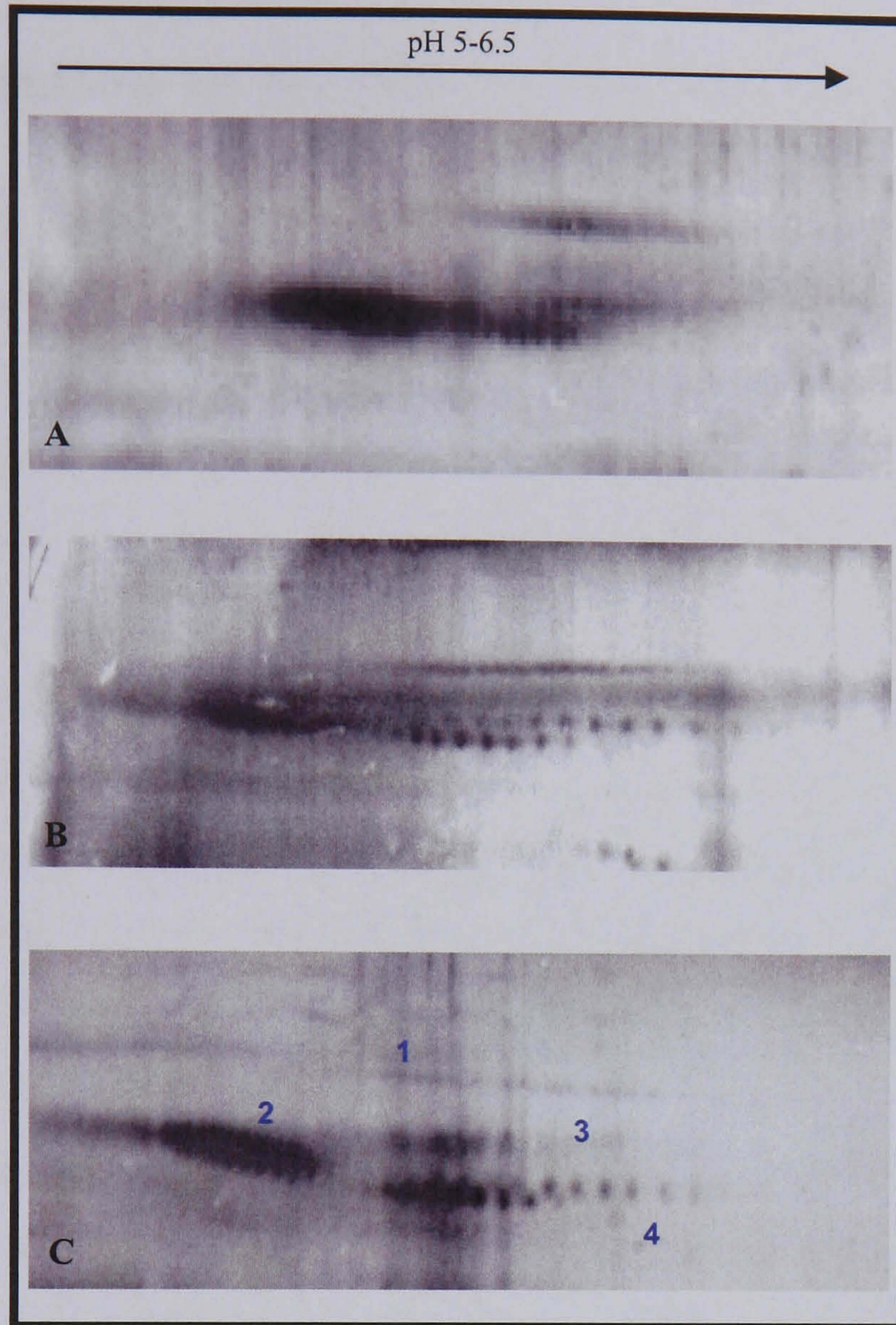
**Figure 5.5:** Inhibition of HPA binding to GP130 in HT29. 90  $\mu$ g of HT29 membrane-enriched proteins were separated on pH 3-10 2-DE and probed with HPA (panel A), HPA preincubated with 250 mM GlcNAc (panel B), HPA preincubated with 250 mM SA (panel C). In the last experiment (panel D) HPA was omitted and highlighted the presence of streptavidin binding proteins in HT29 (dotted box). The binding of HPA to GP130, shown in the black boxes, was totally inhibited by GlcNAc and SA. The negative control where HPA was omitted highlighted the presence of streptavidin binding proteins in HT29.



## 5.7 Analysis of GP130

The data obtained in the 1-DE and 2-DE experiments have shown a range of proteins that bind HPA in HT29. Of these, GP130 was recognised by HPA in both the metastatic cell lines HT29 and SW620. We aimed to analyse this protein further, to this end, the same conditions were used to run three 2-DE experiments using a range of pH gradients to improve the separation of the GP130 material (Figure 5.6). The zoom view of the region containing GP130 shown in Figure 5.6 confirmed that several proteins species comprise GP130. Using the pH3-10 strip the separation was not sufficient to establish a clear map of the polypeptide composition (Figure 5.6, panel A). Using pH 5-8 strip the separation was improved (Figure 5.6, panel B) whilst the pH 4-7 strip afforded the greatest improvement in separation. In this final zoom strip GP130 was found to comprise four clear trails of proteins ranging from pH 5 to 6.5 (numbered 1-4 in Figure 5.6 Panel C). Protein 1 contained approximately 13 faint protein spots each probably representing an isoform of the same protein. The protein 2 contained approximately 10 to 12 spots, the variations between 0.05 and 0.1 pH unit suggest this protein may be modified post-translationally, for example by phosphorylation but there was also a slight variation in the MW which might result from differences in glycosylation. Proteins 3 and 4 contained, respectively, 4 and 10 isoforms. We can also speculate on the affinity of the HPA binding as it seems that HPA binds preferentially to components 2 and 4 of GP130. This series of experiments enabled the separation of the GP130 material and analysis via the Western blotting approach.





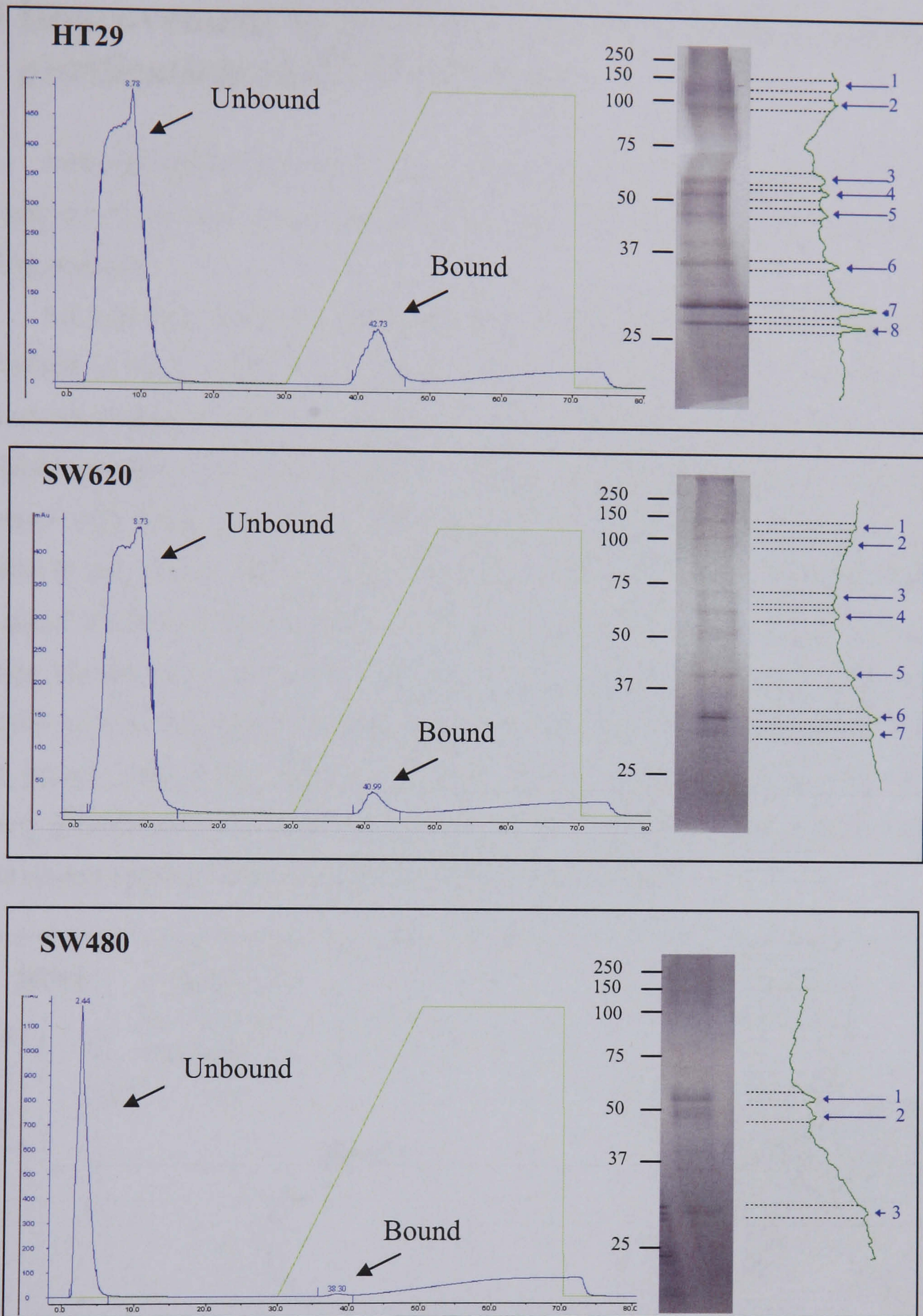
**Figure 5.6:** Separation of GP130 using a zoom 2-D gel strategy. IPG strips with various pH gradient were used to separate GP130. The quality of the separation gradually improved by altering the pH gradient from pH 3-10 (panel A), pH 5-8 (panel B) to pH 4-7 (panel C). Each panel shows a zoom view of the region between pH 5-6.5 containing GP130. In panel C we numbered 1, 2, 3, 4 the polypeptides that comprise GP130.



## 5.8 Purification of the HPA binding proteins of HT29

In the previous section we established the basis of the recognition of membrane-enriched glycoproteins by HPA by probing Western blots with the lectin. In the next step we purified the HPA-binding proteins by HPA affinity chromatography in an attempt to enrich and identify them. The procedure briefly utilised an HPA chromatography column prepared using 1 mg of purified lectin attached to Sepharose beads. Total cell lysate was prepared in 150 mM KCl and 500 µg of proteins were loaded onto the column. Throughout this section each experiment was performed several times (at least three times) and typical results are shown. Figure 5.7 shows the chromatogram obtained with HT29, SW620 and SW480 proteins and the corresponding Coomassie stained 1D gel separation of proteins that bound HPA. The overall pattern of the three separations was in accordance with the previous findings on the Western blots. In HT29, approximately 11% of the protein loaded bound to the column and these appeared to be composed of 8 main protein species ranging from ~25kDa to 150 kDa, these proteins included GP130. In the non-metastatic cell line SW480 approximately 1.2% of the protein loaded bound to the column. Three proteins ranging from ~25 kDa to 55 kDa were visualised after separation on a 1D gel. For the cell line SW620 approximately 3% of the proteins bound to the HPA column. The analysis of the three chromatograms is presented in Appendix 6. The increasing amount of HPA-binding proteins purified from SW480, SW620 and HT29 correlates with the metastatic potential of each of the cell lines and appeared to confirm the importance of GP130 in this model of metastasis.





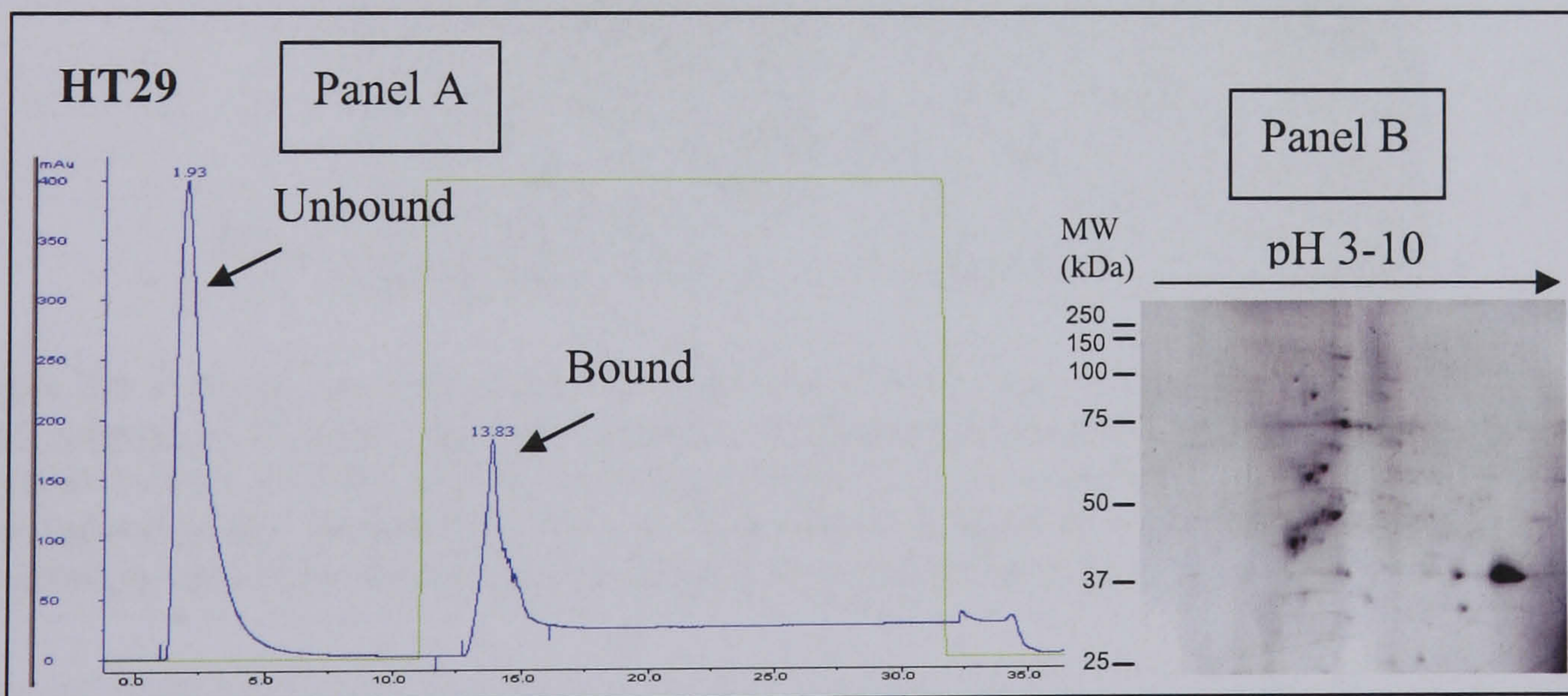
**Figure 5.7:** Affinity purification of HPA binding proteins. Cell lysate was prepared in 150 mM KCl for HT29, SW620, SW480 and 500  $\mu$ g were loaded onto the HPA affinity column. The HPA binding proteins were eluted with a gradient of GlcNAc from 0-1M. The unbound proteins and the proteins which were eluted by competition with GlcNAc (bound) are shown with black arrows. The proteins contained in the eluted fraction were separated on 1D gel and stained with Coomassie Brilliant Blue.



## 5.9 Improvement to the HPA affinity chromatography purification of HT29 proteins

Although sufficient proteins were obtained in the previous experiment to allow 1D analysis, there was not enough material to allow proteomic analysis of the HPA binding proteins.

An improved method in which membrane-enriched proteins were prepared and solubilised in lectin buffer supplemented with 0.1% of CHAPS was developed. This method allowed us to load the column with a sample enriched in HPA binding proteins. To further improve the amount of HPA binding material collected, the column was prepared with 5 mg of purified HPA (instead of 1 mg in the previous experiment) coupled to the column. Figure 5.8 panel A shows the result of an experiment in which 500  $\mu\text{g}$  of HT29 membrane-enriched proteins were loaded onto the column. The HPA binding proteins were eluted from the column with a step gradient of 250 mM GlcNAc. Analysis showed that 28% of the protein loaded onto the column bound HPA (2.5 times more protein than in the previous experiment). This enabled the separation of HPA binding glycoproteins on 2-DE with Coomassie Brilliant Blue staining prior to mass spectrometry analysis of the protein spots (Figure 5.8 panel B).

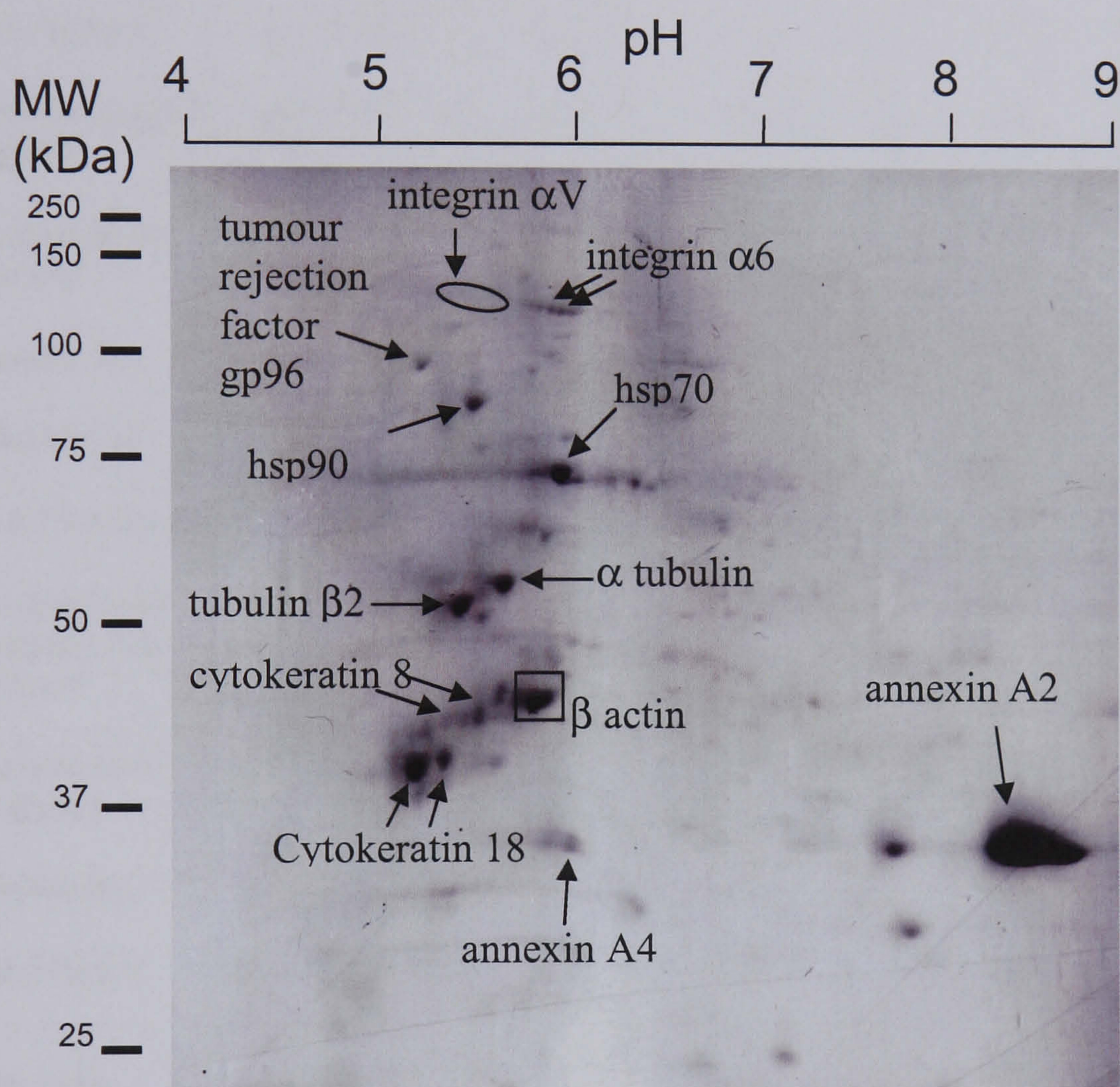


**Figure 5.8:** Affinity purification of HT29 proteins using an HPA-Sepharose column. 500  $\mu\text{g}$  of proteins from HT29 membrane-enriched preparation solubilised in lectin buffer supplemented with 0.1% of CHAPS.

Two peaks are observed (shown with arrows in panel A), these correspond to the flow through and the bound glycoproteins eluted from the column using 250 mM GlcNAc (panel A). Panel B shows the 2-DE separation of the HPA binding glycoproteins after purification by HPA lectin affinity chromatography, the proteins were visualised using Coomassie Blue.



The membrane-enriched proteins purified by HPA affinity chromatography described above were identified using MALDI-MS and MS-MS. The exhaustive list of proteins identified is presented in Table 5.2, the MALDI-TOF mass spectra as well as the sequence coverage of the peptides identified are shown in Appendix 7. The protein spots that were analysed by MALDI-MS and MS-MS are annotated in Figure 5.9.



**Figure 5.9:** Proteomic analysis of the affinity purified HPA binding proteins of HT29. 2-DE separation of HT29 membrane proteins pre-fractionated by HPA affinity chromatography was achieved on a pH 3-10 IPG strip followed by SDS-PAGE. Two out of four protein components of GP130 were recovered and identified by mass spectrometry as integrin  $\alpha 6$  and  $\alpha V$ . 13 other proteins were submitted to mass spectrometry and their identity are presented on the gel.



<b>Protein names</b>	<b>Accession number</b>	<b>Mascot score</b>	<b>mass kDa</b>	<b>calculated pI</b>	<b>sequence coverage</b>
Integrin Alpha 6	gi 5726563	389	120.6	6.44	19%
Integrin Alpha 6	gi 5726563	599	120.6	6.35	25%
Cytokeratin 8	gi 181573	758	53.3	5.5	45%
Cytokeratin 8	gi 181573	758	53.3	5.5	43%
Integrin Alpha V	gi 4504763	431	117	5.45	14%
Tumor rejection antigen gp 96	gi 4507677	166	92.7	4.76	9%
90 kDa Heat shock protein	gi 306891	348	83.5	4.97	20%
Cytokeratin 18	gi 30311	127	47.3	5.27	19%
Cytokeratin 18	gi 30311	127	47.3	5.27	15%
Annexin A2 isoform 2	gi 4757756	1010	38.8	7.57	62%
Guanine nucleotide-binding protein Beta 2 subunit	gi 20357529	470	38	5.6	36%
heat shock protein 8 isoform 1	gi 5729877344	344	71	5.37	14%
Alpha tubulin	gi 340021662	662	50.8	4.94	30%
Tubulin Beta 2	gi 20809886419	419	50.2	4.76	22%
Beta Actin	gi 4501885	511	42	5.29	26%
Annexin A4	gi 1703319	563	36	5.9	32%

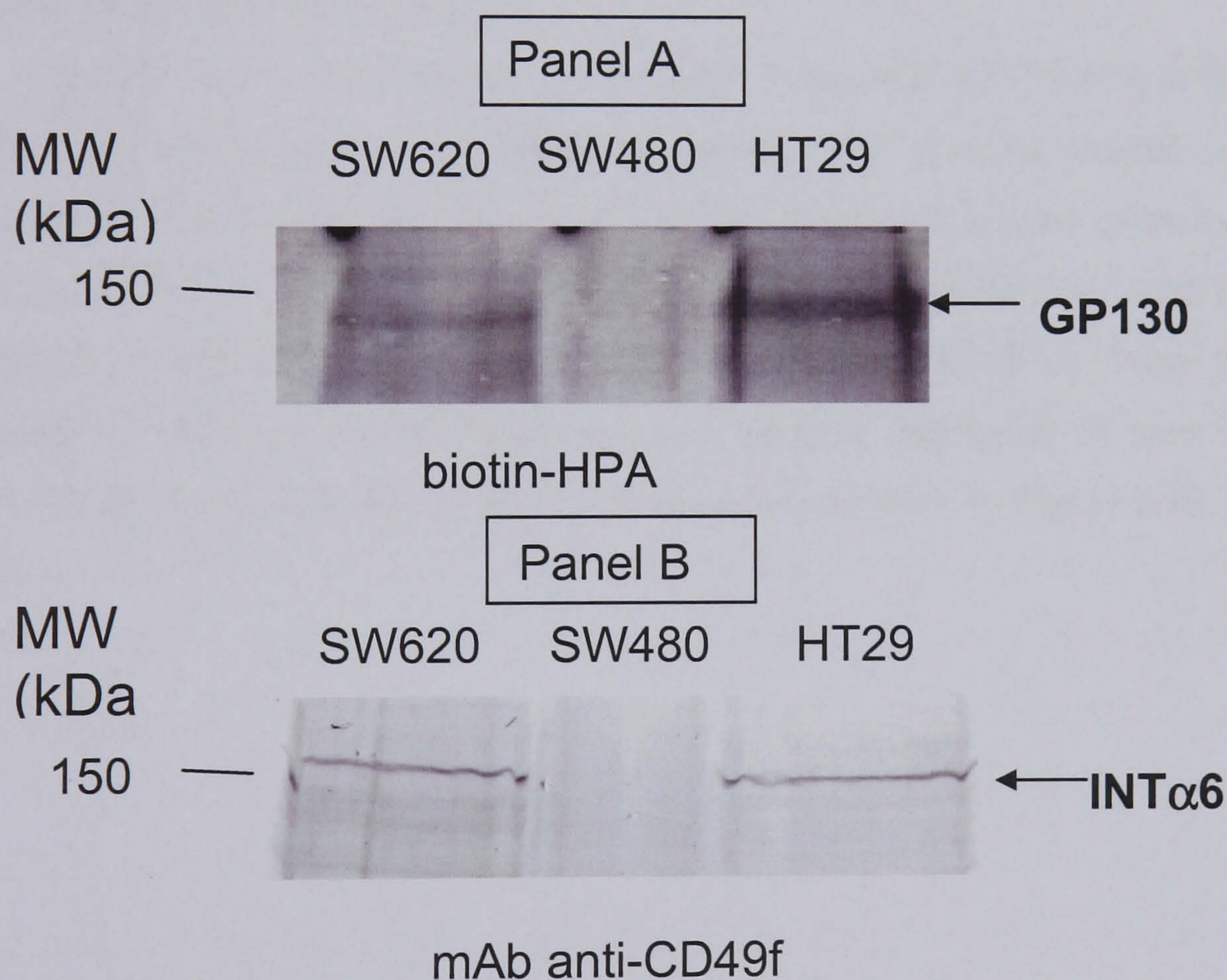
**Table 5.2:** Identification of the HPA binding protein of HT29. The protein spot analysis was compiled in this table and provides protein identities, Mascot score and sequence coverage.

## **5.10 Anti integrin $\alpha 6$ antibody binding to HT29, SW620 and SW480 proteins**

Purification of HPA binding proteins combined with mass spectrometry analysis identified integrin  $\alpha 6$  as an important component of GP130 and this suggests that both the integrin  $\alpha 6$  protein and/or its altered glycovariant may play a role in the metastatic



process. Two experiments were performed and typical results are shown in Figure 5.10. We used an anti-integrin  $\alpha 6$  antibody (anti-CD49f) to compare the level of integrin  $\alpha 6$  in the three cell lines HT29, SW620 and SW480 and we observed that the binding of HPA to the three cell lines HT29, SW620 and SW480 correlated with the binding of the mAb anti-CD49f (Figure 5.10), although the HPA binding band (GP130) was consistently more diffuse and therefore presumably comprises a wider range of epitopes including integrin  $\alpha 6$ . It is important to note that the changes of expression as detected by the mAb anti-CD49f may partly explain the binding of HPA to the metastatic cell lines HT29 and SW620 however it seems that the expression of glycoforms of the integrin  $\alpha 6$  carrying changes in their glycans repertoire is more important for the metastatic phenotype than the level of expression of the “normal” integrin  $\alpha 6$  itself.



**Figure 5.10:** Integrin  $\alpha 6$  antibody and HPA binding to Western blots of HT29, SW620 and SW480 proteins. 20  $\mu$ g of membrane proteins from SW480 and HT29 cell lines were separated on a 12% SDS-PAGE, transferred to nitrocellulose membrane and incubated with either 5  $\mu$ g/ml of biotinylated-HPA (panel A), or 4  $\mu$ g/ml of mAb anti integrin  $\alpha 6$  (panel B), before incubation with 2  $\mu$ g/ml of streptavidin-HRP and detection. GP130 is detected with the lectin only on HT29 and SW620, similarly, integrin  $\alpha 6$  is detected on HT29 and SW620 but not on SW480.

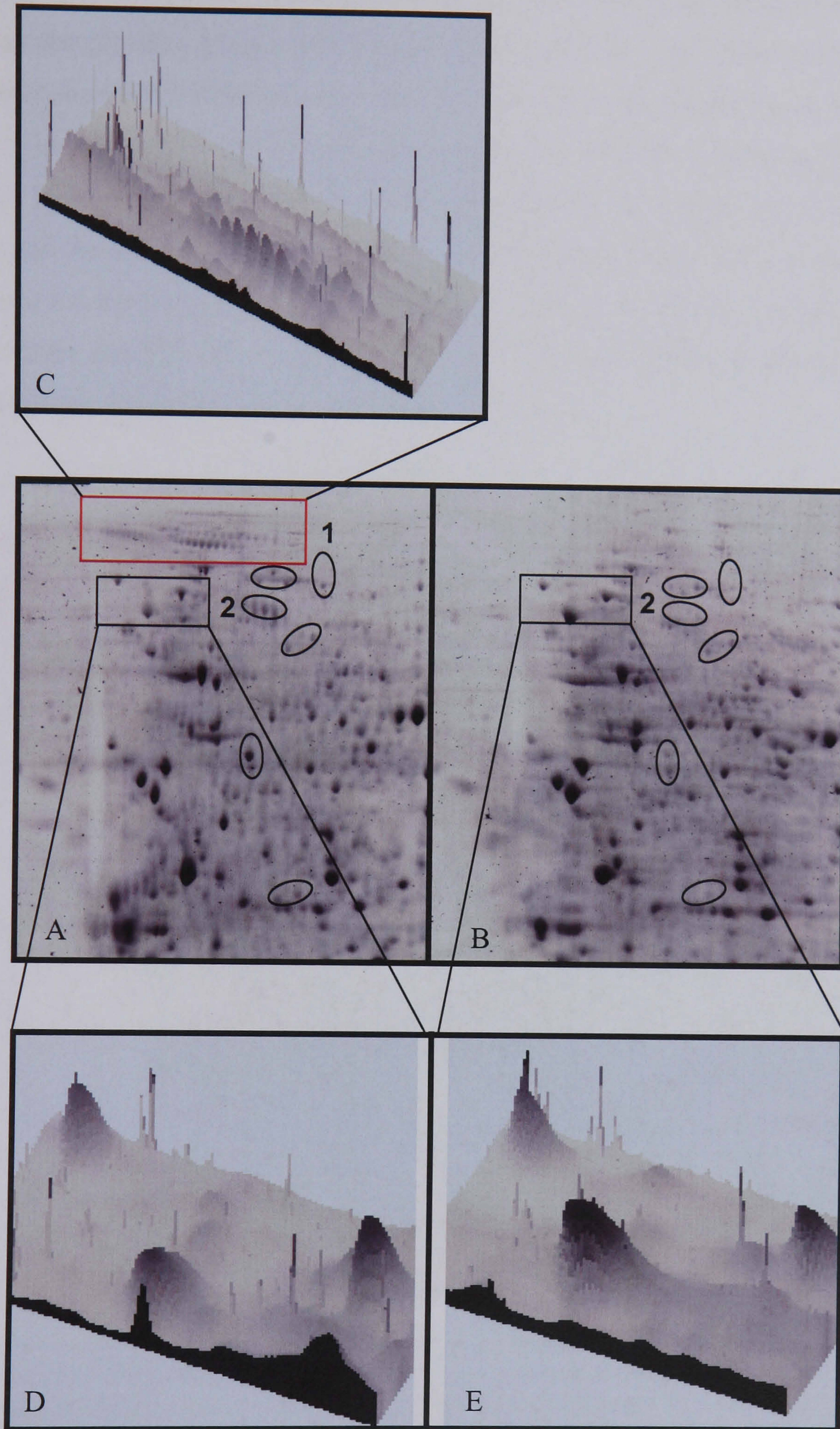


## 5.11 Protein expression analysis of HT29 and SW480

As a further evaluation of the metastatic and non-metastatic cell line models, the overall protein expression was compared using a 2-DE based proteomics approach. This experiment aimed to determine whether the HPA-binding glycoproteins detected in HT29 are ubiquitous proteins showing changes in glycosylation, or if they are glycoproteins found at different levels under particular conditions such as transformation leading to a metastatic phenotype. We prepared two reproducible gels per cell line and we analysed two typical gels, one from HT29 and one from SW480 using membrane proteins separated on an IPG strip of pH 3-10 followed by SDS-PAGE, stained with Sypro Ruby and used a software-assisted approach to compare and match the proteins from SW480 and HT29.

Figure 5.11 shows the region between pH 5-7 and MW 50-150 kDa of HT29 and SW480 2-DE gels (Figure 5.11, panel A and B). The proteins framed in box 1 comprising GP130 are expressed only in HT29. The separation and the intensity of each component of GP130 is illustrated in panel C. Several others proteins (circled in Figure 5.11, panels A and B) are also differentially expressed in HT29 but these were not recognised by HPA on 2-DE. There are also proteins expressed in near identical quantity in HT29 and SW480 as shown on the example (Box 2, Figure 5.11, panel D and E).

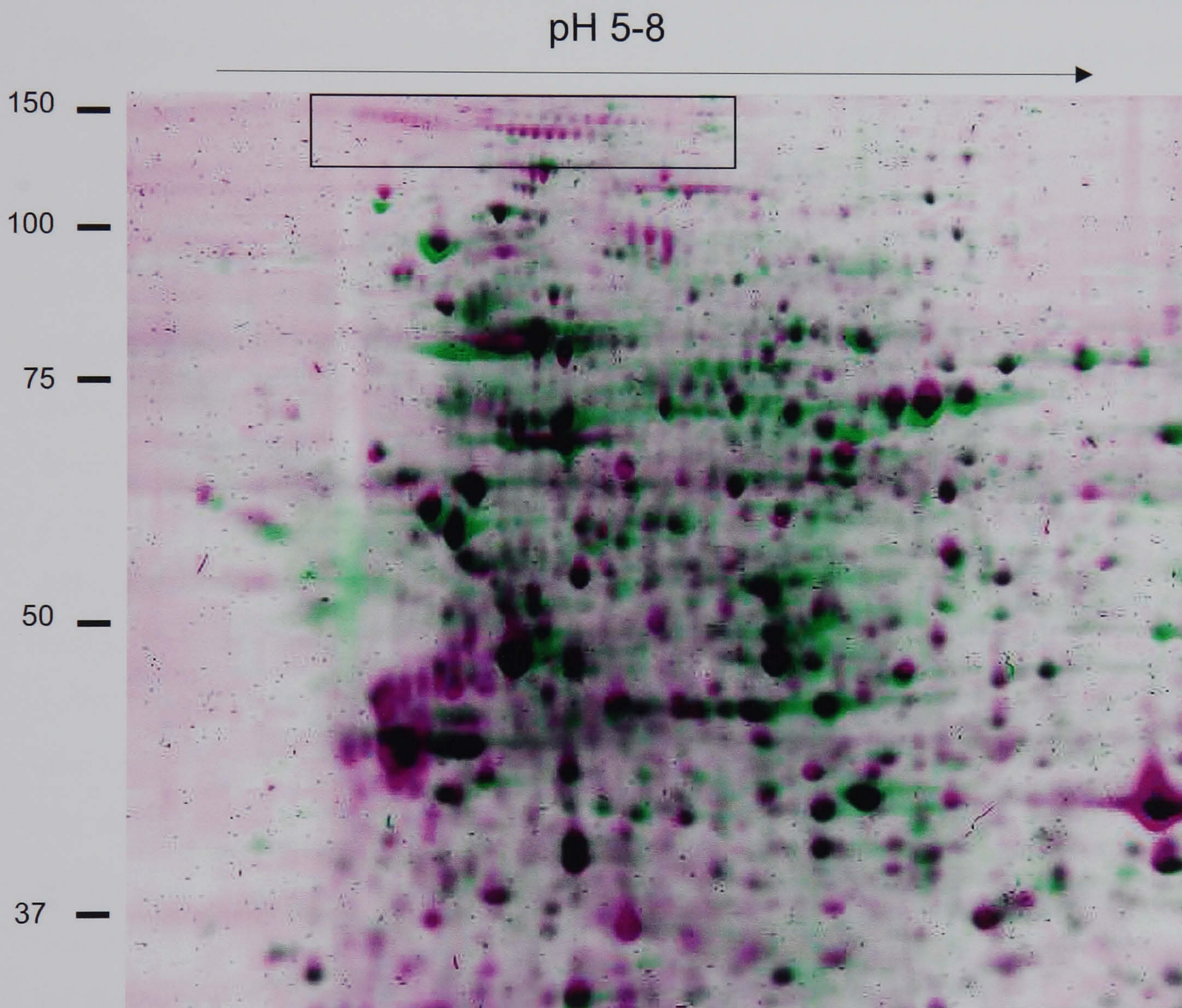




**Figure 5.11:** Comparison of the proteins from HT29 and SW480. 90  $\mu$ g of protein from HT29 and SW480 were separated by 2DE and stained with Sypro ruby. The region between pH5 and 7 and MW from 50 to 150 is presented as it contained a protein of interest, GP130 (panel A and B). Some proteins were found in equivalent quantities in HT29 and SW480 and the 3D view of the region 2 (panel D and E) illustrates this observation. GP130 sustained changes in expression (red box 1) and was expressed in HT29 but not in SW480.



The software package Phoretix Evolution, from Nonlinear Dynamics was used to overlay the proteins maps of HT29 and SW480 (Figure 5.12). 1096 proteins spots were detected on the SW480 gel and 1125 on the HT29 of which 827 were matched to SW480. In HT29, 298 spots were not matched to SW480, including the GP130 material. This suggests a difference in the regulation of the expression of the GP130 proteins and the other proteins in HT29. The GP130 proteins appeared to be of major importance for the binding of HPA. The findings using the Phoretix software reinforce the hypothesis that GP130 is a key element in the binding of HPA and probably in the transformation of tumour cells to a metastatic phenotype.



**Figure 5.12:** Overlay of 2-DE separation from HT29 and SW480. 2-DE was performed with 90  $\mu$ g of proteins from HT29 and SW480: the first dimension was undertaken using a pH 3-10 IPG strip and a zoom in the pH range 5-8 is shown, the second dimension was performed on a 10% SDS-PAGE. The software Phoretix Evolution (Nonlinear Dynamics) was used to analyse the gels. Spots were detected automatically, filter applied and gel matching was performed. SW480 is shown in green and was set as the master gel, HT29 is shown in purple and was matched to SW480. 1096 spots were detected in SW480 and 1125 in HT29. 827 spots were matched and appear in black on the image. Purple spots, including GP130 are shown in the boxed area at the top of the gel and represent proteins spots found exclusively in the metastatic cell line HT29.



## 5.12 Discussion

### *1-DE analysis*

In this study we used a well characterized model of human colorectal cancer cell lines with defined *in vivo* behaviours and lectin binding properties to discern the glycoproteins bound by the lectin HPA. Using 1-DE we found 17, 18 and 18 protein bands were recognised by HPA in HT29, SW620 and SW480 respectively. Many of the proteins of MW <80 kDa were found in all three cell lines and were the weaker bands in terms of intensity of lectin binding. A non specific signal due to biotinylated proteins which bound to streptavidin-HRP was observed in each cell line and had MW between 71 and 74 kDa. The comparative analysis also showed there were considerable differences between the HPA binding protein species in the three cell lines, HT29, SW620 and SW480. Two major HPA binding partners of high molecular weight (MW: 130 kDa and MW: 80 kDa) were identified by lectin blotting of membrane proteins separated by 1DE in the metastatic cells HT29 and SW620. Interestingly these two protein bands were most strongly detected in HT29 and SW620 (GP130 being stronger than GP80) but were not detected in the non-metastatic cell line SW480. Brooks *et al.* (2001) identified 11 major protein bands recognised by HPA in a variety of breast cancer cell lines and they noticed changes in the intensity of HPA binding to some bands but did not identify the proteins. Previous experiments by Schumacher *et al.* (1995) and Mitchell *et al.* (1998) identified 4 and 7 protein bands recognised by HPA and two of these were identified as an N-acetylgalactosaminylated form of albumin and the transferrin receptor. De Albuquerque Garcia Redondo *et al.* (2004) were the first group to evaluate the HPA binding proteins of membrane fractions of three colorectal cancer cell lines. They observed variations in the composition of HPA binding protein of three cell lines (IEC-6, CaCo2, and HCT116). The protein bands observed ranged from 14-60 kDa but they did not establish a clear difference between the cell lines

### *Inhibition experiments*

Inhibition experiments showed that the interaction between HPA and the two predominant protein bands was via a lectin-like interaction that could be inhibited with both GalNAc and GlcNAc and, somewhat surprisingly, SA. Analysis using an anti blood group A antibody has shown that the glycan epitopes that are recognized by HPA



include the blood group A antigens but there was also a range of other protein species that were not recognised by the blood group A antibody and that, presumably, exhibit different glycosylation (Hakomori, 1999). The experiments showed that GP130 did not bear the blood group A antigen but competitive inhibition experiments allowed us to conclude that this protein did contain GlcNAc and SialAc motifs. A proteomic approach was developed to analyse GP130. We demonstrated that GP130 is composed of four glycoproteins which undergo variations in their expression and that differentially bind HPA, a strong correlation was noted between expression of GP130 and binding of HPA.

#### *MS analysis of proteins binding HPA*

The proteins of GP130 and other HPA binding proteins are known to be present in small quantities which did not enable identification by mass spectrometry MALDI-TOF therefore we used a strategy comprising pre-fractionation by HPA-affinity chromatography to unravel the glycoproteins recognized by HPA in the metastatic HT29 cancer cells. This experiment did not allow a complete recovery of all the four members of GP130 protein band but two of the proteins (number 2 and 4) were identified as integrin  $\alpha 6$  and  $\alpha V$ . Thirteen other HPA binding proteins were also identified. The proteins that eluted in the HPA binding affinity chromatography fraction included molecules involved in cell adhesion and migration (integrin  $\alpha 6$ , integrin  $\alpha V$ , annexins) re-modeling (filament proteins including  $\alpha$  tubulin,  $\beta$  tubulin, cytokeratins, actin) and anti-apoptotic pathways (Hsp-70, Hsp-90, Hsp-96 and TNFR-1) (Juliano and Varner, 1983; Prasad *et al.*, 1999; Ciocca and Calderwood, 2005). Some of the proteins are present because they bind directly with HPA and others are found as complexes with HPA binding proteins. Whilst many of these proteins have previously been described in cancer we are not aware of any reagent – other than HPA - which will bind these proteins simultaneously. HPA may recognize these proteins by virtue of changes in the normal glycan repertoire and the data obtained using the anti blood group A antibody supports this hypothesis. It is important to note that the MASCOT search program which compared the resulting peptide mass fingerprint of a sample analysed to the theoretical peptide fingerprint generated *in silico* by the search program, and produces a statistically based Mowse score that evaluates the significance of the



matches (Pappin *et al.*, 1993). In our experiment proteins were identified with Mowse scores >163 (with the exception of the TNFR-1) which confers a high degree of confidence to our results.

### *Integrins*

The results obtained in this study showed that the binding of HPA to HT29 and SW620 is via the recognition of epitopes carried by several proteins including integrin  $\alpha 6$  and integrin  $\alpha V$ . There are many reports of altered integrin expression in carcinoma as compared to their “normal” counterpart, but some of them are conflicting and are reviewed in Gilcrease, 2007. The best studied integrins in cancers include the laminin receptors  $\alpha 2\beta 1$ ,  $\alpha 3\beta 1$ ,  $\alpha 6\beta 1$  and  $\alpha 6\beta 4$  which all showed altered expression in cancerous tissues of the colon as compared to their healthy counterpart (Chao *et al.*, 1996). The presence/absence of integrins and in particular  $\alpha 6\beta 4$  appear to be a common feature in invasive CRC. Indeed  $\alpha 6\beta 4$  is highly involved in several stage of cancerous progression and its expression during metastasis is no longer localised in hemidesmosomes but at the invasive front and in cell protrusions (Rabinovitz *et al.*, 1999; Rabinovitz and Mercurio, 1997).  $\alpha 6\beta 4$  is also involved in the enzyme-mediated degradation of the ECM. In migrating cancerous cells  $\alpha 6\beta 4$  triggers the production of ECM degradative proteases (Ivaska and Heino, 2000). Not only does the level of expression of integrins and in particular  $\alpha 6\beta 4$  seem to be important in metastasis, but changes in their properties and localisation appear essential which could be explained by changes in their glycan repertoire as detected by HPA. Also  $\alpha V$  integrins binds to vitronectin, a constituent of platelets (Hynes, 1987), and its presence in the metastatic CRC cell lines may explain how CRC cells invade and spread to distant organs by attaching to platelets. HPA binds to glycoproteins which play a central role in invasion but more importantly it seems that the glycosylation changes detected by the lectin may be of importance to understand the mechanism of metastasis. Work in our laboratory is being undertaken to study the glycoconjugates of the HPA-binding glycoproteins.

The results obtained in this chapter enabled us to determine the binding specificity of HPA. Also the proteins that bind to HPA were identified and this showed that the lectin recognises a diverse range of proteins with a range of different roles in cell



adhesion, movement, signalling and apoptosis. However, these results were obtained using cell line models and we sought to determine whether the HPA binding glycoproteins of the cell lines were also the same in human tissue samples collected from patients with CRC.



## **CHAPTER SIX:**

### **Preliminary work on tissue samples**



## 6.1 Introduction

We have previously investigated the basis for the recognition of colorectal cancer cells by HPA using three cell lines, chosen for their biological differences in order to construct an *in vitro* model of metastasis. Next we extended our analysis to tissue samples from patients with colorectal cancer. Frozen tissues were kindly provided by Dr Juliette Smith-Ravin (Université de Guadeloupe). The tissues were collected after informed consent under the auspices of the University Hospital Martinique Ethics Committee as described in the materials and methods section. Prior to selection a range of the samples were taken, 5µm frozen sections cut using a cryostat and the presence of cancer cells confirmed by staining with haematoxylin and eosin and viewing under the light microscope. Samples containing >60% cancer cells and free of bloody infiltrate were selected for the proteomic study. The clinical features of the samples selected are presented in Table 6.1.

We grouped the tissue samples into four categories, “low stage” colorectal cancer (C1), corresponding healthy tissue taken away from the tumour tissue (N1), “advanced” colorectal cancer (C2) and corresponding healthy tissue (N2) these represented pools of four tissue samples per category. The proteins of each sample were solubilised individually in U7M/T2 buffer and the protein concentration was determined. The four pools were made by mixing 100 µg of each of the cancer/normal proteins of the four appropriate samples. We investigated the HPA-binding glycoproteins in each group of samples and verified if the proteins found in the cell lines that bound HPA were also present in the tissue samples and to what extent. In this study several replicates of each experiment were reproducibly performed (at least two replicates) and typical results are presented in this section.



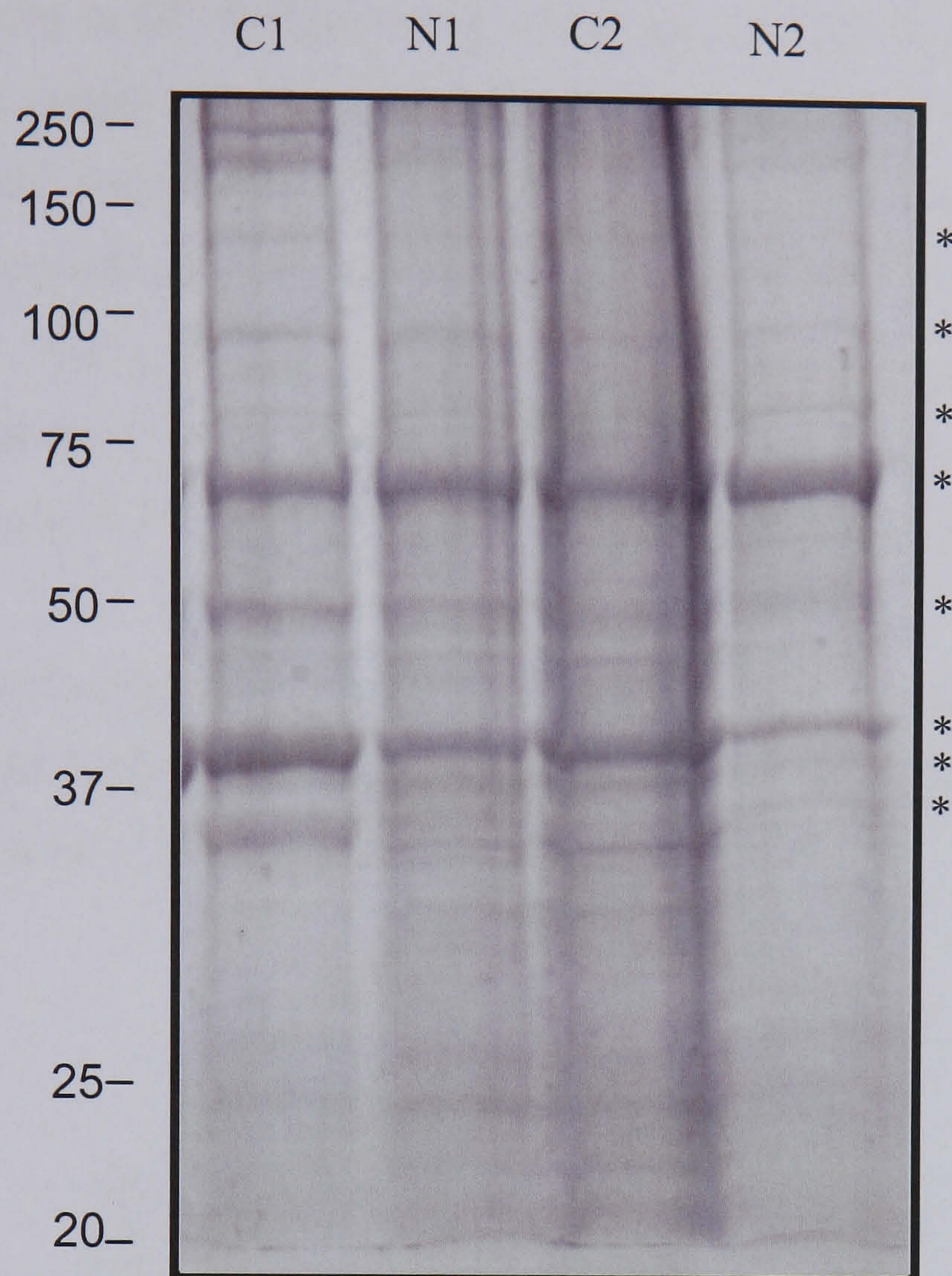
Categories	Tissue samples		
	TNM status	Gender	Age
C1	T2N0	Female	44
	T2N0	Female	62
	T2N0	Female	54
	T3N0	Male	70
N1	Healthy tissues from each patients used in C1		
C2	T4N1	Female	64
	T4N2	Male	70
	T3N2	Female	49
	T3N2	Female	57
N2	Healthy tissues from each patients used in C1		

**Table 6.1:** Description of the pooled tissue samples C1, N1, C2 and N2. An identical amount of proteins (100  $\mu$ g) solubilised from four patients were used to prepare each pooled samples C1, N1, C2 and N2. The TNM status, the gender and the age of each patients are compiled in this table.

## 6.2 Separation of tissue sample proteins by 1-DE

The proteins of C1, N1, C2 and N2 solubilised in U7M/T2 buffer were separated by 1-DE. In this separation the protein composition did not appear to differ significantly across the different pooled samples whether from cancerous colon or normal tissues. Eight proteins were consistently detected in all four samples and these ranged from 30 kDa to 130 kDa (Shown by asterisks in Figure 6.1), the protein expression was slightly altered in the proteins of 30 kDa, 40 kDa and 50 kDa which showed intense staining in C1 and lower staining in the three other samples. Proteins of 250 kDa and 190 kDa were found in C1 and not detected in any other of the three samples. The proteins of sample C2 appeared to have “sticky edges”, this may be attributed to the glycosylation of the proteins although further work would be required to substantiate this hypothesis.





**Figure 6.1:** Protein composition of C1, N1, C2 and N2 analysed by SDS-PAGE. 20 $\mu$ g of proteins of C1, N1, C2, N2 were separated on a 10% SDS-PAGE gel and stained with Coomassie Brilliant Blue. Eight proteins are consistently detected in each sample and are shown by asterixes.

### 6.3 HPA binding glycoproteins

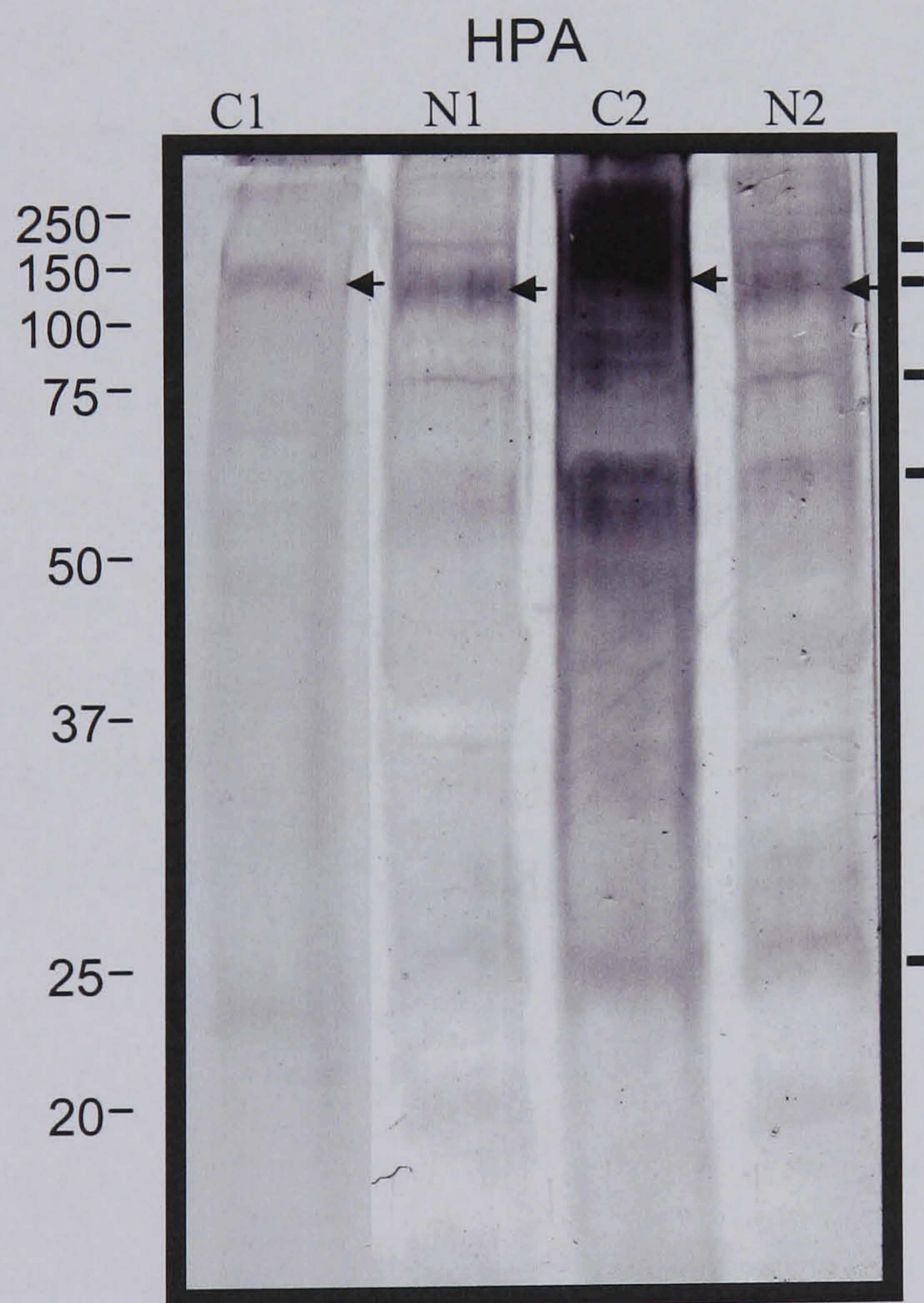
The four samples used in the 1-DE experiments were separated as before, blotted onto nitrocellulose and probed with HPA (Figure 6.2). In this analysis proteins were recognised by HPA in each of the samples, cancerous and normal. The protein bands were diffuse and this is consistent with extensive glycosylation of the proteins as heterogeneity in glycosylation gives subtle differences in MW that are not resolved by 1-DE.

HPA recognised epitopes across the molecular weight range from 20-200 kDa. In samples C1, N1 and N2 the intensity of HPA binding was consistently weak whereas in the metastatic cancer tissues the binding was more intense. This observation of HPA binding to cancer pool C2 supports the hypothesis that metastatic cancer is associated with glycoproteins carrying altered glycan epitopes recognised by the lectin. A band of



130 kDa, similar to GP130 that we identified in CRC cell lines, was observed in every pooled tissue sample (arrows on Figure 6.2) and was the main HPA-binding protein band of all four samples. In C2 this band was very intense and broad and seemingly composed of several diffuse protein bands, a further protein band of 60 kDa in C2 was absent in C1, N1 and faint in N2. In C1 the poor resolution did not enable the visualisation of other minor HPA binding proteins. HPA bound to minor proteins of 170 kDa and 75 kDa in N1, C2 and N2. In C2 and N2 a diffuse band of 25 kDa was also detected.

The preliminary results concurred with the observations that we made using the cell lines as the higher MW proteins were found to bind HPA more in the metastatic cancer tissue pool as compared to low stage cancer pool.



**Figure 6.2:** Lectin blotting to pools of colorectal cancer proteins C1, C2 and corresponding “normal” tissue N1 and N2. 20 µg of proteins from C1, N1, C2, N2 were separated by SDS-PAGE and submitted to detection by HPA-biotin. Streptavidin-HRP was then added and colorimetric detection with DAB/H<sub>2</sub>O<sub>2</sub> was performed. This enabled visualisation of the HPA-binding partners in each of the samples, these are indicated by the black dashes on the right hand side of the western blot and the arrow indicate GP130 band.



## 6.4 Reactivity of CRC tissue samples with antibody directed against the blood group A antigen

This experiment was designed to visualise the proteins recognised by HPA that carry the blood group A antigen. 20 µg of pooled proteins of C1, N1, C2, and N2 were loaded on a 10% SDS-PAGE, transferred to nitrocellulose by Western blotting and probed with an antibody directed against blood group A antigen as before.

The tissue samples consistently showed four major glycoprotein bands carrying the blood group A epitope. These four glycoproteins were 75 kDa, 50 kDa, 40 kDa and 15 kDa and are indicated with red asterisks on Figure 6.3.

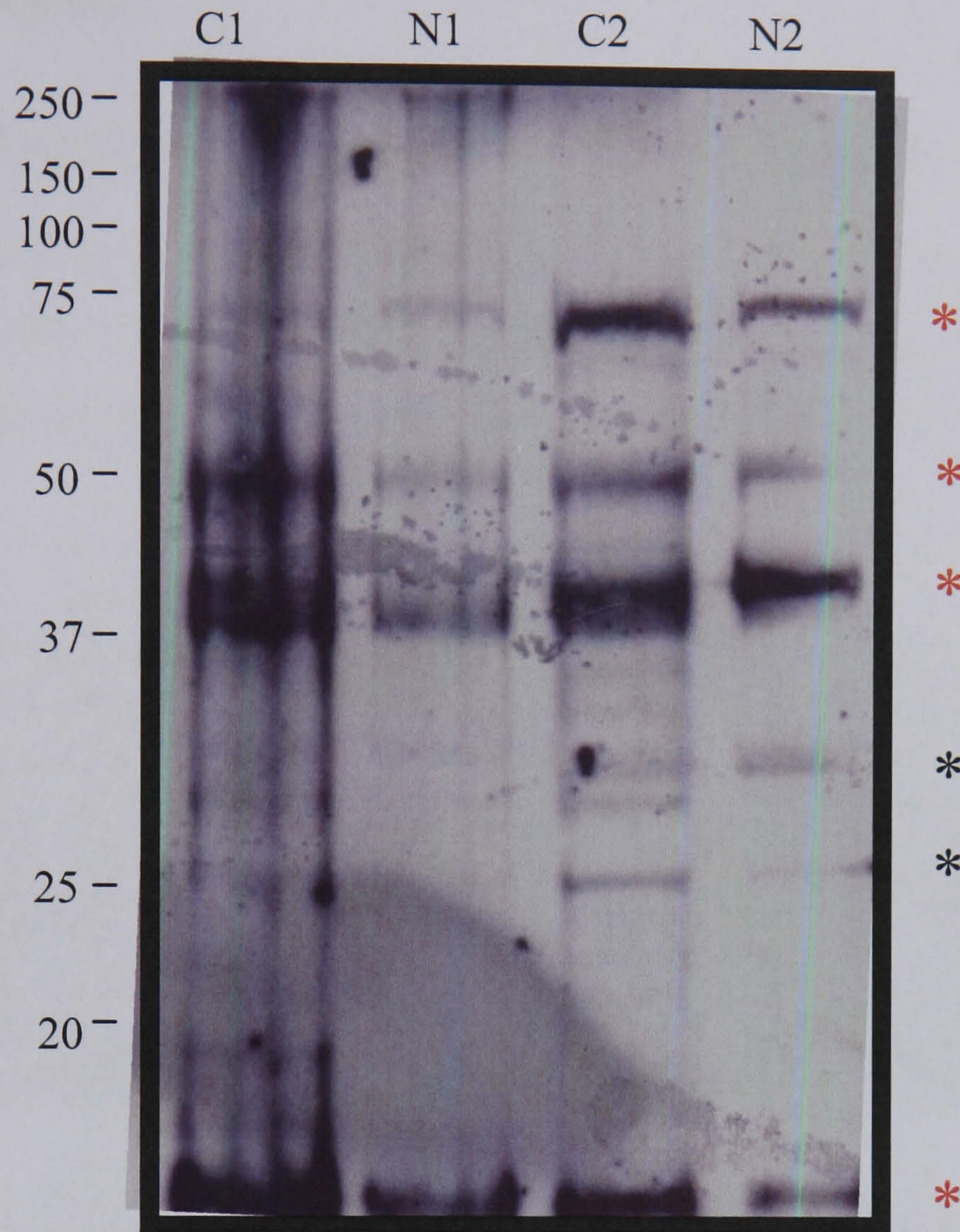
Two other weaker binding glycoprotein bands (27 kDa, 30 kDa), were detected in C1, C2, N1 but not in N2. These two proteins were shown by black asterisks in Figure 6.3.

Compared with the HPA blot we observed that the higher MW proteins that are recognised by the lectin in the tissue samples (Figure 6.2) do not carry the blood group A antigen. Equally the protein band of 60 kDa detected by HPA in C2, N2 and the band of 170 kDa found in N1, C2 and N2 do not exhibit the blood group A determinant. However the relatively weak HPA binding protein of 70 kDa found in N1, C2 and N2 (Figure 6.2) as well as the 30 kDa protein band detected in C1 and N1 both bound to the antibody against the blood group A antigen (Figure 6.3).

The results obtained in this experiment indicated that some, but not all, of the HPA binding glycoproteins from the tissue samples carry the blood group A antigen.



### Anti blood group A



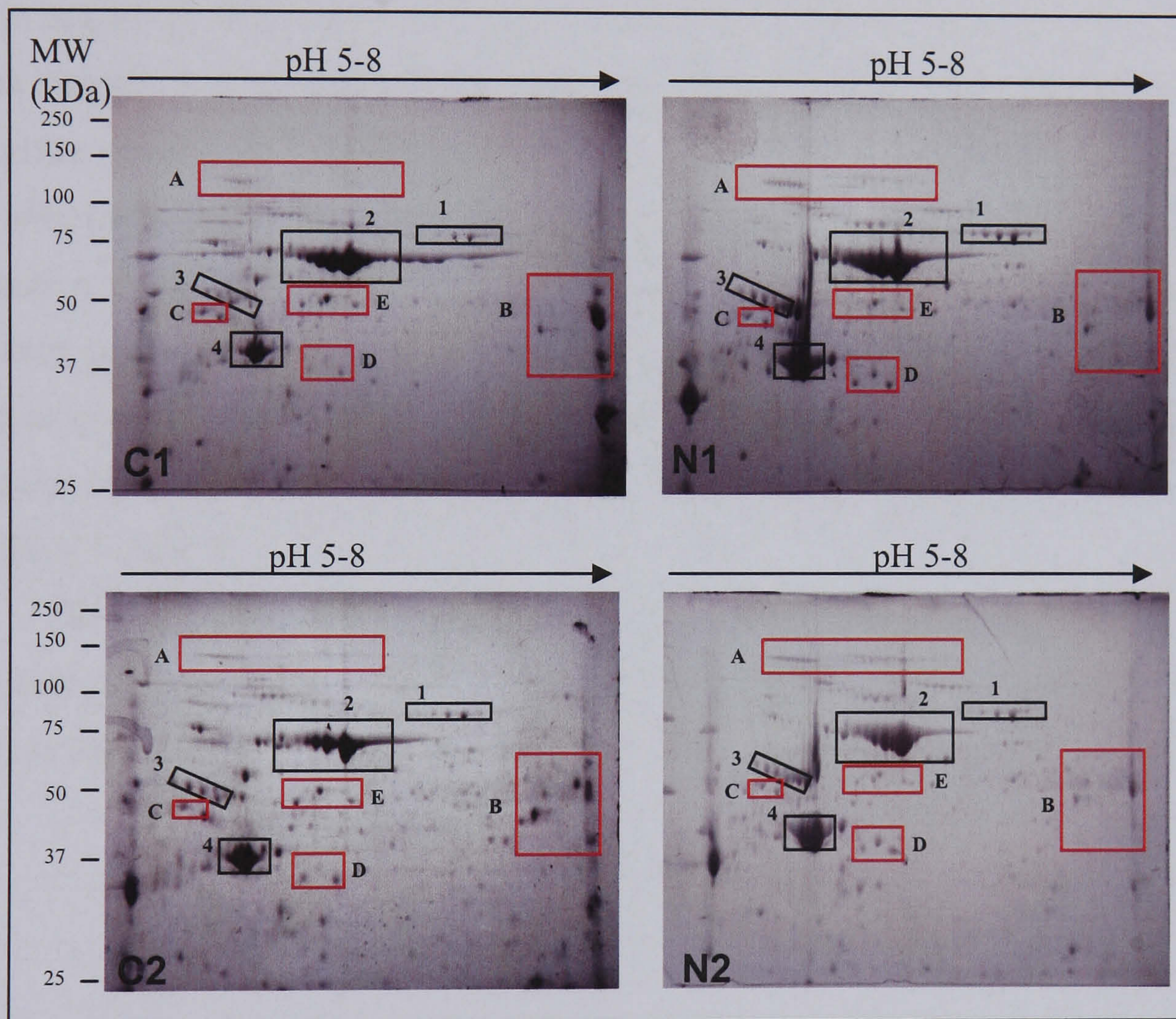
**Figure 6.3:** Reactivity of CRC tissue samples with blood group A antibody. 20  $\mu$ g of proteins from C1, N1, C2, N2 were separated on 10% SDS-PAGE, transferred to nitrocellulose and probed with a monoclonal anti-blood group A antibody. Four proteins recognised by the anti-blood A antibody (marked with red asterisks) were common to all of the pooled tissue samples four samples and two others (marked by black asterisks) were only present in C1 and N1.



## 6.5 HPA binding glycoproteins: 2-DE and lectin blotting analysis

### 6.5.1 Separation of tissue sample proteins by 2-DE

The proteins from the pooled tissue samples were separated by 2-DE on a pH 5-8 IPG strip and a 10% SDS-PAGE, and were stained with Coomassie Brilliant Blue. The migration of the proteins is similar in the four gels as shown in Figure 6.4.



**Figure 6.4:** Separation by 2-DE of pooled proteins from normal (N) or cancerous (C) tissue samples of CRC patients with high stage (C2 and N2) and low stage (C1 and N1) disease. The gel was stained with Coomassie blue. The areas shown by the black boxes represent serum proteins whilst the areas shown by the red boxes indicate proteins showing differential protein levels in the cancer or high stage samples.

There are proteins present in large quantities, reproducibly found in all the four gels and shown in the black boxes numbered 1-4. By comparison with 2-D gels loaded with serum proteins we deduced that these proteins may be serotransferrin (1), albumin (2), haptoglobin (3) and apolipoprotein (4) (Steel *et al.*, 2003) and presumably these are present in the samples from blood infiltrate. Although the serum proteins are present in

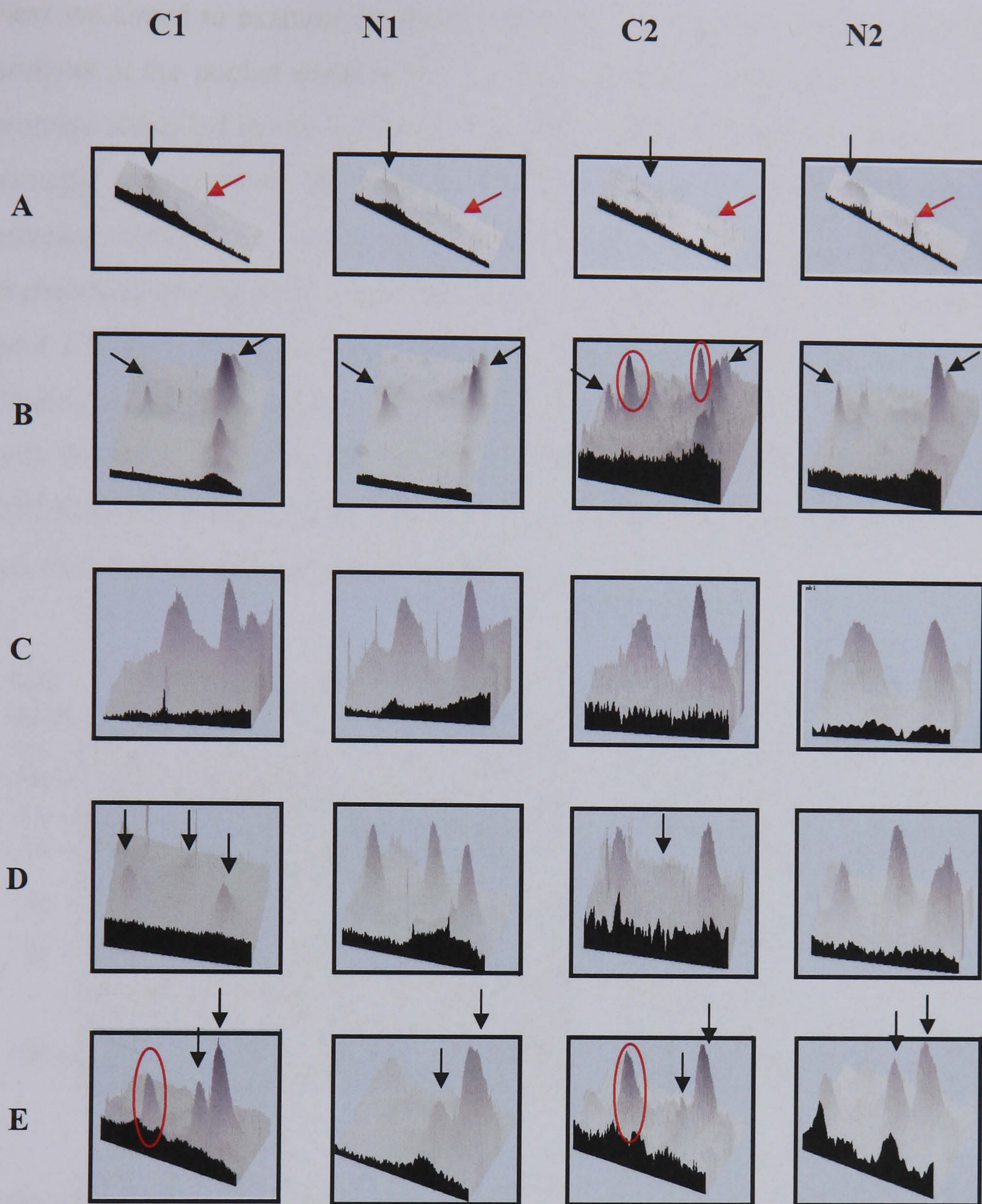


relatively large quantities we were also able to observe several differences in the low abundance proteins (shown in the red boxes of Figure 6.4 labelled A-D) which are further analysed in 3D views (Figure 6.5).

The 3D view for each of the boxes shown in Figure 6.4 was generated to help assess the expression levels in all the four samples (Figure 6.5). In Figure 6.5, region A showed a range of protein spots (black arrow) consistently observed in all four samples. A second range of protein spots, shown with a red arrow was observed only in the normal tissues N1 and N2 and these were not observed in the cancerous samples C1 and C2. In region B, there were two proteins consistently found in all the four samples (black arrow) but interestingly two proteins (circled) were only expressed in the metastatic sample. In region C two proteins were detected consistently across all the four samples in slightly varying quantities. In region D three proteins were consistently detected in the normal tissues (N1 and N2) whereas all three proteins in C1 and one of the three proteins in C2 showed reduced expression levels. Finally in region E, two proteins were detected in all four samples (black arrows) with a variation in the levels of the lower MW protein and one of the proteins (circled) was expressed only in cancerous samples C1 and C2.

The separation of the proteins by 2-DE enabled us to observe several proteins only detectable in the cancerous samples. Two proteins observed in region B were found in higher levels in the protein pool derived from the high stage CRC samples





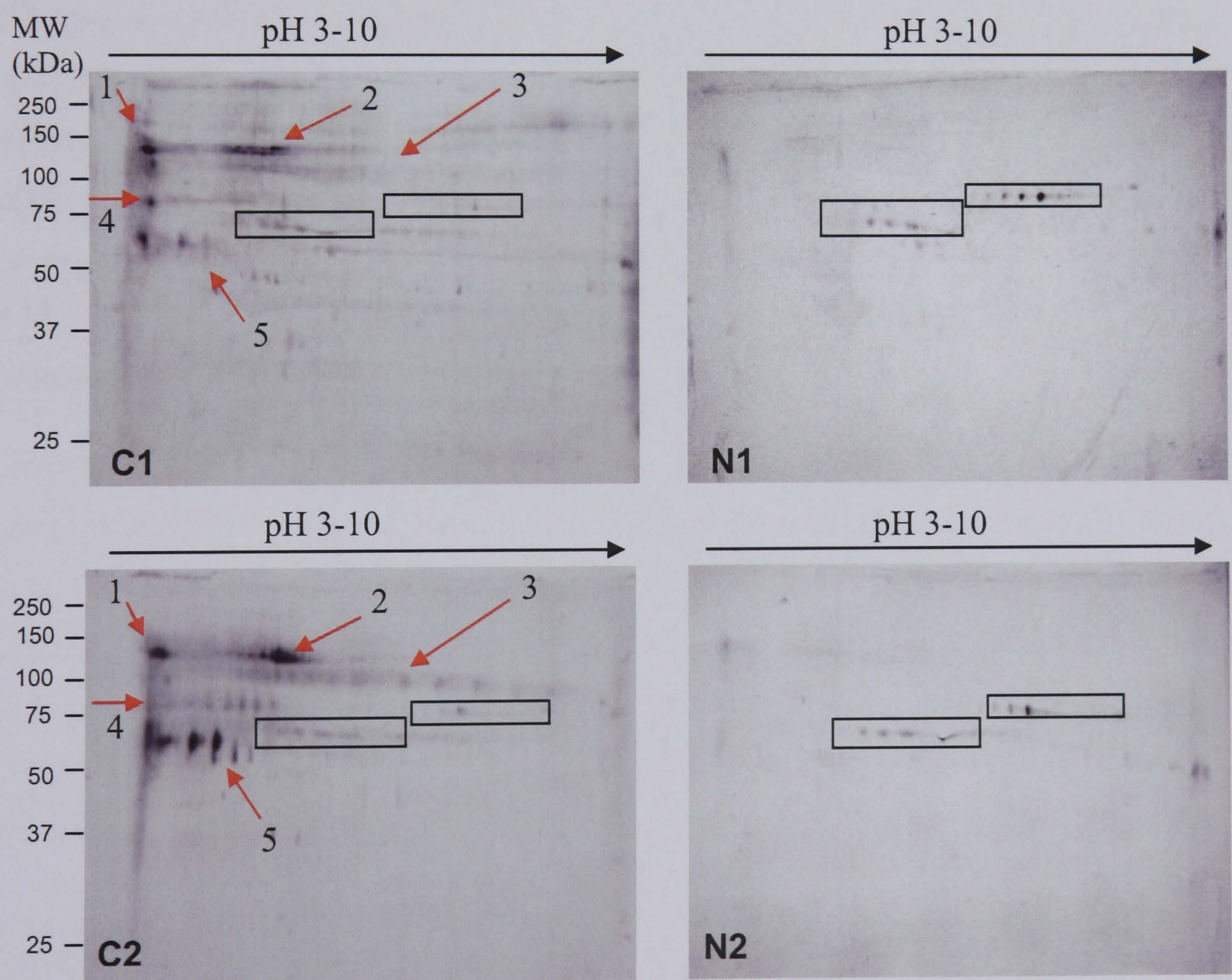
**Figure 6.5:** 3D view of proteins showing altered expression levels by 2-DE in C1, N1, C2 and N2. In panel A the black arrows show a group of proteins consistently expressed in the four samples whereas the red arrows show proteins detected only in N1 and N2. In panel B two proteins are expressed in the four samples (black arrows) and two proteins (circled in red) are present only in the metastatic sample C2. In panel C two proteins are detected in each sample. In the region shown in panel D three proteins are detected in N1 and N2 in similar quantities whereas all three in C1 and one (lowest MW) in C2 showed a reduced expression (black arrows). In panel E two proteins are detected in all four samples with slight variations (black arrows) and one protein circled in red is detected only in C1 and C2.

### 6.5.2 Analysis of the HPA binding proteins separated by 2-DE

The data obtained when the tissue samples were separated by 1-DE showed that there were several proteins that bound to HPA, although these were poorly resolved.



Next we aimed to examine in depth, using 2-DE and lectin blotting, the HPA binding proteins in the pooled samples C1, N1, C2, N2 with a particular focus on the GP130 proteins identified in the cell lines. Samples C1 and C2 contained many glycoproteins strongly recognised by HPA whereas in their healthy counterparts N1 and N2 only 7 proteins spots were consistently detected, (Figure 6.6). It appears that HPA preferentially binds to glycoproteins of the cancerous cells and in the metastatic cancer pool C2 there was more intense binding of the lectin. There were five main HPA binding areas when Western blots of the pooled proteins from C1 and C2 were probed with the lectin. These included a range of proteins spots of 130 kDa believed to be the GP130 proteins observed in cell lines. The five main HPA binding regions on the 2-D gel were further analysed using the Melanie 6 software.

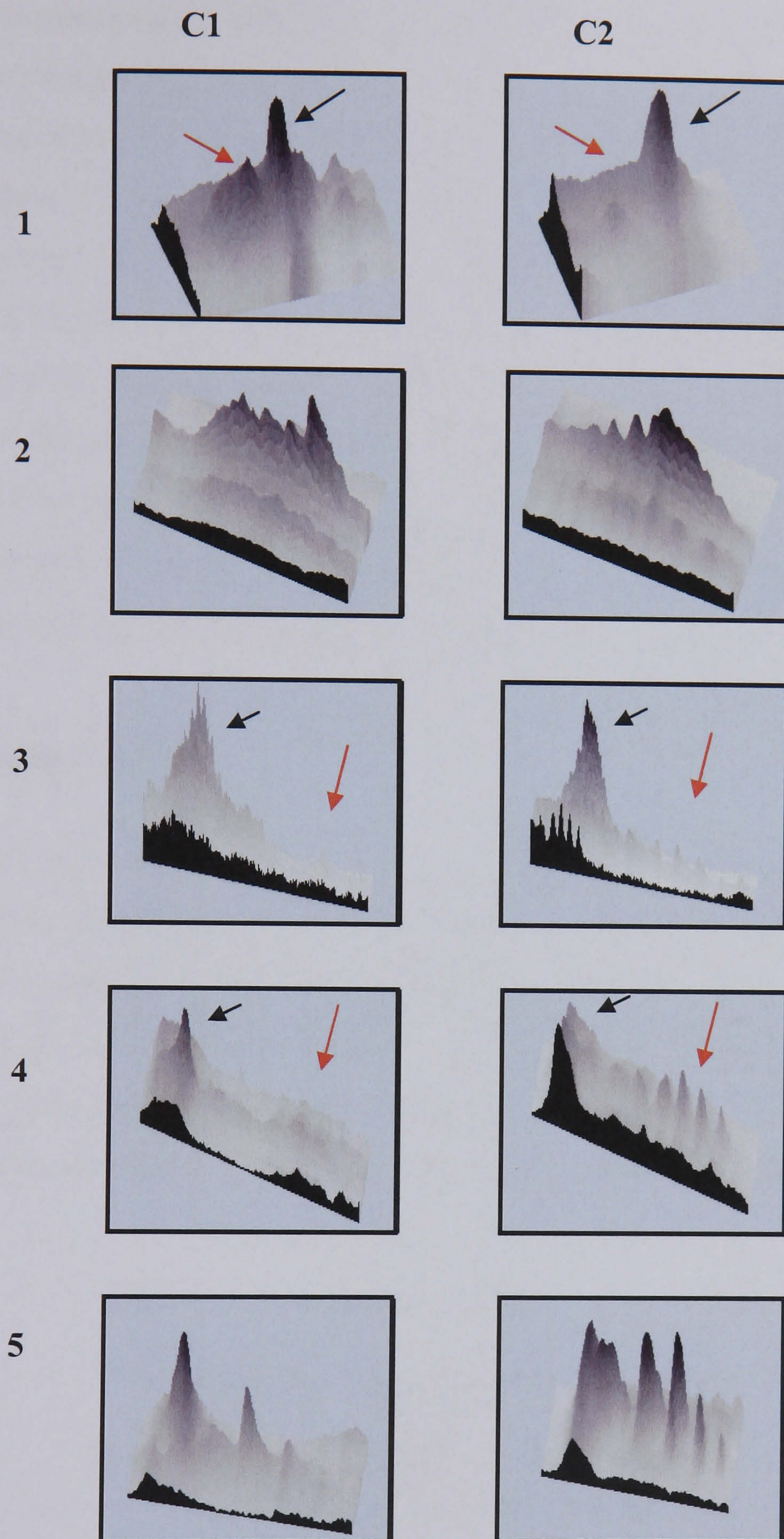


**Figure 6.6:** Detection of the HPA-binding proteins of C1, N1, C2 and N2 after separation of protein by 2-DE. The area labelled 1, 2, 3, 4, 5 contain the HPA binding protein of the samples C1 and C2 and were investigated further. The boxed area represent the non specific signal due to streptavidin binding.



Figure 6.7 shows the 3D view of the five HPA binding regions from the pooled tissue-derived proteins. Region 1 comprises one glycoprotein that strongly bound to HPA in C1 and C2 and is viewed as a sharp peak. C1 contains one other glycoprotein that was a less intense HPA binding protein and is represented as a poorly resolved peak in the 3D view (Figure 6.7 red arrow), this acidic glycoprotein bound by HPA was absent in the C2 material. The HPA binding glycoproteins shown by arrows 2 and 3 in Figure 6.6 showed a similar pattern of migration in C1 and C2 but the intensity of the binding was increased in C2 as illustrated by the 3D view (Figure 6.7, panels 2 and 3), The MW and pI of these proteins allowed us to correlate these with the GP130 proteins found on the metastatic cell line HT29 (Chapter 5). Two components of GP130 that we identified as integrin alpha V and integrin alpha 6 in cell lines were detectable in regions 2 and 3 of C1 and C2, these proteins strongly bound HPA in the metastatic pool, C2. Region 4 and 5 also contained proteins more strongly recognised in C2 as compared to C1. In region 4 a sharp peak is detected in C1 whereas the same peak appeared more diffuse in C2 (shown by black arrows). In C1 this region also comprises a trail of diffuse proteins weakly bound by HPA (shown by a red arrow). In C2 these proteins are more strongly recognised by HPA and appeared as 6 successive protein spots of similar MW. Finally in region 5 HPA recognised three proteins in C1 and five of higher intensity in C2.





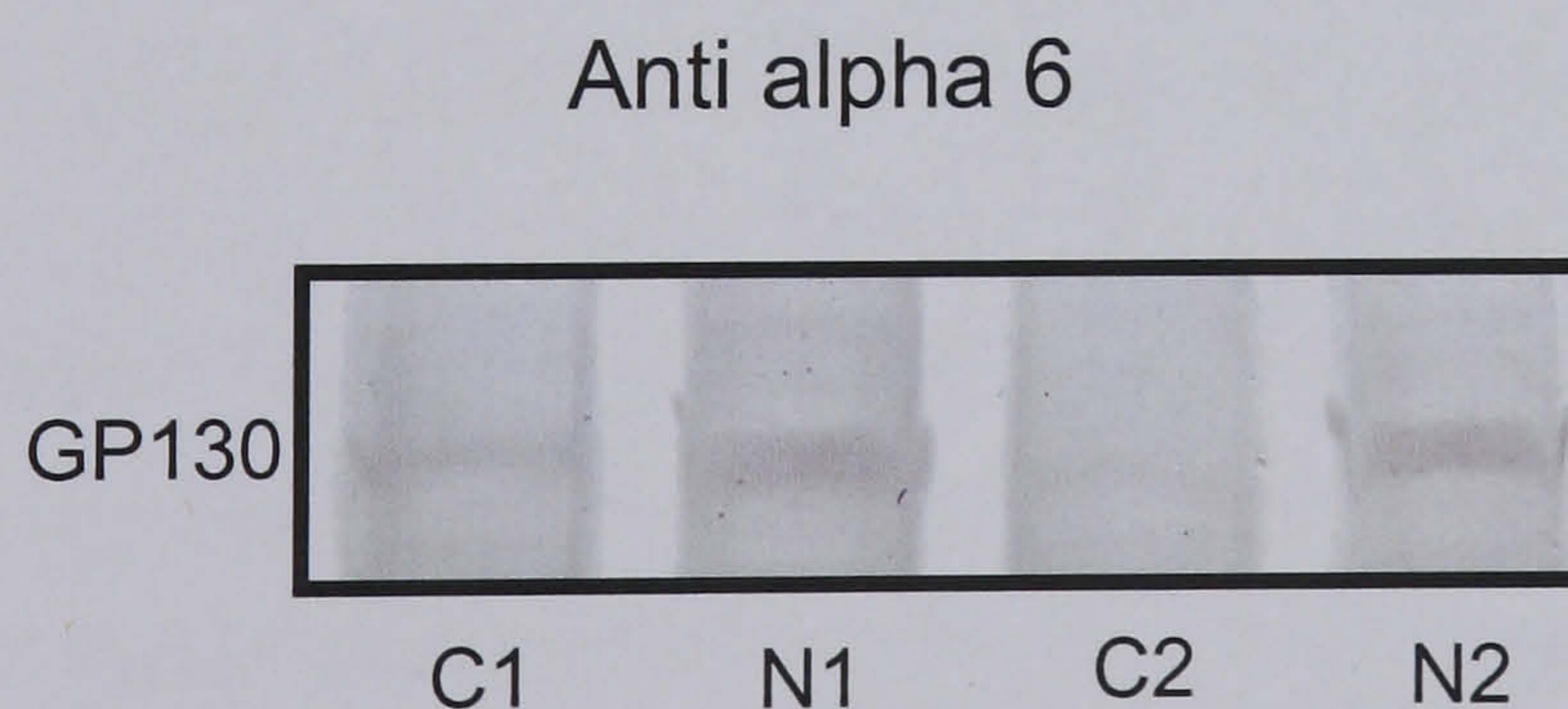
**Figure 6.7:** 3D view of HPA-binding proteins of the region 1, 2, 3, 4, 5 of C1 and C2 indicated by the red arrows on Figure 6.6. In panel 1 one glycoprotein is recognised by HPA in C1 and C2 (black arrows) and one protein shown by red arrows is bound in C1 only. In panel 2 a similar pattern of HPA binding protein is observed although the intensity is higher in C2. In panel 3 there is one common protein in C1 and C2 (black arrows) and a series of glycoprotein isoforms appear to be present only in C2 (red arrows). In panel 4 one protein peak appear more diffuse in C2 (black arrows) and a trail of protein isoforms is present in higher intensity in C2 (red arrows). The proteins of panel 5 are more intense in C2.



The results of the 2-DE separation showed that the binding of HPA was stronger in the cancerous tissue than in the normal tissue counterpart, but this analysis also showed that HPA bound significantly more strongly to proteins derived from the high stage cancers (C2) as compared with the pooled proteins from the low stage cancers (C1). We observed five main regions on the 2-DE blot from the two cancerous samples C1 and C2 for which there were differences with respect to HPA binding. Two of the areas on the blots (zones 2 and 3) may represent components of GP130 and these were elevated in the pooled protein from the high stage cancers (C2). Based on the MW, pI and protein migration pattern we hypothesised that the proteins of region 2 and region 3 may be integrin  $\alpha V$  and integrin  $\alpha 6$  respectively. In the next part of the work we used an antibody directed against integrin  $\alpha 6$  to test this hypothesis.

### 6.5.3 Integrin $\alpha 6$

Proteins from C1, N1, C2, N2 were separated by 1-DE, transferred to nitrocellulose membrane by Western blotting and probed with an antibody against integrin  $\alpha 6$  (Figure 6.8). The results obtained showed there was a reduction in the level of integrin  $\alpha 6$  in the cancerous sample and in particular in the metastatic sample C2 compared with the normal counterpart and the expression of integrin  $\alpha 6$  did not correlate with the binding of HPA as the latter was increased in the metastatic sample.



**Figure 6.8** Anti-integrin  $\alpha 6$  antibody probing of Western blots of cancer and normal tissue proteins. 20  $\mu\text{g}$  of proteins C1, N1, C2, N2, separated on 10% SDS-PAGE were probed with either HPA or antibody anti-integrin  $\alpha 6$ . The profile of the binding intensity was created. Integrin  $\alpha 6$  seemed to be present in higher quantity in normal tissue as compared to their cancerous counterpart. Again integrin  $\alpha 6$  sustained a dramatic decrease in C2.



## 6.6 Discussion

In this chapter we used human tissue samples to compare the overall 2-DE protein map and the HPA binding glycoproteins with those obtained from the CRC cell lines. We used 16 human colorectal tissues samples divided in four sub-categories according to their TNM status: C1 (early stage cancer), C2 (advanced cancer), N1 and N2 were chosen as control samples; therefore in each category there were four tissue samples. Our previous findings in cell lines (SW480, SW620 and HT29) of varying metastatic phenotype from non metastatic to highly metastatic, enabled us to determine that the lectin HPA recognised several glycoproteins including GP130 in the metastatic cell lines. Two of the GP130 components were identified as integrin  $\alpha 6$  and integrin  $\alpha V$ . It has been shown that the binding of HPA to cell lines and human tissues correlates with a metastatic phenotype. Our observations on the cell lines suggested that the main HPA binding partner was GP130 and we concluded that the expression of GP130 could be an important feature towards the acquisition of the metastatic phenotype in cell lines. These findings are supported by various studies which attribute a role for integrin  $\alpha 6$ , in its dimeric form  $\alpha 6\beta 1$ ,  $\alpha 6\beta 4$ , in progression and metastasis, this has been observed in tumours originating from the colon, breast, prostate, lung and melanoma (Chao *et al.*, 1996; Shaw *et al.*, 1996; Rabinovitz *et al.*, 1995; Yamamoto *et al.*, 1996; Kuphal *et al.*, 2005).

The present chapter investigated the glycoprotein partners of HPA in the tissue samples. The 1-DE/lectin blot showed that glycoproteins of the cancer samples were very heavily glycosylated with HPA binding glycans. The multitude of proteins bearing the HPA binding epitopes did not allow clear evaluation of all the HPA binding partners present in the samples, although the binding of HPA was observed to be stronger in the proteins pooled from the metastatic samples. The same analysis using proteins separated by 2-DE gave a better resolution enabling a more detailed analysis. Five groups of HPA-binding proteins were consistently observed in the C1 and C2 protein pools and the intensity of the binding varied between the two pools, with several proteins more intensely recognised by HPA in the metastatic pool, C2. These included a high molecular weight protein which was of MW consistent with GP130. The Western



blotting experiment with an antibody directed against integrin  $\alpha 6$ , however failed to confirm the presence of this protein in the metastatic cancer sample.

HPA binds proteins that exhibit altered glycosylation in metastatic cancer. In the cell line model GP130 was one of the main HPA-binding components but this was not the case in the tissue samples. There are several possible reasons for this apparent discrepancy in results, for example, cancer tissue samples are a heterogeneous mixture of cells, some will possess a metastatic phenotype whilst other will not, also pooled samples were used in this analysis and in some of the samples GP130 may be of relevance with respect to HPA binding whilst in other samples it may not. Finally we compared GP130 levels in both normal and cancerous tissue, the integrin  $\alpha 6$  antibody bound to both of these tissues but this does not presuppose that the glycosylation of this epitope is the same in normal and cancer tissue. Indeed, it may be the case that whilst the GP130 material was the main HPA binding band in the cancer samples the protein level was less than in the normal samples, perhaps indicating a “hyper-glycosylated” form of this protein in the cancer samples. Further analysis at the protein and glycan level will be required before the similarity between the cell line model and tissue samples can be determined at the molecular level. Other approaches could have been used to investigate the importance of integrin  $\alpha 6$  and its glycosylation in tissue samples. Indeed the double blotting of 1-DE separated proteins with anti-integrin  $\alpha 6$  antibody followed by HPA could confirm that HPA recognise the abnormal glycosylation carried by integrin  $\alpha 6$  rather than the protein itself. Also in order to verify our hypothesis the removal of the contaminating serum proteins and an improvement of the solubilisation of proteins could decrease the complexity of the pooled samples. The use of pooled samples constituted an easy way to evaluate our observations made in cell lines, however the complexity and heterogeneity of the tissue samples appeared to limit our analysis. Lectin staining of the tissues and selection of cells using laser capture microdissection technology prior to protein analysis of the proteins composition and glycosylation by MS-based approaches could be a way forward to evaluate the importance of the glycosylation changes carried by integrin  $\alpha 6$  and other membrane glycoproteins in cancer progression.

In this analysis HPA has proved to be an interesting tool to detect proteins potentially involved in metastasis and further work needs to be carried out in order to



identify these tissue derived proteins and their glycosylation. The prognostic value of HPA was confirmed on tissue samples but it seemed that HPA binding does not rely only on the presence of integrin  $\alpha 6$  but probably on a much larger range of HPA binding proteins.



**CHAPTER SEVEN:  
General discussion**



## 7.1 HPA as a tool for cancer prognostication

Over the past 30 years many studies have demonstrated the ability of the lectin HPA to bind to poor prognosis primary tumours, including those of the colon. Although the precise nature of the HPA-binding glycans involved in cancer cell metastasis have yet to be discovered, variations in the expression of a range of glycoconjugates on the surface of epithelial cancers have been shown to occur during malignant transformation and to play an important role in the metastatic process. The value of HPA as a prognostic marker has been demonstrated in retrospective studies of resected primary tumour tissues, cell lines and in SCID mice (Ikeda *et al.*, 1994; Schumacher *et al.*, 1994 a, b). The lectin HPA which has a nominal monosaccharide binding specificity for  $\alpha$ -linked GalNAc and GlcNAc (Hammarstrom and Kabat, 1969) detects changes in glycosylation associated with tumour progression and is thought to recognise glycan epitopes that are involved in cell adhesion either locally in the primary tumour or on endothelial cells at sites of metastases (Springer and Lasky, 1991). Although changes in the glycan composition of cancer cells have been observed, little is known about the cancer specific HPA binding partners (Lescar *et al.*, 2007, Brooks, 2000). In the current study our aim was to characterise the glycoprotein binding partners of the lectin HPA. We examined HPA binding to CRC cell lines and evaluated the intensity of the binding; we assessed the effect of preincubating the lectin with a range of monosaccharides at different concentrations. Next we used a proteomic approach to isolate and identify the HPA binding proteins present in the cell membrane of the metastatic cell line HT29, finally we tested our findings on CRC tissue samples.

## 7.2 The binding of HPA to CRC cells

The localisation of HPA binding to the CRC cell lines HT29 and SW480 was investigated at the level of confocal microscopy (chapter 3). Direct observations as well as 2D and 3D reconstructions enabled us to localise the binding to the surface of the metastatic cell line HT29 with some granular intracellular staining consistent with the Golgi labelling (Brooks, 2000). We measured the binding intensity and the effect of competitive inhibition by relevant (GalNAc, GlcNAc, SA) and irrelevant sugars (Man) on a large population of cells using a microtitre plate assay developed in our laboratory.



We found that GalNAc, GlcNAc and SA almost completely inhibit the binding of HPA at a concentration of 50 mM. Although it seems that GalNAc, the nominally specific sugar for HPA was the most suitable monosaccharide for inhibiting the binding at both the cellular and Western blotting level, only limited differences were observed between the three sugars suggesting a more complex binding specificity. The interaction between the cancer-cell derived glyconjugates and HPA could be more fully investigated in studies utilising surface plasmon resonance (BiaCore system) or isothermal titration calorimetry and such an approach would allow the affinity and avidity of the HPA interaction to be mapped in the presence / absence of a range of competing sugars.

Somewhat surprisingly (as discussed above) we observed that HPA binding to HT29 membrane glycoproteins can be inhibited by SA. This constitutes a new binding specificity for the lectin in CRC. Other reports have suggested that the HPA binding glycans from breast cancer tissues contain charged residues (Chen *et al.*, 1995; Dwek *et al.*, 2001) and that HPA binding to breast cancer tissue sections can be abrogated by pretreatment with neuraminidase (Fenlon *et al.*, 1987). In further support of this observation a glycan microarray experiment has shown that HPA can bind to sialyl-T antigen (Sanchez *et al.*, 2006). Reports describing the SA composition of colorectal tumours are rather contradictory; a recent study reported a decrease in sialylation (Konno *et al.*, 2002) whilst another study reported an increase in sialylation in CRC (De Albuquerque Garcia Redondo *et al.*, 2004), and this may be attributed to increased sialyltransferase activity (Dall'Olio *et al.*, 1989; Akamatsu *et al.*, 1996). The idea that HPA interacts with metastatic cancer cells via recognition of SA residues is supported by the results in this study and also by unpublished observations from our laboratory showing that one of the polypeptide components of HPA has 54% sequence similarity with a SA binding lectin from the garden snail *Cepea hortensis* (Markiv unpublished data). The presence of isolectins and other components in the lectin preparation might explain the differences observed in the pattern of HPA-binding to cancer tissues compared with that of other GalNAc binding lectins, for example the lectins from *Helix aspersa* (HAA) and *Dolichos biflorus* (DBA).

It seems likely that HPA recognises a range of oligosaccharide structures including the blood group A antigen, the Cad antigen, the Forssman determinant (Baker



*et al.* 1983), sialyl-T, STn and / or Tn antigen (Springer 1989). It has been suggested that HPA simply recognises the Tn antigen that is often elevated in solid tumours (Springer 1988, 1989) but histochemical studies with breast cancer tissues and the anti-Tn antibodies BRICIII and BRIC66 have shown that HPA binds to different cells within breast tumour tissue, thereby supporting the idea that HPA binding encompasses a wider range of glycans epitopes (Brooks and Leathem, 1995). It is likely that other factors such as the presence of other monosaccharides within the complex oligosaccharide structure, the availability of divalent cations ( $\text{Ca}^{2+}$ ,  $\text{Mg}^{2+}$ ) and the conformation of the protein backbone of the glycoproteins harbouring the HPA binding glycans may all be required for the binding of HPA (Brooks *et al.*, 1997 b). Also, HPA is a glycoprotein and it is possible that the interaction between the lectin and cancer cells is via cancer cell proteins binding to the glycans of HPA (which are likely to include N-linked structures with xylose / fucose residues, van Kuik *et al.*, 1985) Studies with recombinant or non-glycosylated lectin would be required to either support or refute this hypothesis.

Our findings have illustrated the complexity of HPA recognition of cancer cells. The exposure of terminal GalNAc often described as the main HPA binding partner, is unusual in normal cells and is a commonly found alteration in cancer cells either due to up-regulation of GalNAc transferases (Brockhausen, 2005) or down-regulation of sialyl transferases (Shang *et al.*, 1999). The inhibition of HPA binding with SA is an interesting feature of this study and we hypothesise that HPA may recognise the STn antigen which is an indicator of poor prognosis CRC. The data appears contradictory as, on the one hand, HPA recognises glycan epitopes that may be uncovered by a decrease in sialylation and, on the other hand, HPA appears to recognise sialylated epitopes. One potential explanation for this apparent dichotomy may be that HPA can bind to core GalNAc / Tn and another lectin or an isolectin of HPA binds to sialic acid. Further work would be required to determine if this is the case preferably in conjunction with experiments to measure the glycosyltransferase activity (perhaps using a microarray methodology such as the one that is currently being piloted by the Consortium for Functional Glycomics <http://www.functionalglycomics.org>). The mechanism by which HPA interacts with the cancer glycans also needs further characterisation and, as



mentioned above, it may be the case that the presence of isolectins in the HPA preparation enables the recognition of a range of different glycosylated structures.

### **7.3 HPA-binding glycoproteins in CRC cell lines**

Proteomic analysis of HT29 cell membrane proteins revealed that the basis for HPA recognition is via its ability to bind to a range of glycoproteins with diverse functions in cancer biology (chapter 5). The analysis of the proteins separated by SDS-PAGE and following lectin blotting showed that HT29, SW620 and SW480 expressed different levels of HPA binding proteins with a large number of the protein species of varying size and intensity recognised in the three cell lines. The proteins included several species that bound the lectin only weakly (MW<75 kDa), these were sometimes recognised by the anti-blood group A antibody and were not essential for the specific recognition of the metastatic cells by HPA. Quantitative differences in levels of higher MW protein species for example GP130 and GP80 were also noted. The GP130 and GP80 glycoproteins were present in higher amount in the metastatic cell lines HT29 and SW620 compared with SW480. Preliminary results on the cell lines suggested that GP130 and GP80 (to a lesser extent) is a metastasis-specific HPA-binding glycoconjugate containing GlcNAc, GalNAc and SA. GP80 was recognised by HPA and the blood group A antibody, whilst GP130 did not appear to contain the blood group A epitope. A table was constructed (Appendix 8) that gives an overview of the results of the 1-DE and Western blotting experiments. From the cell line work it seems likely that the binding of HPA to metastatic cell lines was, in part, due to recognition of GP130 and GP80 as their intensity across the cell lines was correlated with the general HPA binding intensity. GP130 was shown to comprise a mixture of four glycoproteins and the 2-DE work suggested that each of the GP130 polypeptide species contains several differentially glycosylated / phosphorylated isoforms. A comparison of the levels of this group of proteins in HT29 and SW480 showed that GP130 is expressed in the metastatic cell line alone. Inhibition of HPA binding to GP130 on Western blots showed that the nominal specificity of HPA, GalNAc, is too restrictive to explain HPA binding to this group of proteins and probably does not clearly describe the interaction between HPA and the surface of metastatic CRC cells. Further inhibition work and



glycan release / mapping would be needed to characterise the glycans involved in HPA binding to GP130 and the other proteins found to bind HPA in this study. One of the main components of GP130 was integrin  $\alpha 6$ , this protein has previously been correlated with aggressive cancer and has been shown to facilitate invasion of cancer cells through the ECM (Chao *et al.*, 1996). The discovery that some members of the integrin family are amongst the proteins most recognised by HPA in aggressive cancer cells is not surprising as such proteins (e.g.  $\alpha 2\beta 1$ ,  $\alpha 3\beta 1$ ,  $\alpha 6\beta 1$ ,  $\alpha 6\beta 4$ ) have previously been described to be associated with aggressive phenotype in colorectal cancer cell lines (Chao *et al.*, 1996). For example integrin  $\alpha 6\beta 4$  expressed in CRC cell lines promote cell invasion on matrigel and its redistribution from hemidesmosome to cell protrusion suggest a role in cancer cell invasion by regulating cell movement (Chao *et al.*, 1996; Rabinovitz *et al.*, 1999; Rabinovitz and Mercurio, 1997). Also integrins have been involved in other aspect of cancer progression such as cell growth, survival and ECM degradation (reviewed by Mercurio and Rabinovitz, 2001). However we suggested that not only the expression of integrins play a role in cancer but also changes in their glycan composition as detected by HPA. Previous studied have found that the  $\alpha 6\beta 4$  expressed in cancer cells do not form hemidesmosome suggesting that glycovariant of the integrin could be expressed in cancer cells and this could be correlated to our findings. Moreover Ichikawa *et al.* (1998) described how changes of blood group A determinant carried by members of the integrin family ( $\alpha 3$ ,  $\alpha 6$ ,  $\beta 1$ ) modulate motility and proliferation. The presence of HPA-binding glycans on integrin  $\alpha 6$ , may modulate its function in HT29 cells and the role of this protein in the invasion of HT29 cells in our *in vitro* model will be the subject of future studies using the matrigel technology. Also unravelling the glycan composition of the integrins and other proteins recognised by HPA using a MS-based approach may provide new insights into the understanding of cancer progression and invasion.

In this study we have established that the variation in HPA binding in cell lines relies on the presence of several proteins including GP130 and GP80. Our findings partly correlate with the observations made by Prokopishyn *et al.* (1999) who used a different approach based on 3A7 antibody binding to HT29 cells. The 3A7 antibody detects oncodevelopmental changes and  $\alpha 3\beta 1$  integrin was identified as a component of a protein entity (GP140), which appeared to be the major carrier of oncodevelopmental



carbohydrates in HT29. The Prokopishyn study and our own used different approaches but the results were very similar suggesting that members of the integrin family, possessing abnormal glycosylation, may play a role in the metastatic process.

The HPA binding glycoproteins that have been studied in breast (Brooks *et al.*, 2001) and CRC (De Albuquerque Garcia Redondo *et al.*, 2004) cell lines were poorly characterised. We pre-fractionated the HT29 and SW480 cell membrane proteome by affinity-purification on an HPA-Sepharose column and we analysed the proteins by 2-DE (chapter 5). The proteins that were eluted in the HPA binding fraction were present either by virtue of their ability to bind directly to HPA or as protein complexes with HPA binding partners. Identification of the HPA binding proteins by MALDI-MS / MS-MS showed that all of the proteins had previously been described as altered in cancer and the binding of HPA to the metastatic cancer cells could not be explained by the presence or absence of a single HPA-binding glycoprotein. HPA binding to the cancer cells was explained by the lectin binding to a range of glycoproteins with varying expression levels and / or glycosylation states.

## 7.4 HPA-binding glycoproteins in CRC tissue samples

The analysis of the colorectal tissue sample proteins (cancerous and normal) by both 1-DE and 2-DE highlighted differences in the levels of several proteins (chapter 6). The 2-DE and lectin blotting approach enabled us to isolate several HPA binding partners found exclusively in the cancerous samples. Some of these proteins are present in higher levels in the protein pool from the metastatic tissue samples as compared to the non metastatic tissue sample pool. The comparative analysis showed that one of the HPA binding proteins might be GP130 but immunodetection using an antibody directed against integrin  $\alpha 6$  failed to establish a relationship between the integrin  $\alpha 6$  level and the intensity of HPA binding. This brings to light the limitations of cell line models and the difficulty of transposing findings from an *in vitro* model to cancer tissue samples. Our approach allowed us to separate five groups of proteins recognised by HPA in the proteins from the cancer tissue samples. The levels of the proteins were compared using the protein pools each containing proteins derived from four different tissue samples. Clearly the variation in the level of the proteins in the individual tumour samples would



need to be established. A higher throughput approach to evaluate the use of the protein as a biomarker of metastasis in low stage cancer, perhaps using an antibody directed against the proteins or glycopeptides would also be useful for testing larger numbers of CRC tissue samples.

The proteins recognised by HPA could play a role in the metastatic process and their identification is needed to complement the findings that were made with the cell line model. The identification of these proteins and their glycosylation might also increase our understanding of the metastatic process and may be of interest as cancer biomarkers.

## 7.5 Conclusions and future directions

The work presented in this thesis confirmed that HPA is a useful tool for identification of proteins in metastatic cancer. The proteomic analysis showed that the value of HPA relies in its recognition of complex carbohydrates carried by specific proteins differentially expressed in metastatic cells. More work is required to confirm and extend our findings to determine if the HPA binding glycoproteins observed in CRC are the same as those found in other epithelial tumours and also to characterise the glycans involved.

One of the main reasons that HPA has not been used for diagnostic purposes is the variability in the methodology for HPA histopathology (Brooks *et al.*, 1996). Other important factors that might render the HPA material useful in clinical decision making include the need for recombinant lectin (for use in diagnostic kits), lack of clarity regarding the cancer-associated HPA-binding glycans and limited knowledge of the structural basis for HPA-binding to metastatic cancer cells. Recombinant HPA lectin would be of considerable value in driving forward method development efforts and would allow multi-centre trials to be conducted to evaluate the lectin in a diagnostic setting. A detailed knowledge of either the cancer or lectin biology would open avenues based around targeting the HPA binding epitopes in solid tumours. The data that we have obtained has enabled us to make significant inferences regarding the basis for HPA recognition of metastatic cancer cells.



## **APPENDICES**



## APPENDIX 1: TNM staging

T describes the extent of the cancer spread through the colon wall. The wall of the colon is formed of several layers described in the section “anatomy of the colon” of this thesis. These layers are the following from the inner to the outer, mucosa, muscularis mucosa, submucosa, muscularis propria, subserosa and serosa.

Tx: description impossible

Tis: the cancer is named “in situ” and is confined in the mucosa. It has not invaded beyond the muscularis mucosa.

T1: The cancer has invaded through the muscularis mucosa and extent to the submucosa

T2: The cancer has invaded through the submucosa and extent to the muscularis propria

T3: The cancer has invaded through the muscularis propria and subserosa without affecting any nearby organs or tissues.

T4: The cancer has spread through the wall of the colon into the nearby tissues.

N indicates the degree of involvement of the regional lymph node

Nx: no description possible

N0: No lymph node involvement is found

N1: Cancer cells found in 1-3 neighbouring lymph node

N2: Cancer cells found in 4 or more nearby lymph node

M indicates whether or not the cancer has spread to distant organs.

Mx: no description possible

M0: no distant spread

M1: distant spread observed



## **APPENDIX 2: Protein assays**

### **Bradford protein assay**

50  $\mu$ l of BSA standard solution prepared in buffers 1 to 4 (table 3) or appropriate dilution of sample in a final volume of 50  $\mu$ l, were incubated for 5 min at room temperature with 1.5 ml of ready to use Bradford reagent (Sigma), consisting of Brilliant blue G in methanol and phosphoric acid. The absorbance was measured at 595 nm on a spectrophotometer.

### **DC protein assay:**

50  $\mu$ l of BSA standard solution prepared in buffers 1 to 4 (table 3) were incubated with 200  $\mu$ l of reagent A, consisting in alkaline copper tartrate solution supplemented with SDS solution, and 2 ml of Folin reagent (reagent B) for 15 min before the absorbance was read at 750 nm in spectrophotometer.

### **RCDC protein assay:**

25  $\mu$ l of BSA standard solution prepared in buffers 1 to 4 (table 2.5) were incubated for 1 min at room temperature with 125  $\mu$ l of RC reagent 1 before 125  $\mu$ l of RC reagent 2 was added and the mixture centrifuged at 15000 g for 5 min. 127  $\mu$ l of DC reagent A (alkaline copper tartrate supplemented with SDS) was added to the pellet and incubated for a further 5 min before being incubated for 15 min with 1 ml of Folin reagent. The absorbance was then read at 750 nm.



## APPENDIX 3: Recipes for buffers used in 1-DE and 2-DE experiments.

### Resolving gel Buffer (1M Tris-HCl pH 8.8)

---

Tris (FW 121.1)	1M
dH <sub>2</sub> O	150 ml
HCl	to pH 8.8
dH <sub>2</sub> O	to 200 ml

### Stacking gel Buffer (0.5M Tris-HCl pH 6.8)

---

Tris (FW 121.1)	0.5M
dH <sub>2</sub> O	150 ml
HCl	to pH 6.8
dH <sub>2</sub> O	to 200 ml

### Water-saturated n-butanol

---

N-butanol	50 ml
dH <sub>2</sub> O	50 ml

### 15 % Ammonium persulfate

---

Ammonium persulfate (FW 228.2)	15% w/v
dH <sub>2</sub> O	1 ml

### 10 % SDS

---

SDS (FW 288.38)	10%
dH <sub>2</sub> O	to 100 ml

### Running buffer

---

Tris (FW 121.1)	0.025 M
Glycine (FW 75.07)	0.192 M
SDS (FW 288.38)	0.1% w/v
dH <sub>2</sub> O	to 1L



<b>Stacking gel (2.5 ml per minigel)</b>	<b>4%</b>
Acrylamide/bisacrylamide 40%	0.25 ml
Stacking buffer	0.63 ml
dH <sub>2</sub> O	1.5 ml
10% SDS	25 $\mu$ l
15% APS	12.5 $\mu$ l
TEMED	2.5 $\mu$ l

<b>Resolving gel (5 ml per minigel)</b>	<b>10%</b>	<b>12%</b>
Acrylamide/bisacrylamide 40%	1.25 ml	1.5 ml
Resolving buffer	1.9 ml	1.9 ml
dH <sub>2</sub> O	1.8 ml	1.55 ml
10% SDS	50 $\mu$ l	50 $\mu$ l
15% APS	25 $\mu$ l	25 $\mu$ l
TEMED	2.5 $\mu$ l	2.5 $\mu$ l

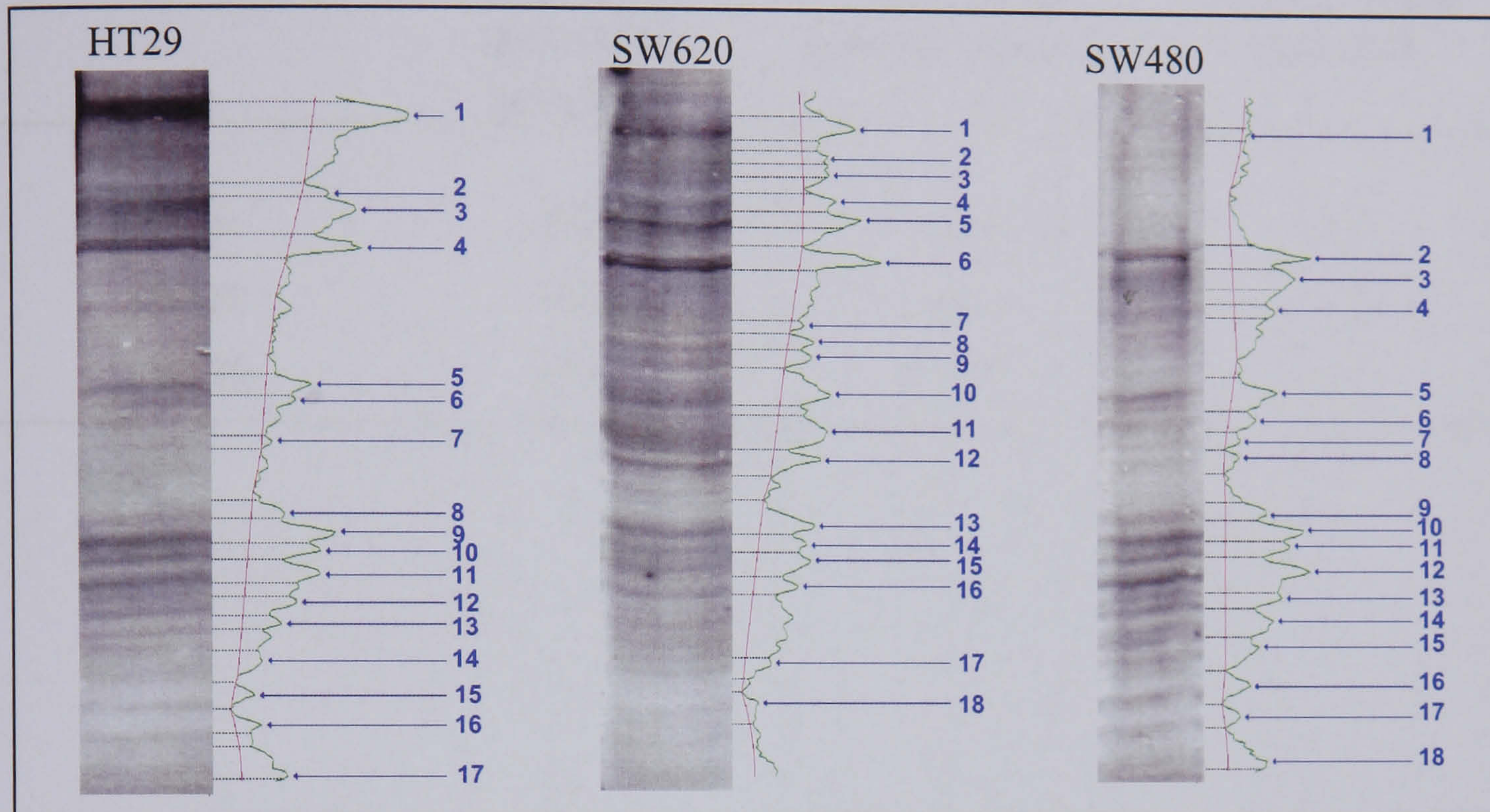


## APPENDIX 4 : Microtitre plate assay

	HT29			SW620			SW480		
	Fluorescence/ 100000 cells (Au)	Mean	Standard deviation	Fluorescence/ 100000 cells (Au)	Mean	Standard deviation	Fluorescence/ 100000 cells (Au)	Mean	Standard deviation
1	6699			4816			1337		
2	6862			5204			1001		
3	7524	7014	539	5281	4985	233	1181	1216	132
4	7048			4772			1199		
5	6265			4755			1376		
6	7688			5080			1202		



## APPENDIX 5: List of HPA-binding proteins in HT29, SW620 and SW480



### HPA binding protein bands

HT29		SW620		SW480	
Band number	MW	Band number	MW	Band number	MW
1	135	1	130	1	130
2	94	2	113	2	73
3	87	3	105	3	67
4	74	4	92	4	60
5	45	5	85	5	44
6	43	6	71	6	41
7	38	7	55	7	38
8	33	8	52	8	37
9	31	9	50	9	32
10	30	10	44	10	31
11	29	11	40	11	30
12	28	12	37	12	29
13	27	13	32	13	28
14	26	14	31	14	27
15	25.5	15	30	15	26
16	25	16	29	16	25.5
17	24	17	26	17	25
		18	25	18	24



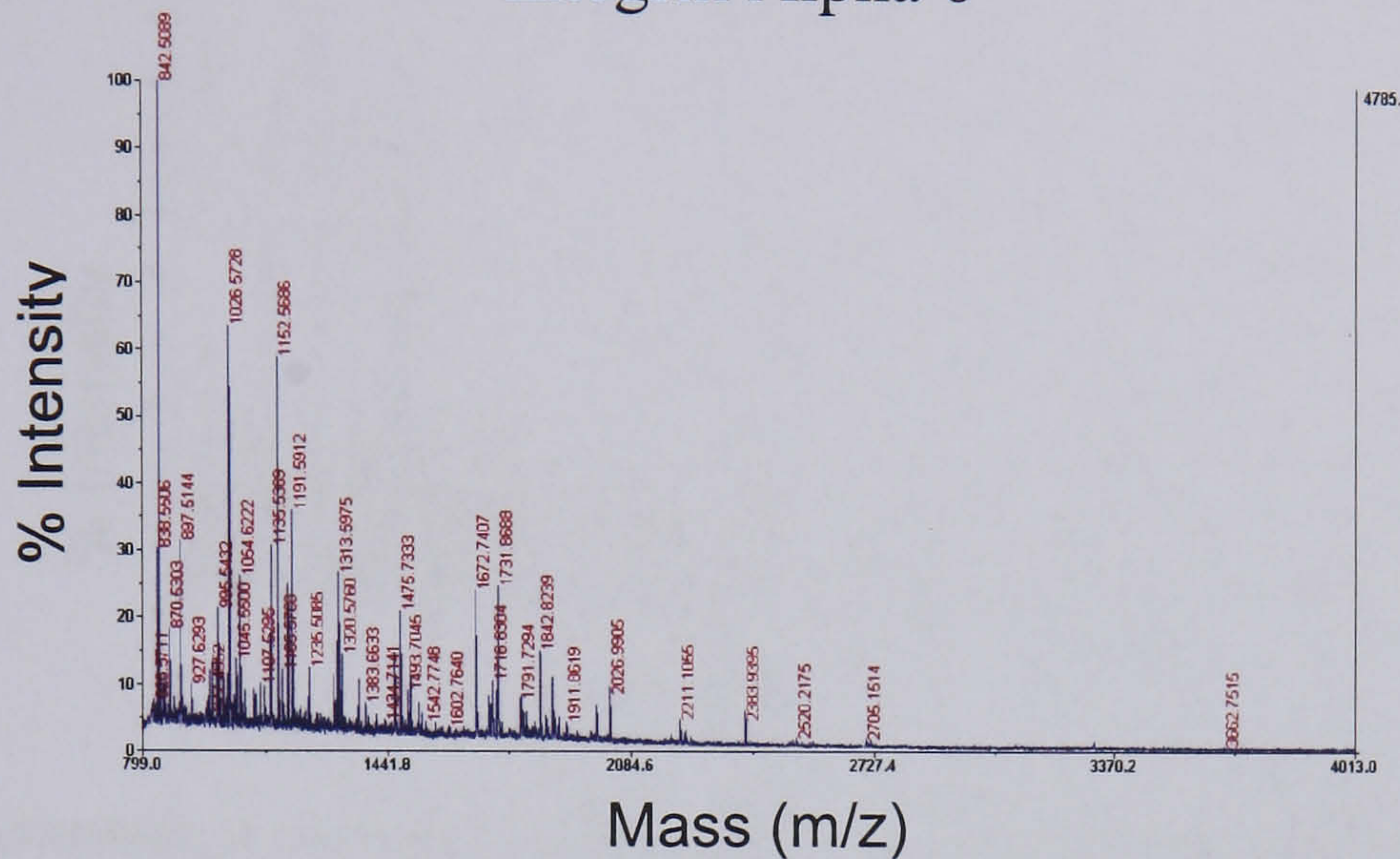
## APPENDIX 6 : Affinity chromatography analysis

<b>Cell lines</b>	<b>Area of the unbound fraction</b>	<b>Area of the bound fraction</b>	<b>% of bound proteins</b>
HT29	2999	388	11
SW480	1963	25	1.2
SW620	2799	107	3



# APPENDIX 7: Mass spectra and sequence coverage

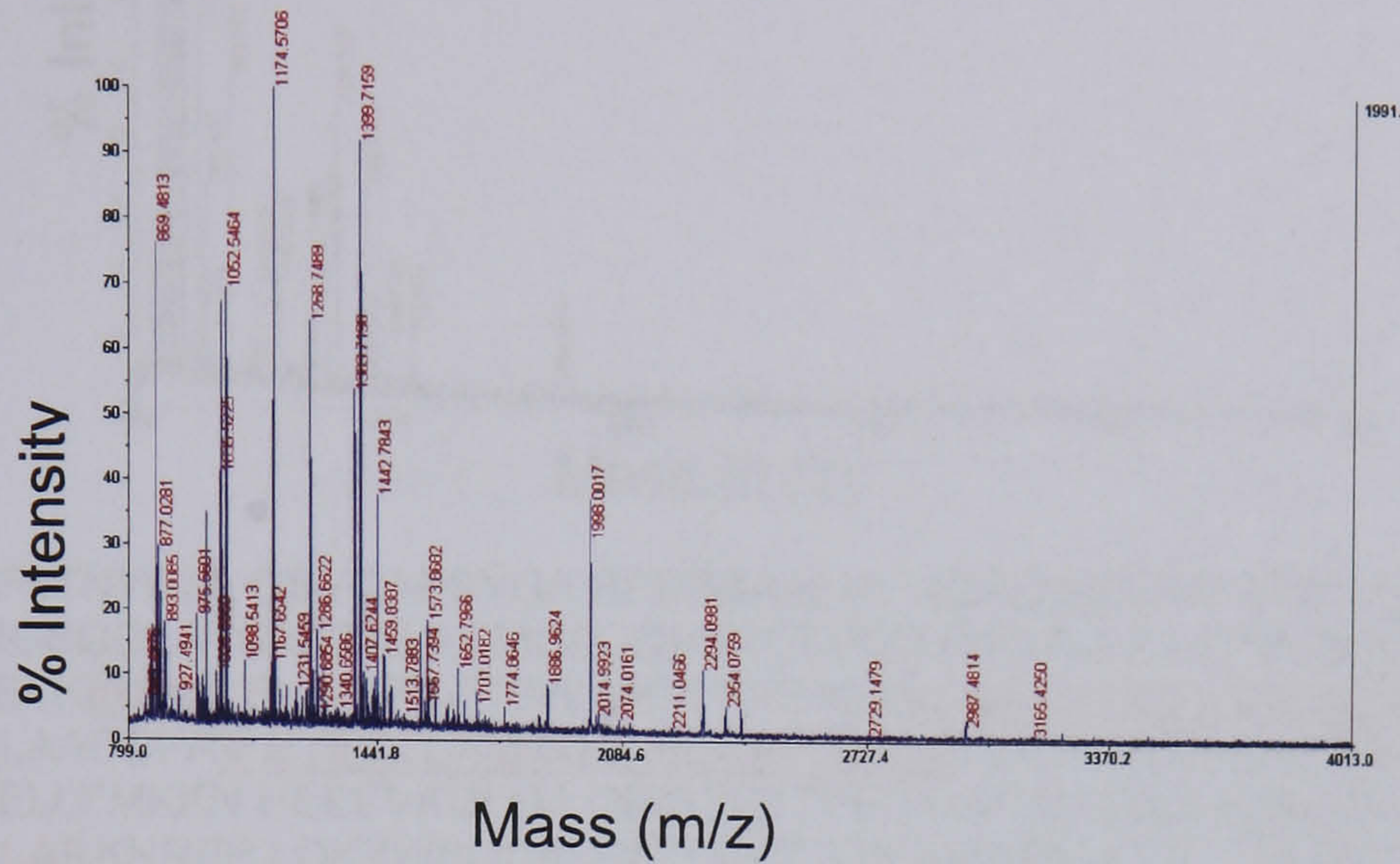
Integrin Alpha 6



MAAAGQLCLL YLSAGLLSRL GAAFNLDTRE DNVIRKYGDP GSLFGFSLAM  
 HWQLQPEDKR **LLVGAPRAE ALPLQR**ANRT **GGLYSCDITA R**GPCTRIEFD  
 NDADPTSESK EDQWMGVTVQ SQPGGKVVV CAHRYEKRQH VNTKQESRDI  
 FGR**CYVLSQN LRIEDDMDGG DWSFCDGR**LR GHEK**FGSCQQ GVAATFTK**DF  
 HYIVFGAPGT YNWKGIVRVE QKNNTFFDMN IFEDGPYEVG GETEHDESLV  
 PVPANSYLG FSLDSGKGI VS **KDEITFVSGA PR**ANHSGAVV LLKRDMKSAH  
 LLPEHIFDGE GLASSFGYDV AVVDLNKDGW QDIVIGAPQY FDR**DGEVGGGA**  
**VVYVMNQGR WNNVKPIR**LN GTKDSMFGIA VKNIGDINQD GYPDIAVGAP  
 YDDLKGVFIY HGSANGINTK PTQVLKGISP YFGYSIAGNM DLDR**NSYPDV**  
**AVGSLSDSVT IFRSRPVINI QK**TITVTPNR IDLRQK**TACG APSGICLQVK**  
 SCFEYTANPA GYNPSISIVG TLEAEKERRK SGLSSRVQFR NQGSEPK**YTQ**  
**ELTLK**RQKQK VCMEETLWLQ DNIRDK**LRPI PITASVEIQE PSSR**RRVNSL  
 PEVLPILNSD EPKTAHIDVH FLKEGCGDDN VCNSNLKLEY KFCTREGNQD  
 KFSYLPKQKG VPELVKLDQK DIALEITVTN SPSNPRNPTK DGDDAHEAK**L**  
**IATFPDTLTY SAYR**ELRAFP EKQLSCVANQ NGSQADCELG NPFKRNSNVT  
 FYLVLSTTEV TFDTPYLDIN LKLETTSNQD NLAPITAKAK VVIELLSVS  
 GVAKPSQVYF GGTVVGEQAM **KSEDEVGSLI EYEF**RVINLG KPLTNLGTAT  
 LNIQWPKEIS NGKWLLYLK VESKGLEKVT CEPQKEINSL NLTESHNSRK  
 KREITEKQID DNRKFSLFAE RKYQTLNCSV NVNCVNIR**CP LRGLDSK**ASL  
 ILRSRLWNST FLEEYSKLN LDILMRAFID VTAAAENIRL PNAGTQVRVT  
 VFPSKTVAQY SGVPWWIILV AILAGILMLA LLVFILWKCG FFKRNKKDHY  
 DATYHKAEIH AQPSDKERLT SDA



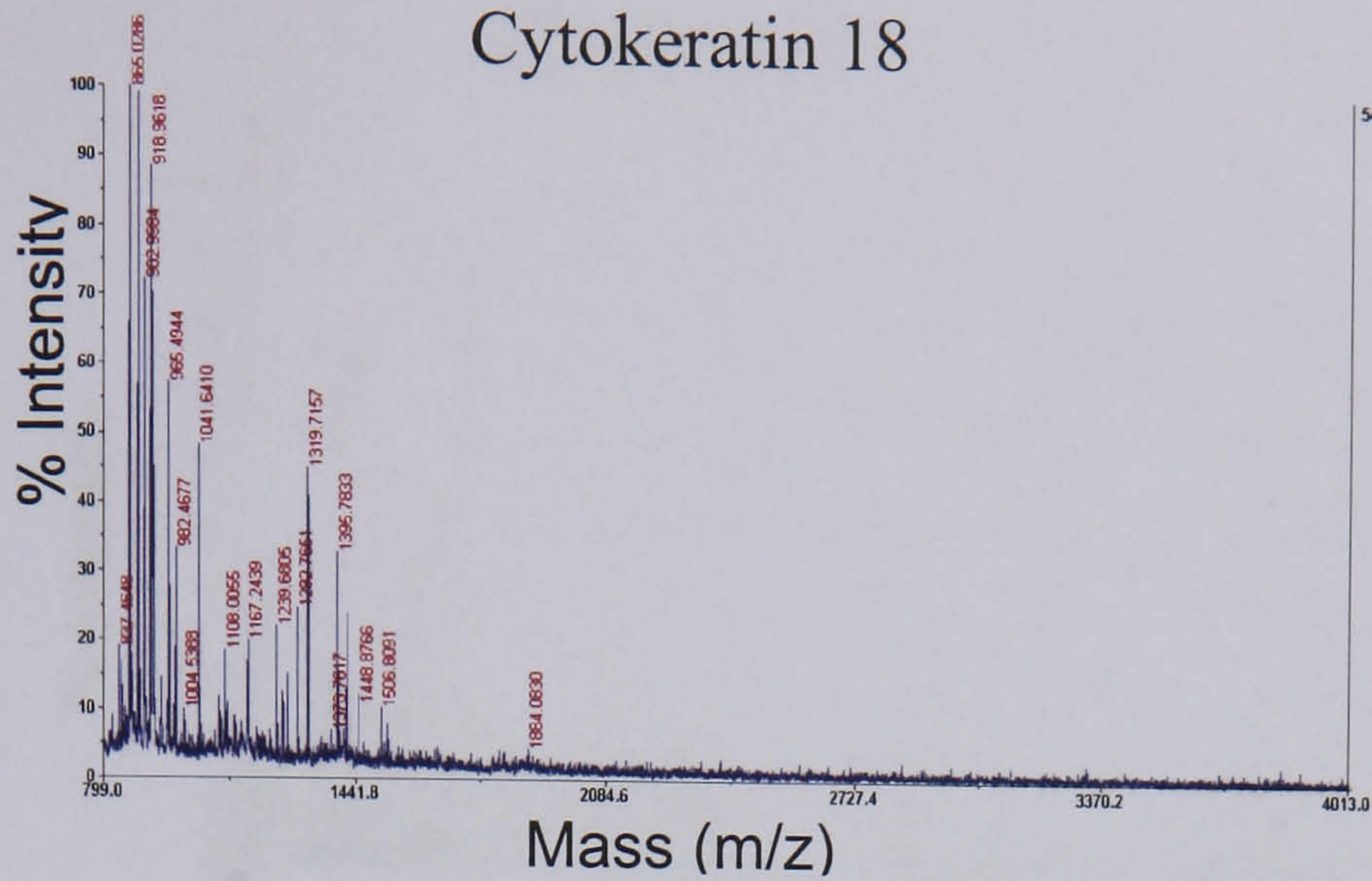
## Integrin alpha V



MAFP~~RRRLR~~ LGPRGLPLLL SGLLCRA FNLDVDSPAE YSGPEGSYFGFAVDFFVPSA  
 SSRMFLVGA PKANTTQPGI VEGGQVLKCD WSSTRRCQPIEFDATGNRDY AKDDPLEFKS  
HQWFGASVRS KQDKILACAP LYHWRTEMKQEREPVGTCLF QDGTKTVEYA  
 PCRSQDIDAD GQGFCQGGFS IDFTKADRVL LGPGGSFYWQ GQLISDQVAE IVSKYDPNVY  
 SIKYNNQLAT RTAQAIFFDDSYLGYSVAVDG FNGDGIDDFV SGVPRAARTL GMVYIYDGKN  
 MSSLYNFTGEQMAAYFGFSV AATDINGDDY ADVFIGAPLF MDRGSDGKLQ  
EVGQVSVSLQ RASGDFQTTK LNGFEVFARF GSAIAPLGDL DQDGFNDIAI AAPYGGEDKK  
GIVYIFNGRS TGLNAVPSQI LEGQWAARSM PPSFGYSMKG ATDIDKNGYP DLIVGAFGVD  
 RAILYRARPV ITVNAGLEVY PSILNQDNKT CSLPGTALKV SCFNVRFLCK ADGKGVLPK  
 LNFQVELLLD KLKQKGAIIR ALFLYSRSPSHSKNMTISRG GLMQCEELIA YLRDESEFRD  
 KLTPITIFME YRLDYRTAAD TTGLQPILNQ FTPANISRQA HILLDCGEDN VCKPKLEVS  
 DSDQKKIYIGDDNPLTLIVK AQNQGEGAYE AELIVSIPLQ ADFIGVVRNN  
 EALARLSCAFKTENQTRQVV CDLGNPMKAG TQLLAGLRFV VHQQSEMDTS  
 VKFDLQIQSNLFDKVSPVV SHKVDLAVLA AVEIRGVSSP DHIFLPIPNW EHKENPETEE  
 DVGPVQHIY ELRNNGPSSF SKAMLHLQWP YKYNNNTLLY ILHYDIDGPM NCTSDMEINP  
 LRIKISSLQT TEKNDTVAGQ GERDHLITKR DLALSEGDIH TLGCGVAQCL KIVCQVGRD  
 RGKSAILYVK SLLWTETFMN KENQNHSSYLKSSASFNIE FPYKNLPIED ITNSTLVTTN  
 VTWGIQPAPM PVPVWVILA VLAGLLLLAV LVFVMYRMGF FKRVRPPQEE QEREQLQPHE  
 NGEENSET

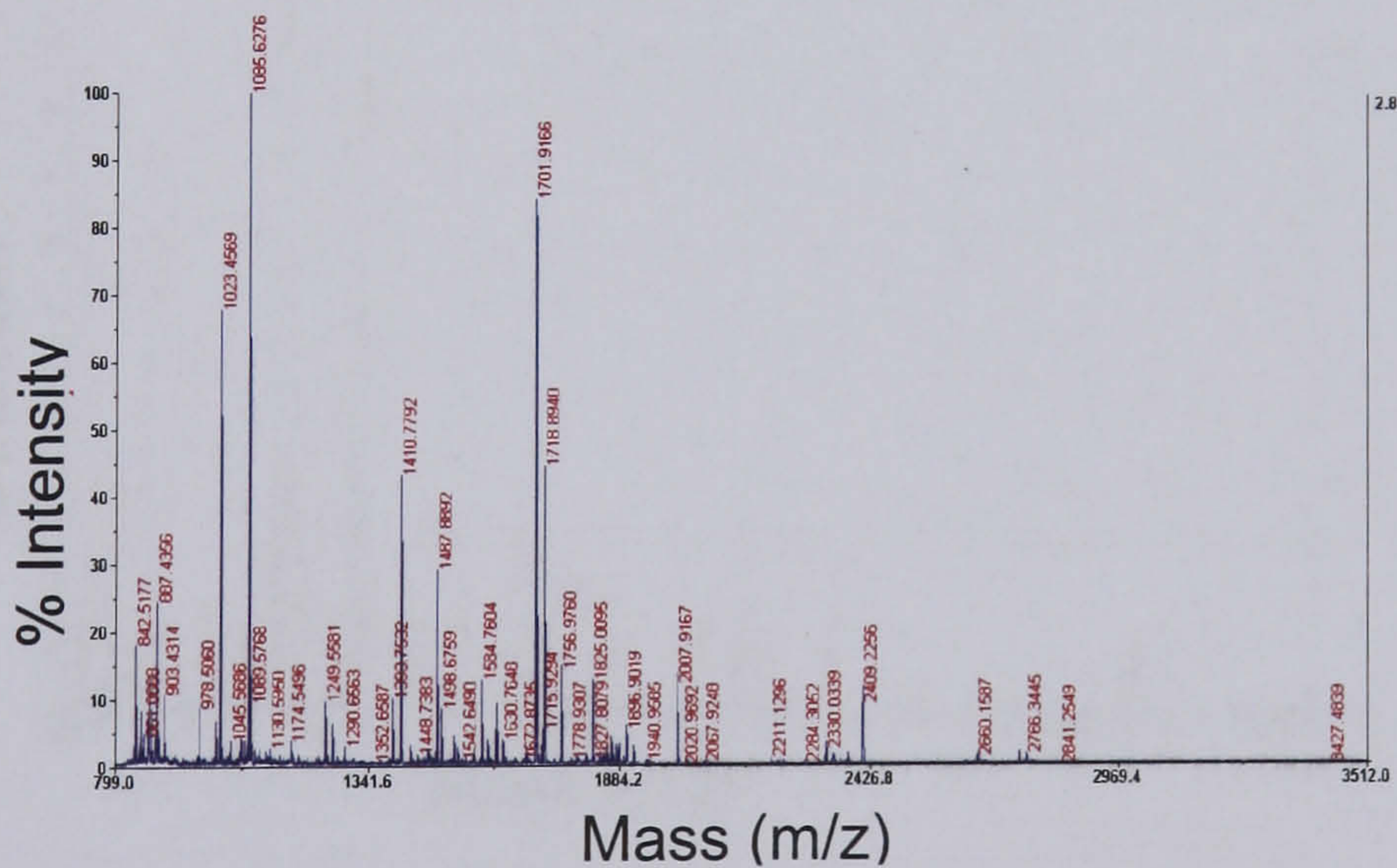


## Cytokeratin 18



STFSTNYRSL GSVQAPSYGA RPYSSAASVY AGAGGSGSRI SVSRSTSFRG  
 GMGSGGLATG IAGGLAGMGG IQNEKETMQS LNDRLASYLD RVRSLTENR  
 RLESKIREHL EKKGPPQVRDW SHYFKIIDL RAQIFANTVD NARIVLQIDN  
 ARLAADDFRV KYETELAMRQ SVENDIHGLR KVIDDTNITR LQLETEIEAL  
 KEELLFMKN HEEEVKGLQA QIASSGLTVE VDAPKSQDLA KIMADIRAQY  
 DELARKNREE LDKYWSQQIE ESTTVTTQS AEVGAAETTL TELRRTVQSL  
 EIDLDSMRNL KASLENSLRE VEARYALQME QLNGILLHLE SELAQTRAEG  
 QRQAQYEAL LNIKVKLEAE IATYRRLLED GEDFNLGDAL DSSNSMQTIQ  
 KTTTRRIVDG KVVSETNDTK VLRH

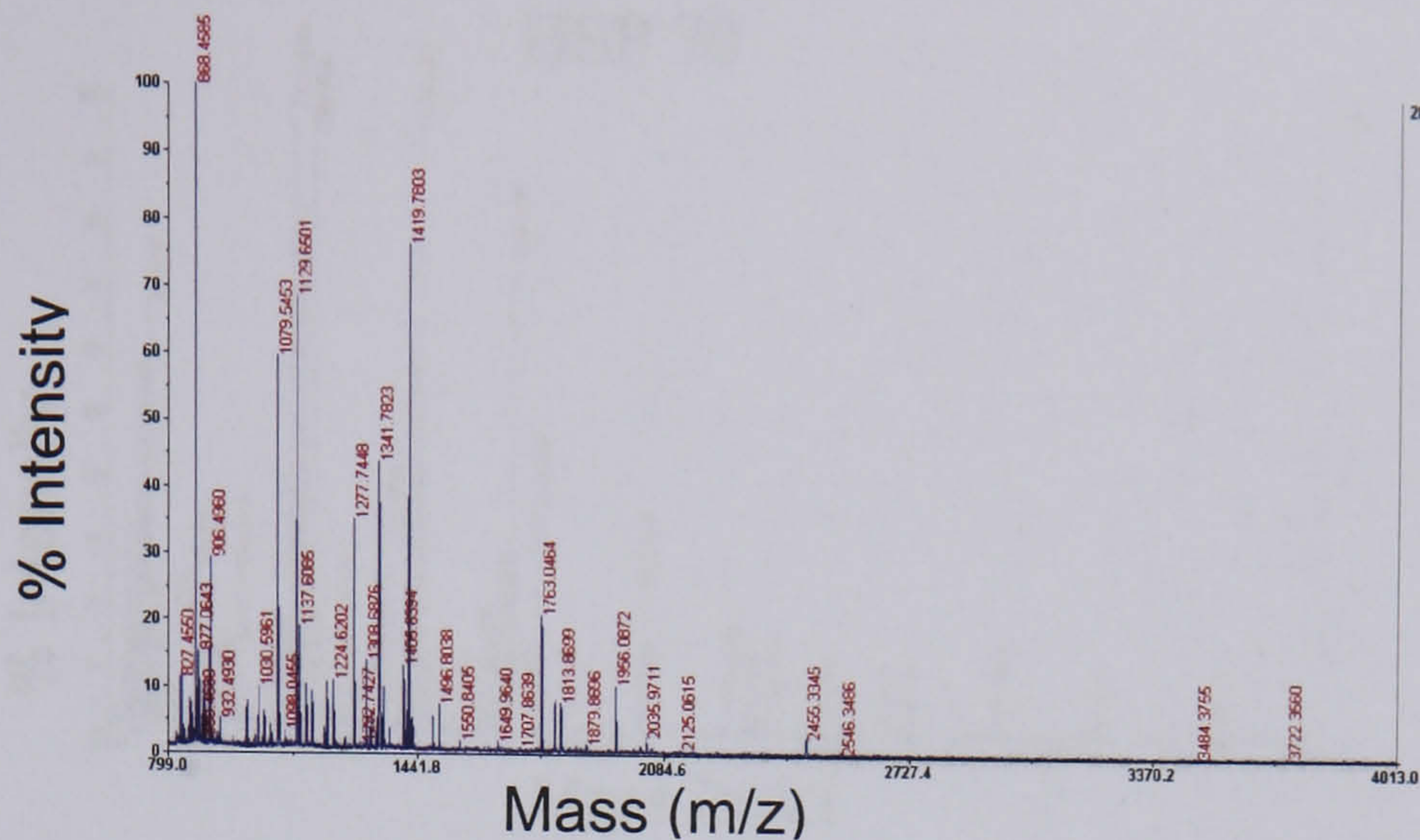
## Alpha tubulin



MRECISIHVG QAGVQIGNAC WELYCLEHGI QPDGQMPSDK TIGGGDSFN  
 TFFSETGAGK HVPRAVFVDL EPTVIDEVR T GTYRQLFHPE QLITGKEDAANNYAR GHYTI  
 GKEIIDLVLD RIRKLAQCT RLQGFLVFHS FGGGTGSGFT SLLMERLSVD YGKSKLEFS  
 IYPAPQVSTA VVEPYNSILT THTTLEHSDC AFMVDNEAIY DICRRNLDIE RPTYTNLNR  
 ISQIVSSITA SLRFDGALNVDL TEFQTNLV PYPRIHFPLA TYAPVISA EK AYHEQLSVAD  
 ITNACFEPAN QMVKCDPGHG KYMACCLLYR GDVVPKDVNA AIATIKTKRT  
 IQFVDWCPTGFKVGINYQPP TVVPGDLAK VQRAVCMLSN TTAIAEAWAR  
 LDHKFDL MYA KRA FVHWYVG EGMEEGEFSE AREDMAALEK DYEEVGVD SV  
 EGE GEE GEE Y

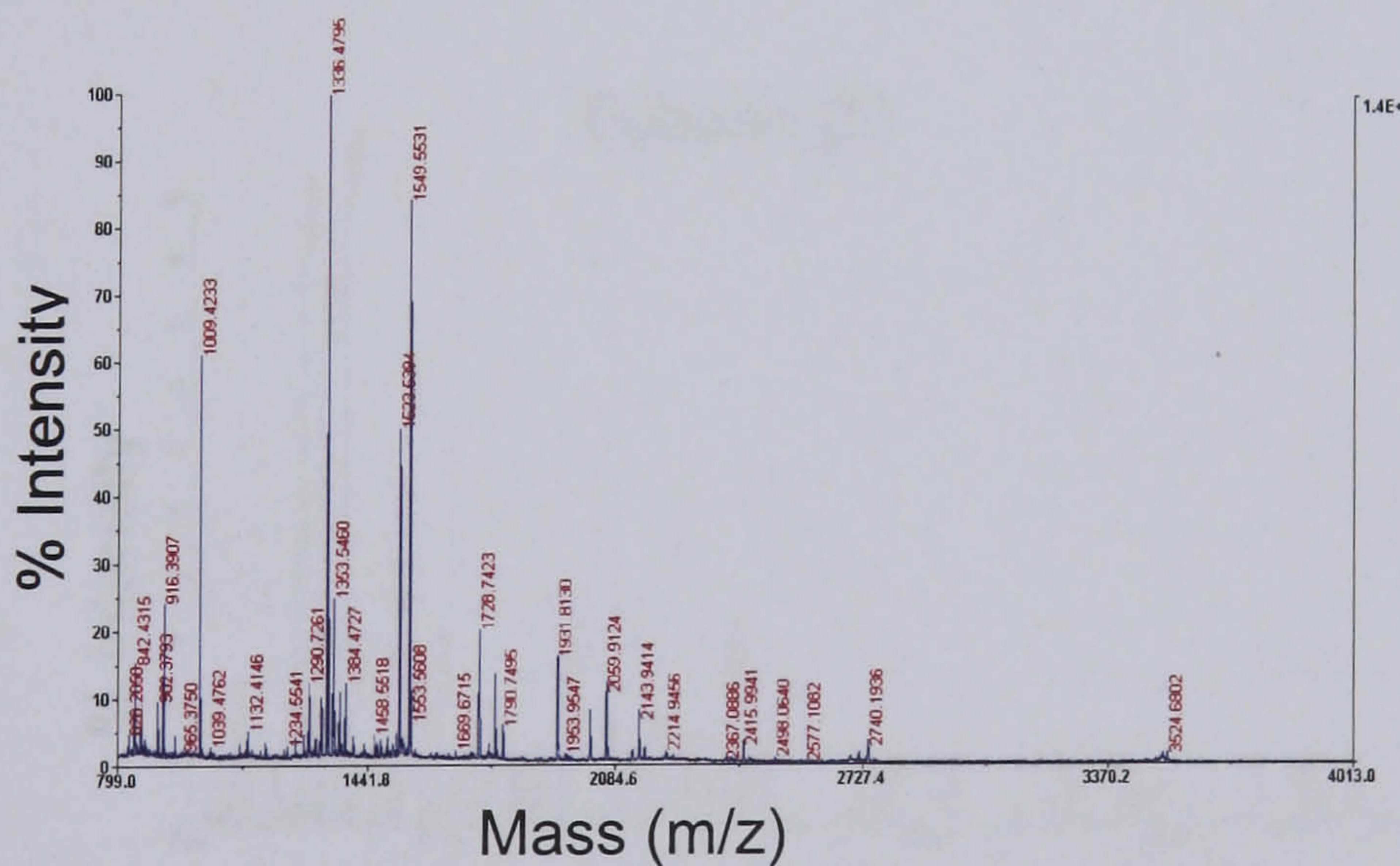


## Cytokeratin 8



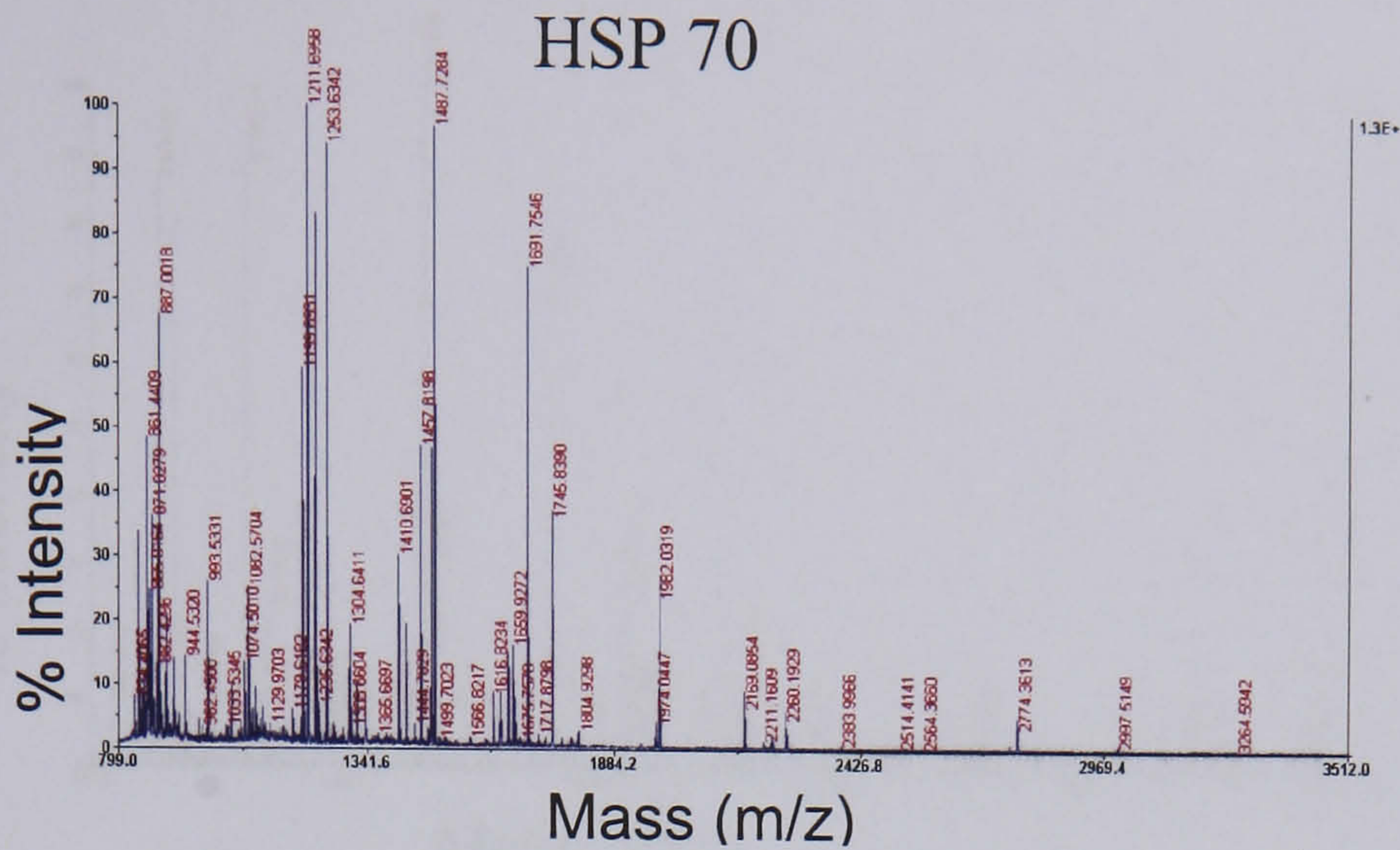
MSIRVTQKSY KVSTSGPRAF SSRSYTSGPG SRISSSSFSR VGSSNFRGGL  
GGGYGGASGM GGITAVTVNQ SLLSPLVLEV DPNIQAVR **TQ EKEQIK** TLNN  
**KFASFIDKVR FLEQQNK** MLE TK **WSELLQQQK** TARSNMDNMF ESYINNLRR **Q**  
**LETLGQEK** LK LEAELGNMQG LVEDFKNKYE DEINKR **TEME NEFVLIKKDV**  
**DEAYMNKVEL ESRLEGLTDE INFLRQLYEE EIR** ELQSQIS DTSVVLSDMN  
SR **SLDMDSII AEVKAQYEDI ANRSRAEAE MYQIKYEELQ SLAGKHGDDL**  
**RRTKTEISEM NR** NISR **LQAE IEGLKGQRAS LEAAIADAEQ RGELAIK** DAN  
AK **LSELEAAL QRAKQDMARQ** LREYQELMN **V KLALDIEIAT YRK** LLEGEES  
RLESGMQNMS IHTKTTGGYA GGLSSAYGGS QAGLSYSLGS SFGSGAGSSS  
FSRTSSSRV VVKKIETRDG KLVSESSDVL PK

## Guanine nucleotide-binding protein, beta-2 subunit

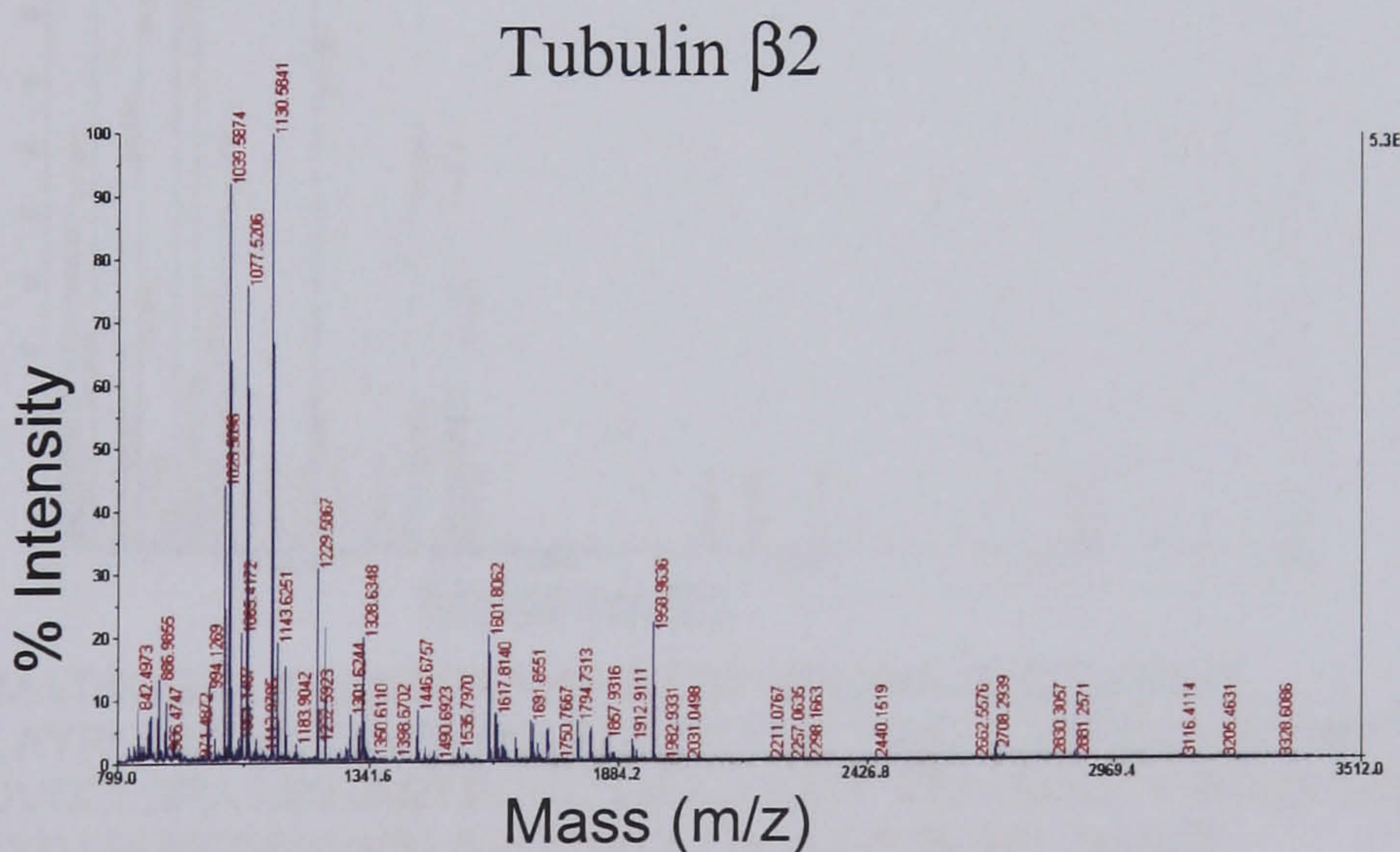


M **SELEQLRQE AEQLR** NQIRD AR **KACGDSTL TQITAGLDPV**  
**GRIQMRTRRTRLRGLAKIYA MHWGTDSR** LL VSASQDGKLI IWDSYTTNKV  
HAIPLRSSWVMT CAYAPSGN FVACGGLDNI CSYSLKTRE GNVRVSR **ELP**  
**GHTGYLSCCR** FLDDNQIITS SGGTTALWD IETGQQTVGF AGHSGDVMSL  
SLAPDGRTFVSGACDASIK **L WDVDRSMCR** Q TFIGHESDIN AVAFFPNGYA  
FTTGSDDATCRLFDLR **ADQE LLMYSHDNII CGITSAFVS** R SGRLLLAGYD  
DFNCNIWDAM KGDR **AGVLAG HDNRVSLGV TDDGMAVATG SWDSFLKI** WNW





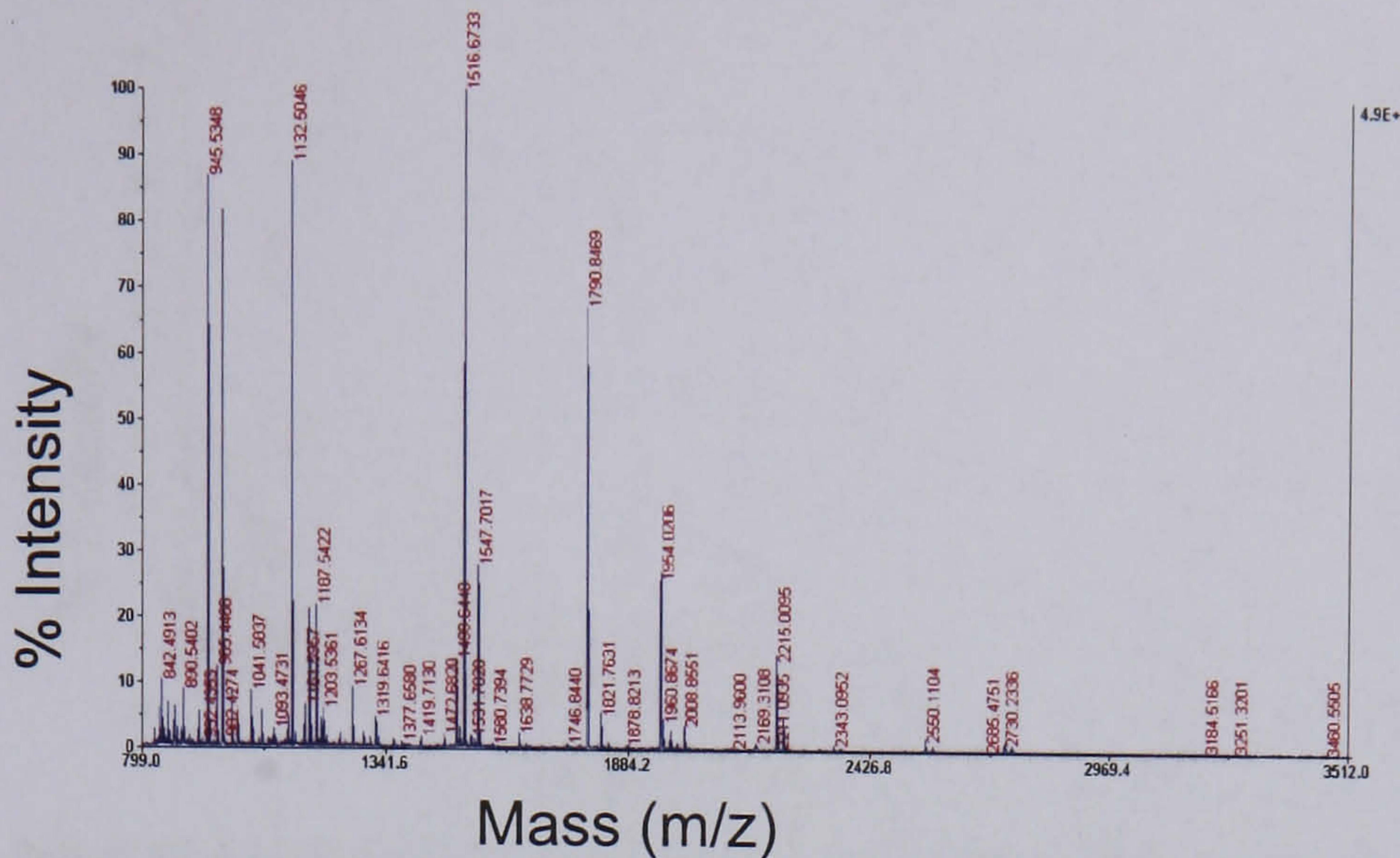
MSKGPVAVGID LGTTYSCVGV FQHGK**VEIIA NDQGNRTTPS YVAFTDTERL**  
 IGDAAKNQVA MNPTNTVFDA KRLIGRRFDD AVVQSDMKHW  
 PFMVVNDAGRPKVQVEYKGE TKSFPYEEVS SMVLTkmKEI AEAYLGK**TVT**  
**NAVVTVPAYFNDSQR**QATK**D AGTIAGLNVL RI**INEPTAAA IAYGLDKKVG  
 AERNVLIFDL GGGTFDVSIL TIEDGIFEVK **STAGDTHLGG EDFDNR**MVNH  
 FIAEFKRKHK KDISENKRAV RRLRTACERA KRTLSSSTQA SIEIDSLYEG  
 IDFYTSITRA R**FEELNADLF R**GTLDPVEKA LRDAKLDKSQ IHDIVLVGGS  
 TRIPKIQKLL QDFFNGKELN KSINPDEAVA YGAAVQAAIL SGDKSENVQD  
 LLLLDVTPLS LGIETAGGVM TVLIKRNTTI PTKQTQFTT YSDNQPGVLI  
 QVYEGERAMT KDNLLGKFE LTGIPPAPRG VPQIEVTFDI DANGILNVSA  
 VDKSTGKENK ITITNDKGRL SKEDIERMVQ EAEKYKAEDE KQRDKVSSKN  
 SLESYAFNMKATVEDEKLQG KINDEDKQKI LDKCNEIINW LDK**NQTAEKE**  
**EFEHQQK**ELEKVCNPIITKL YQSAGGMPGG MPGGFPGGGA  
 PPSGGASSGP TIEEVD



MREIVHLQAG QCGNQIGAKF WEVISDEHGI DPTGTYHGDS  
 DLQLERINVYYNEATGGKYV **PRAVLVDLEP GTMDSVR**SGP FGQIFRPDNF  
 VFGQSGAGNN WAK**GHYTEGA ELVDSVLDV R**KEAESCDCL QGFQLTHSLG  
 GGTGSGMGTL LISK**IREEYP DR**IMNTFSVV PSPKVSDTVV EPYNATLSVH  
 QLVENTDETY CIDNEALYDI CFRTLKLTTP TYGDLNNLVS ATMSGVTTCL  
 R**FPGQLNADLRKLAVNMVPF PR**LHFFMPGF APLTSRGSQQ YRALVPELT  
 QQMFDAK**NMM AACDPR**HGRY **LTVAAVFR**GR MSMKEVDEQM LNVQKNSSY  
 FVEWIPNNVK **TAVCDIPPR**G LKMSATFIGN STAIQELFKR **ISEQFTAMFR**  
 RKAFLHWYTG EGMDEMEFTE AESNMNDLVS EYQQYQDATA EEEGEFEEEA  
 EEEVA

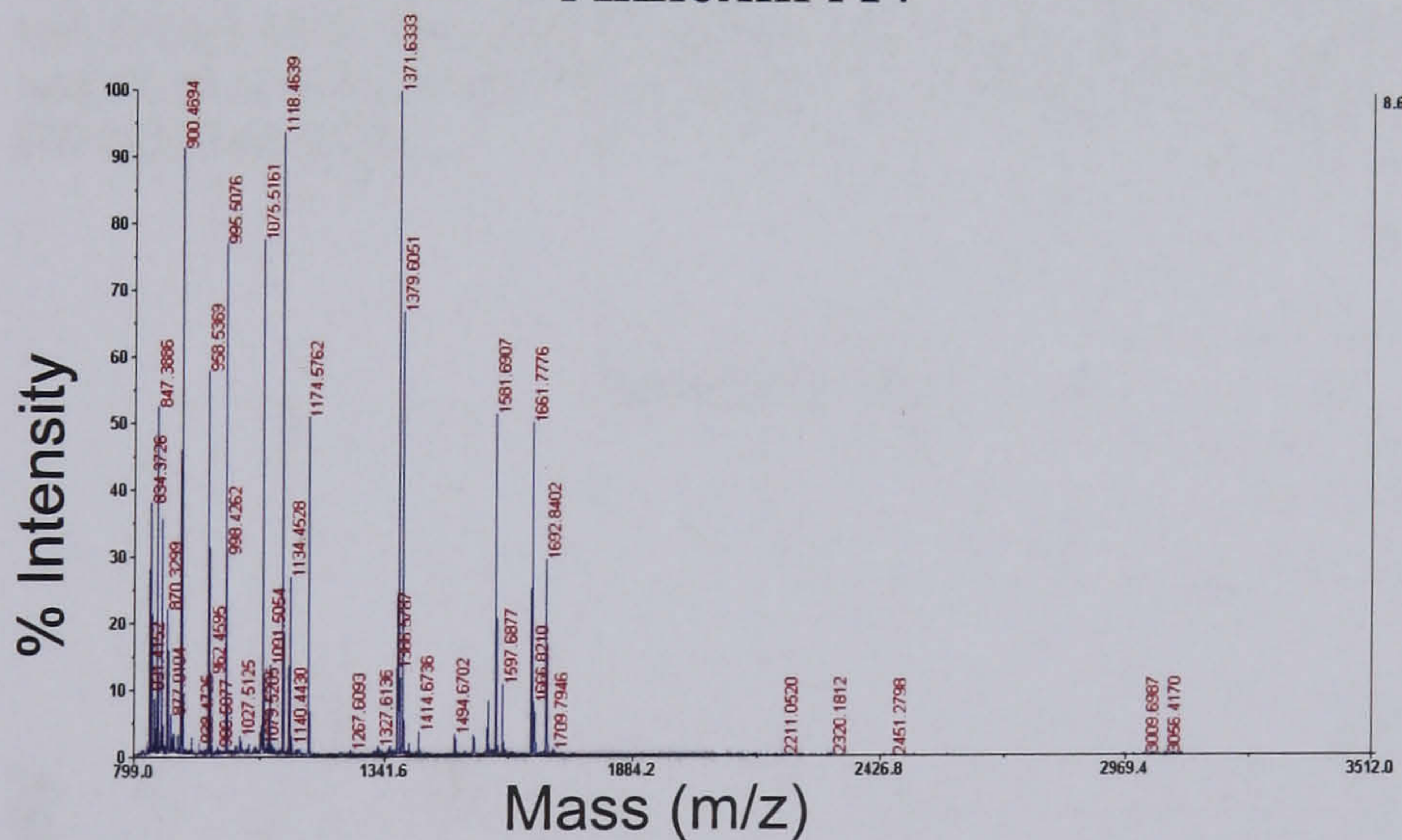


### β actin



MDDDIAALVV DNGSGMCK **AG FAGDDAPRAV** FPSIVGRPR**H**  
**QGVMVGMGQK** DSYVGDEAQS KRGILTLKYP IEHGIVTNWD DMEKIWHHTF  
 YNELR **VAPEEHPVLLTEAPL NPK** ANREKMT QIMFETFNTP AMYVAIQAVL  
 SLYASGRTTGVMDSGDGVT HTVPIYEGYA LPHAILRLDL AGRDLTDYLM  
 KILTER **GYSFTTTAER** EIVR DIKEKLCYVA LDFEQEMATA ASSSSLEK**SY**  
**ELPDGQVITIGNER** FRCPEA LFQPSFLGME SCGIHETTFN SIMKCDVDIR  
 K**DLYANTVLSGGTTMYPGIA DR** MQKEITAL APSTMKIKII APPERKYSVW  
 IGGASILASLS TFQQMWISK **Q EYDESGPSIV HR** KCF

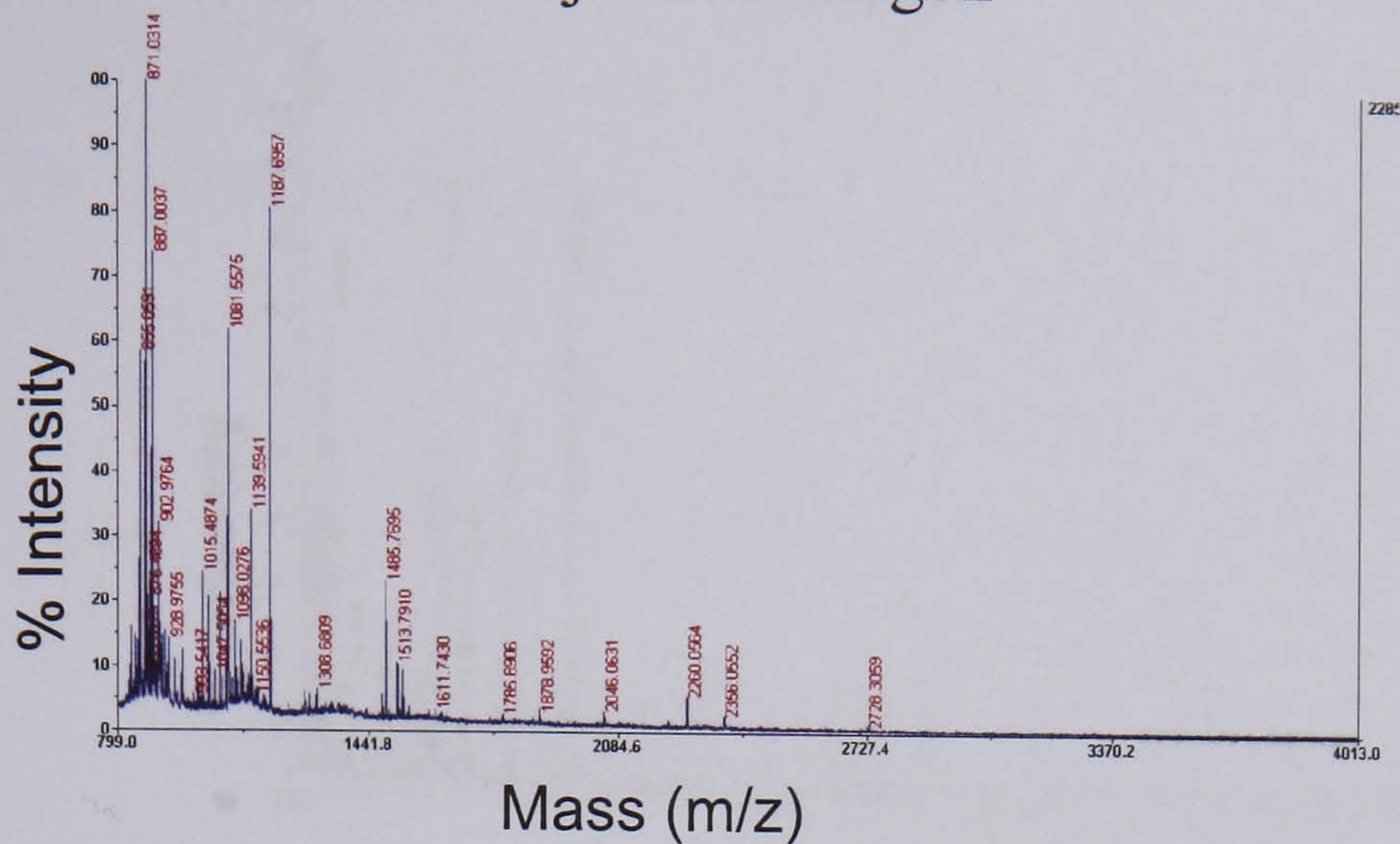
### Annexin A4



MAMATKGGTV **KAASGFNAME DAQTLR** KAMK GLGTDEDAII  
 SVLAYRNTAQ RQEIRTAYKS TIGRDLIDDL KSELSGNFEQ VIVGMMTPTV  
 LYDVQELRRA MK **GAGTDEGC LIEILAS RTP EEIRISQTY QQYGRSLED**  
**DIRSDTSFMFQRLVLSL SAG GRDEGNYLDD ALVRQDAQDL**  
 YEAGEKKWGT DEVK **FLTVLCSR** NNRNHLH V FDEYKRISQK DIEQSIKSET  
 SGSFEDALLA IVKCMRNKSA YFAEKLYKSM KGLGTDDNTL IRVMVSR**AEI**  
**DMLDIR** AHFK RLYGKSLYSFIKGDTS GDYR KVLLVLCGGD D

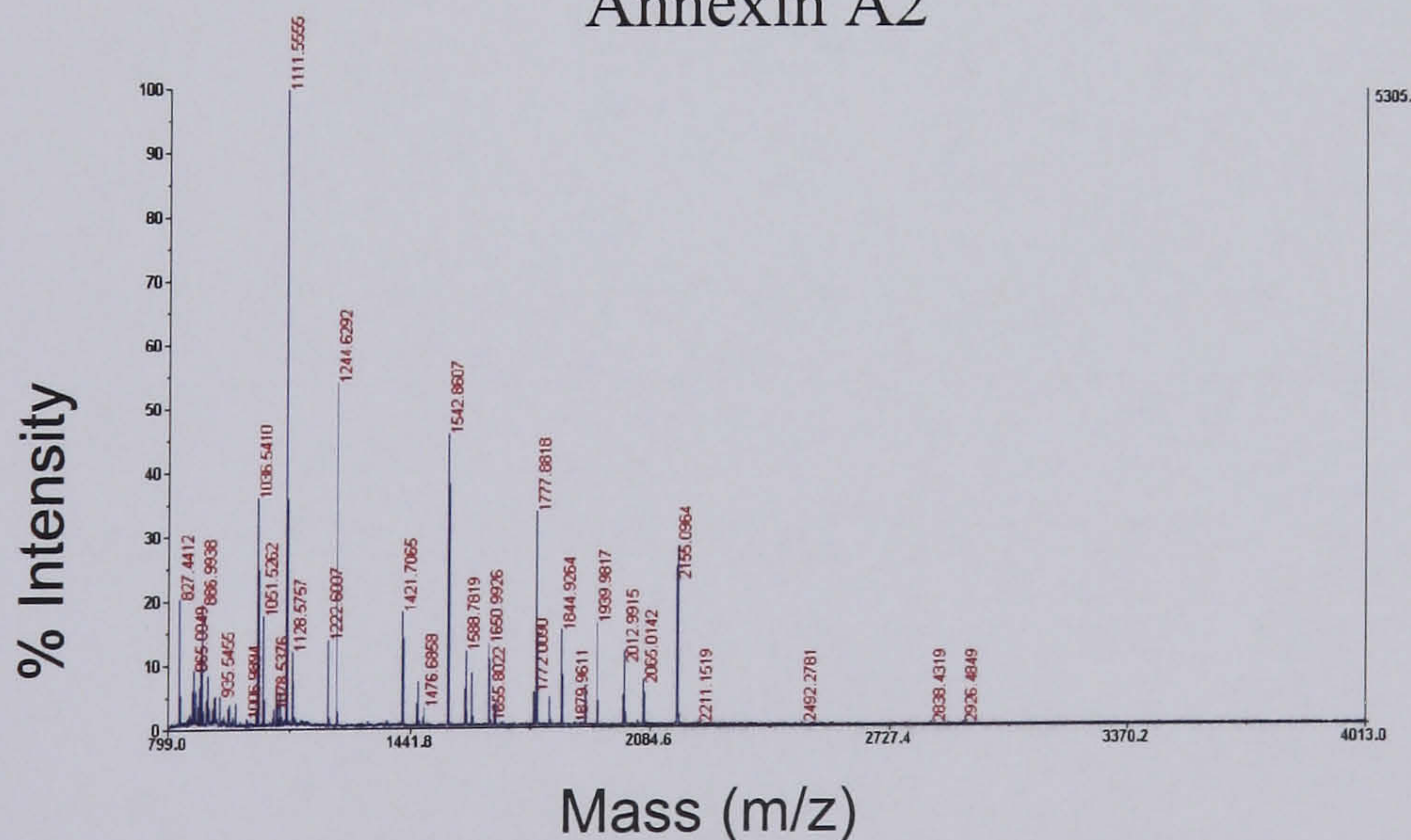


## Tumor rejection antigen



MRALWVLGLC CVLLTFGSVR ADVDGT VEEDLGKSRE  
 GSRTDDEVVQREEEAIQLDG LNASQIRELR EKSEK**FAFQA EVNR**MMKLII  
 NSLYKNKEIFLRELISNASD ALDKIRLISL TDENALSGNE ELTVKIKCDK  
 EK**NLLHVTDTGVGMTR**EELV KNLGTIAKSG TSEFLNKMTE AQEDGQSTSE  
 LIGQFGVGFY SAFLVADKVI VTSKHNDTQ HIWESDSNEF SVIADPRGNT  
 LGRGTTITLV LKEEASDYLE LDTIKNLVKK YSQFINFIY VWSSKTETVE  
 EPMEEEEAAK EEKEESDDEA AVEEEEEEEK PKTKKVEKTV WDWELMNDIK  
 PIWQRPSKEVEEYKAFYK SFSKESDDPM AYIHFTAEGE VTFK**SILFVP**  
**TSAPRGLFDE YGSK**KSDYIK LYVRRVITD DFHDMMPKYL NFVK**GVDSD**  
**DLPLNVS**RETQQHKLLKVI RKKLVRKTLD MIKKIADDKY NDTFWKEFGT  
 NIK**LGVIEDHSNR**TRLAKLL RFQSSHPTD ITSLDQYVER MKEKQDK**IYF**  
**MAGSSR**KEAE SSPFVERLLK KGYEVIYLTE PVDEYCIQAL PEFDGKRFQN  
 VAKEGVKFDE SEKTKESREA VEKEFEPLLN WMKDKALKDK IEKAVVSQRL  
 TESPICALVASQYGWSGNMER IMKAQAYQTG KDISTNYYAS QKKTFEINPR  
 HPLIRDMLRR IKEDDDKT VLDLAVL FETATLRSGYLLP DTKAYGDRIE  
 RMLRSLNIDPDAKVEEPEE EPEETAEDT TEDTEQDEDE EMDVGTDEEE  
 ETAKESTA EK DEL

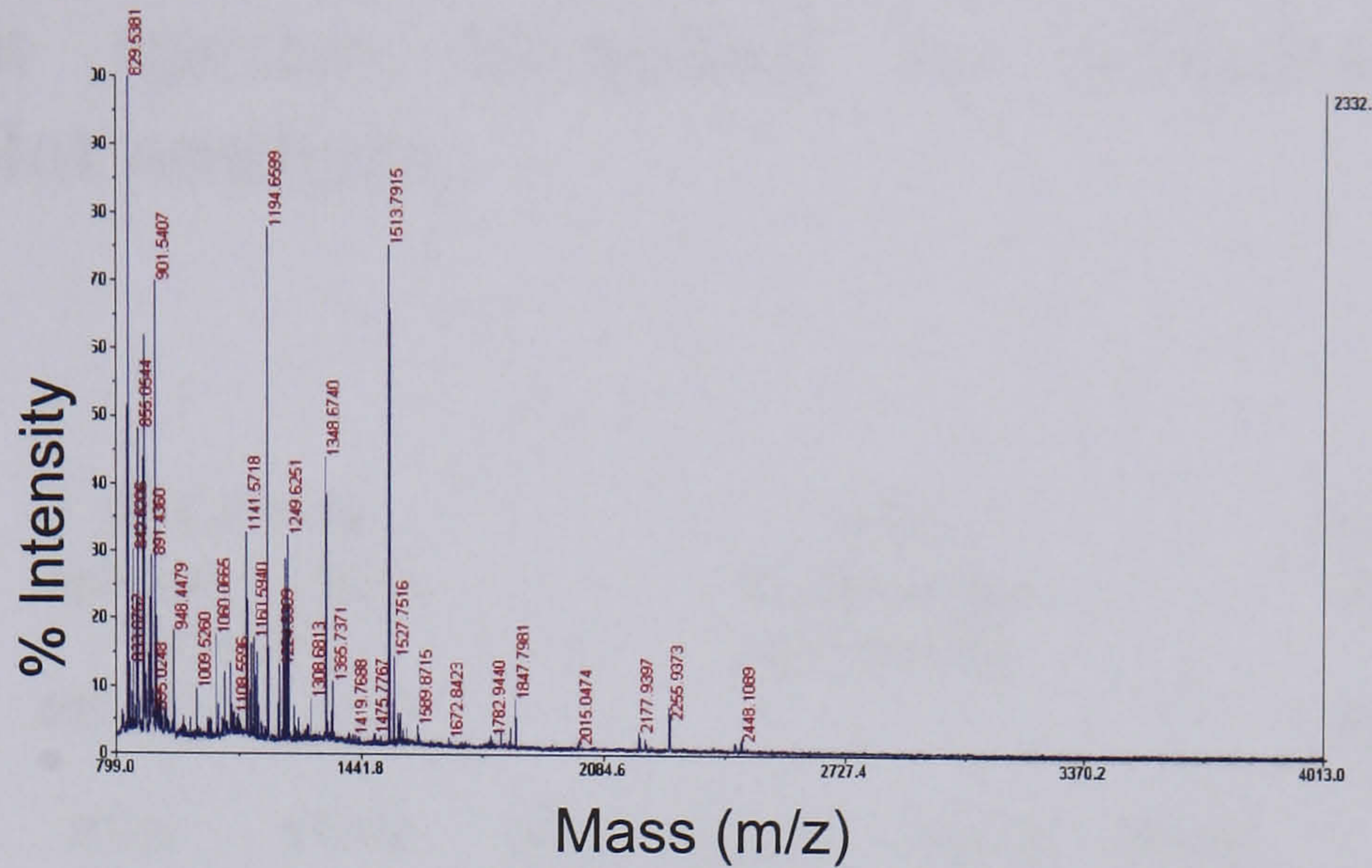
## Annexin A2



MSTVHEILCK **LSLEGDHSTP PSAYGSVKAY TNFDAERDAL NIETAIKTKG**  
**VDEVTVNIL TNRNAQRQD IAFAYQR**RTK KELASALKSA LSGHLETVIL  
 GLLKTPAQYD ASELKASMKG LGTDEDSLIE IICSR TNQEL QEINRVYKEM  
 YKTDLEKDII SDTSGDFRKL MVALAKGRR AEDGSVIDYEL IDQDARDLYD  
 AGVKKRGTDV PKWISIMTER SVP HLQK VFD RYKSYSPYDM LESIRKEVKG  
 DLEN AFLNLV QCIQNKPLYF ADRLYDSMKG KGTRDKVLIR IMVSRSEVDM  
 LKIRSEFKRK YGKSLYYYIQ QDTKG DYQKA LLYLCGGDD



## HSP 90



MPEEVHGGEE EVETFAFQAE IALIIN TFYSNKEIFL RELISNASDA  
 LDKIRYESLT DPSKLDGKE LK**IDIIPNPQ ER**TLTLVDTG IGMTK**ADLIN**  
**NLGTIAK**SGT KAFMEALQAG ADISMIGQFG VGFYSAYLVA EKVVIRK**HN**  
**DDEQYAWESS AGGSFTVR**AD HGEPiGMGTK VILHLKEDQT  
 EYLEERRVKE VVKKHSQFIG YPITLYLEKE REKEISDDEA EEEKGEKEEE  
 DKDDEEKPKI EDVGSDEEDD SGKDKKKKTK KIKEK**YIDQE ELNKTPIWT**  
**RNPDDITQEEYGEFYKSLTN DWEDHLAVKH FSVEGQLEFR**  
**ALLFIPR**RAP FDLFENKKKK NNIKLYVRRV FIMDSCDELI PEYLN**FIRGV**  
**VDSEDLPLNI SR**EMLQQSKI LKVIRKNIVK KCLELFSELA EDKENYK**KFY**  
**EAFSKNLKLG IHEDSTNR**RRLSELLRYHTS QSGDEMTSLS  
 EYVSRMKETQ K**SIYYITGES**KEQVANSFAV ERVRKRGFEV VYMTEPIDEY  
 CVQQLKEFDG KSLVSVTKEG LELPEDEEEK KKMEESKAKF ENLCKLMKEI  
 LDKKVEKVTI SNRLVSSPCC IVTSTYGWTA NMERIMKAQA  
 LRDNSTMGYM MAKKHLEINP DHPIVETLRQ KAEADKNDKA VKDLVLLFE  
 TALLSSGFSL EDPQTHSNRI YRMIKLGLGI DEDEVAAEPPNAAVPDEIPP  
 LEGDEDASRM EEVD



## APPENDIX 8: Relative intensity and molecular weight of protein species identified by SDS-PAGE and Western blot analysis.

Relative MW (kDa)	RF value	SDS-PAGE analysis of HPA affinity chromatography		HPA Western blot HT29 cells				SDS-PAGE analysis of HT29 membrane proteins	Blood Group A antibody	
		HT29	SW480	HPA alone	250mM GalNAc	250mM GlcNAc	250mM sialic acid		HT29	SW480
141	0.04	8.64		4.29			4.29	1.67		
133	0.06	12.24							3.60	
108	0.08	12.90		0.53			0.53	2.97		
89	0.13	2.26		0.53			0.53	5.33		7.26
78	0.18	4.18		6.78	6.22	1.33	6.78	2.81	2.92	
77	0.20							1.15		
73	0.24			16.07	1.70	11.51	16.07	2.55	7.59	7.24
68	0.27			2.54	1.36	2.27	2.54			
67	0.28	4.75		0.35	0.29	0.56	0.35	4.01		
62	0.31			0.11	0.16	0.28	0.11	4.04		
62	0.34			2.85	2.50	1.70	2.85			
62	0.35							0.94		
62	0.36	6.28						2.13		
59	0.39							2.60		
58	0.41	2.00	17.63					1.20	11.40	8.31
58	0.44							8.29	15.78	
57	0.46		16.11	5.13	3.44		5.13			2.14
55	0.48	6.50		8.46	3.90		8.46	4.65		
49	0.53				13.67	1.65	26.88	1.37	6.13	7.90



48	0.56	3.51		27.85	13.00	18.63	0.98	3.06		1.91
47	0.57	5.68						4.98		
43	0.63	5.67						5.72	10.25	4.63
40	0.66							11.40		
40	0.67							0.67	12.25	5.84
39	0.70	4.82						2.11		16.46
36	0.76		24.55	13.24	19.16	27.85	13.24	22.28	8.61	2.99
35	0.77	11.18	41.72							
35	0.78									2.44
34	0.80									
33	0.82	5.29		10.04					5.30	11.68
32	0.84			10.41				0.66		
31	0.86			0.62	33.47	6.48	0.62			
30	0.87			10.66				10.66		
29	0.89	4.10		10.78	1.14			3.36		
28	0.93								8.73	21.20
27	0.97	8.64							7.44	



## **REFERENCES**



Ajioka, Y., Allison, L.J., Jass, J.R. (1996). Significance of MUC1 and MUC2 mucin expression in colorectal cancer. *J Clin Pathol* 49, 560–564.

Akamatsu, S., Yazawa, S., Zenita, K., Matsumoto, H., Tachikawa, T., Kannagi, R. (1996). Elevation of an alpha(1,3)fucosyltransferase activity correlated with apoptosis in the human colon adenocarcinoma cell line, HT-29. *Glycoconj J* 13, 1021–1029.

Alam, S.M., Whitford, P., Cushley, W., George, W.D., Campbell, A.M. (1990). Flow cytometric analysis of cell surface carbohydrates in metastatic human breast cancer. *Br J Cancer* 62(2):238-242.

Altevogt, P., Fogel, M., Cheingsong-Popov, R., Dennis, J., Robinson, P., Schirmacher, V. (1983). Different patterns of lectin binding and cell surface sialylation detected on related high- and low-metastatic tumor lines. *Cancer Res.* 43(11), 5138-5144.

American Joint Committee on Cancer (AJCC) (2002). Cancer staging manual. 6 edn. Philadelphia: Lippincott-Raven.

Altmann, G. G., Enesco, M. (1967). Cell number as a measure of distribution and renewal of epithelial cells in the small intestine of growing and adult rats. *Am. J. Anat.* 121, 319-336.

Aoki T, Kawano J, Oinuma T, Haraguchi Y, Eto T, Suganuma T (1993) human colorectal carcinoma-specific glycoconjugates detected by pokeweed mitogen lectin. *J Histochem Cytochem* 41:1321-1330

Astler, V.B., Coller FA. (1954). The prognostic significance of direct extension of carcinoma of the colon and rectum. *Ann. Surg.* 139:846-851.

Aub, J.C., Sanford, B.H., Cote, M.N. (1965). Studies on reactivity of tumor and normal cells to a wheat germ agglutinin. *Proc Natl Acad Sci* 54(2),396-399.



Aub, J.C., Tieslau, C., Lankester, A. (1963). Reactions of normal and tumor cell surfaces to enzymes wheat-germ lipase and associated mucopolysaccharides. *Proc Natl Acad Sci* 50,613-619.

Baker, D.A., Sugii, S., Kabat, E.A., Ratcliffe, R.M., Hermentin, P., Lemieux, R.U. (1983). Immunochemical studies on the combining sites of Forssman hapten reactive hemagglutinins from *Dolichos biflorus*, *Helix pomatia*, and *Wistaria floribunda*. *Biochemistry*. 22(11), 2741-2750.

Baldus S. E., Zirbes T. K., Monig S. P., Engel S., Monaca E., Rafiqpoor K., Hanisch F. G., Hanski C., Thiele J., Pichlmaier H., Dienes H. P. (1998). Histopathological subtypes and prognosis of gastric cancer are correlated with the expression of mucin-associated sialylated antigens: sialosyl-Lewis(a), sialosyl-Lewis(x) and sialosyl-Tn. *Tumour Biol.*, 19, 445-453.

Baldus, S.E., Monig, S.P., Hanisch, F.G., Zirbes, T.K., Flucke, U., Oelert, S., Zilkens, G., Madejczik, B., Thiele, J., Schneider, P.M., Holscher, A.H., Dienes, H.P. (2002). Comparative evaluation of the prognostic value of MUC1, MUC2, sialyl-Lewis(a) and sialyl-Lewis(x) antigens in colorectal adenocarcinoma. *Histopathol* 40, 440–449.

Baldus, S.E., Park, Y.O., Kotlarek, G.M., Hell, K., Fischer, R. (1996). Expression of Galb1-4GlcNAc sequences by human gastrointestinal neoplasms and their precursors as detected by *Erythrina cristagalli* and *Erythrina corallodendron* lectins. *Int J Oncology* 9, 43–48.

Banks, R., Craven, R., Harnden, P., Selby, P. (2003). Use of a sensitive EnVision + based detection system for Western blotting: avoidance of streptavidin binding to endogenous biotin and biotin-containing proteins in kidney and other tissues. *Proteomics* 3, 558-561.

Benchimol, S., Fuks, A., Jothy, S., Beauchemin, N., Shirota, K., Stanners, C.P. (1989). Carcinoembryonic antigen, a human tumor marker, functions as an intercellular adhesion molecule. *Cell* 57(2), 327-334.



Bhavanandan, V.P., Furukawa, K. (1995). Biochemistry and oncology of sialoglycoproteins. In *Biology of the sialic acids*; Rosenberg A, Ed.; Plenum Press: New York and London, 145-197.

Bingham, S.A., Day, N.E., Luben, R., Ferrari, P., Slimani, N., Norat, T., Clavel-Chapelon, F., Kesse, E., Nieters, A., Boeing, H., Tjonneland, A., Overvad, K., Martinez, C., Dorronsoro, M., Gonzalez, C.A., Key, T.J., Trichopoulou, A., Naska, A., Vineis, P., Tumino, R., Krogh, V., Bueno-de-Mesquita, H.B., Peeters, P.H., Berglund, G., Hallmans, G., Lund, E., Skeie, G., Kaaks, R., Riboli, E. (2003). European Prospective Investigation into Cancer and Nutrition. Dietary fibre in food and protection against colorectal cancer in the European Prospective Investigation into Cancer and Nutrition (EPIC): an observational study. *Lancet* 3;361(9368),1496-1501.

Birch, M., Mitchell, S., Hart, I.R. (1991). Isolation and characterization of human melanoma cell variants expressing high and low levels of CD44. *Cancer Res.* 51(24), 6660-6667.

Birchmeier, W., Behrens, J. (1994). Cadherin expression in carcinomas: role in the formation of cell junctions and the prevention of invasiveness. *Biochim Biophys Acta.* 1198(1), 11-26

Blackwell, K., Hurwitz, H., Lieberman, G., Novotny, W., Snyder, S., Dewhirst, M., Greenberg, C. (2004). Circulating D-dimer levels are better predictors of overall survival and disease progression than carcinoembryonic antigen levels in patients with metastatic colorectal carcinoma. *Cancer.* 1, 77-82.

Bloom, E.J., Itzkowitz, S.H, Kim, Y.S. (1990). Carbohydrate tumor markers in colon cancer and polyps. In *Colon Cancer Cells*, Academic Press Inc., New York, eds. Moyer, M.P. and Poste, G.H. pp. 429-451.

Boland, C.R., Deshmukh, G.D. (1990). The carbohydrate composition of mucin in colonic cancer. *Gastroenterology*, 98, 1170-1177.



- Boland, C.R., Montgomery, C.K., Kim, Y.S. (1982). Alterations in human colonic mucin occurring with cellular differentiation and malignant transformation. *Proc. Natl. Acad. Sci. USA* 79, 315-321.
- Bottini, C., Miotti, S., Fiorucci, S., Facheris, P., Menard, S., Colnaghi, M.I. (1993). Polarization of the alpha 6 beta 4 integrin in ovarian carcinomas. *Int. J. Cancer* 54, 261-267.
- Bouck, N., Stellmach, V., Hsu, S.C. (1996). How tumors become angiogenic. *Adv. Cancer Res.* 69, 135-174.
- Bouvard, D., Brakebusch, C., Gustafsson, E., Aszodi, A., Bengtsson, T., Berna, A., Fassler, R. (2001). Functional consequences of integrin gene mutations in mice. *Circ Res.* 89(3), 211-23.
- Bradford, M.M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem.* 72, 248-254.
- Bresalier, R.S., Byrd, J.C., Brodt, P., Ogata, S., Itzkowitz, S.H., Yunker, C.K. (1998). Liver metastasis and adhesion to the sinusoidal endothelium by human colon cancer cells is related to mucin carbohydrate chain length. *Int. J. Cancer* 76(4), 556-562.
- Brockhausen, I. (2000). O-linked chain glycosyltransferases. *Methods Mol Biol.* 125, 273-293.
- Brockhausen, I. (2006). Mucin-type O-glycans in human colon and breast cancer: glycodynamics and functions. *EMBO Rep.* 7(6), 599-604.
- Brooks, S. A., Lymboura, M., Schumacher, U., and Leathem, A. J. C. (1996). Histochemistry to detect Helix pomatia lectin binding in breast cancer: methodology makes a difference. *The Journal of Histochemistry and Cytochemistry* 44, 519-524.



Brooks, S.A. (2000). The involvement of *Helix pomatia* lectin (HPA) binding N-acetylgalactosamine glycans in cancer progression. *Histol Histopathol* 15,143–158.

Brooks, S.A., Hall, D.M., Buley, I. (2001). GalNAc glycoprotein expression by breast cell lines, primary breast cancer and normal breast epithelial membrane. *Br J Cancer* 85, 1014–1022

Brooks, S.A., Leathem, A.J. (1991). Prediction of lymph node involvement in breast cancer by detection of altered glycosylation in the primary tumour. *Lancet* 338(8759):71-74.

Brooks, S., Leathem, A. (1998-99). Expression of N-acetyl galactosaminylated and sialylated glycans by metastases arising from primary breast cancer. *Invasion metastasis* 18(3), 115-121.

Brooks, S.A., Leathem, A.J.C., Schumacher, U. (1997). Lectin histochemistry – a concise practical handbook. *Publ. Bios Scientific*. Publishers Oxford, UK. pp. 8-12.

Brooks, S.A., Lymboura, M., Schumacher, U., Leathem, A.J. (1996). Histochemistry to detect *Helix pomatia* lectin binding in breast cancer: methodology makes a difference. *J Histochem Cytochem* 44,519–524.

Brooks, S.A., Wilkinson, D. (2003). Validation of a simple avidin-biotin detection method for *Helix pomatia* lectin (HPA) binding as a prognostic marker in cancer. *Acta Histochem*. 105(3), 205-212.

Brooks, S.A., Leathem, A.J.C. (1995). Altered expression of alpha-GalNAc glycoproteins by breast cancers. *British Journal of Cancer* 71, 1033-1038.



Bryan, T.M., Englezou, A., Gupta, J., Bacchetti, S., Reddel, R.R. (1995). Telomere elongation in immortal human cells without detectable telomerase activity. *EMBO J.* 14, 4240–4248.

Bubb, V.J., Curtis, L.J., Cunningham, C., Dunlop, M.G., Carothers, A.D., Morris, R.G., White, S., Bird, C.C., Wyllie, A.H. (1996). Microsatellite instability and the role of hMSH2 in sporadic colorectal cancer. *Oncogene.* 12, 2641-2649

Bufill, J.A. (1990). Colorectal cancer: evidence for distinct genetic categories based on proximal or distal tumor location. *Ann Intern Med* 113, 779-788.

Burger, M.M., Goldberg, A.R. (1967). Identification of a tumor-specific determinant on neoplastic cell surfaces. *Proc Natl Acad Sci U S A.* 57(2):359-366.

Burns, T.F., El-Deiry, W.S. (1999). The p53 pathway and apoptosis. *J Cell Physiol.* 181(2), 231-239.

Byrd, J.C., Bresalier, R.S. (2004). Mucins and mucin binding proteins in colorectal cancer. *Cancer Metastasis Rev.* 23(1-2),77-99.

Byrd, J.C., Sternberg, L., Yan, P., Ho, S.B., Bresalier, R.S. (1998). Ectopic expression of MUC5 gastric mucin in colorectal adenocarcinoma. *Gastroenterol.* 114, A573.

Calderwood, D.A., Tuckwell, D.S., Humphries, M.J. (1995). Specificity of integrin I-domain-ligand binding. *Biochem Soc Trans.* 23(4), 504S-508S.

Calvete, J.J., Henschen, A., Gonzalez-Rodriguez, J. (1989). Complete localization of the intrachain disulphide bonds and the N-glycosylation points in the alpha-subunit of human platelet glycoprotein IIb. *Biochem J.* 261(2):561–568.



Cao, Y., Blohm, D., Ghadimi, M., Stosiek, P., Xing, P.X., Karsten, U. (1997). Mucins (MUC1 and MUC3) of gastrointestinal and breast epithelial reveal different and heterogeneous tumor-associated aberrations in glycosylation. *J Histochem Cytochem* 45, 1547–1557.

Cao, Y., Karsten, U. R., liebrich, W., Haensch, W., Springer, G. F., Schlag, P. M. (1995). Expression of Thomsen - Friedenreich - related antigens in primary and metastatic colorectal carcinomas: A re-evaluation. *Cancer* 76, 1700 – 1708.

Carter, W.G., Wayner, E.A., Bouchard, T.S., Kaur, P. (1990). The role of integrins alpha 2 beta 1 and alpha 3 beta 1 in cell-cell and cell-substrate adhesion of human epidermal cells. *J Cell Biol.* 110(4), 1387-404.

Chao, A., Thun, M.J., Connell, C.J., McCullough, M.L., Jacobs E.J., Flanders, W.D., Rodriguez, C., Sinha, R., Calle, E.E. (2005). Meat consumption and risk of colorectal cancer. *JAMA.* 12;293(2),172-182.

Chao, C., Lotz, M.M., Clarke, A.C., Mercurio, A.M. (1996). A function for the integrin alpha6 beta4 in the invasive properties of colorectal carcinoma cells. *Cancer Res.* 56(20), 4811-4819.

Chatterjee, S.K., Zetter, B.R. (2005). Cancer biomarkers: knowing the present and predicting the future. *Future Oncol.* 1, 37-50.

Chen, Y., Jain, R., Chandrasekaran, E., Matta, K. (1995). Use of sialylated or sulfated derivatives and acrylamide copolymers of Gal beta 1,3GalNAc alpha- and GalNAc alpha- to determine the specificities of blood group T- and Tn-specific lectins and the copolymers to measure anti-T and anti-Tn antibody levels in cancer patients. *Glycoconjugate Journal* 12, 55-62.



Chevallet, M., Santoni, V., Poinas, A., Rouquié, D., Fuchs, A., Kieffer, S., Rossignol, M., Lunardi, J., Garin, J., Rabilloud, T. (1998). New zwitterionic detergents improve the analysis of membrane proteins by two-dimensional electrophoresis. *Electrophoresis*. 11, 1901-1909.

Christofori, G. (2003). Changing neighbours, changing behaviour: cell adhesion molecule-mediated signalling during tumour progression. *EMBO J*. 22(10), 2318-2323.

Ciocca, D., Calderwood, S. (2005). Heat shock proteins in cancer: diagnostic, prognostic, predictive, and treatment implications. *Cell Stress Chaperones* 10, 86-103.

Citarda, F., Tomaselli, G., Capocaccia, R., Barcherini, S., Crespi M. (2001). Efficacy in standard clinical practice of colonoscopic polypectomy in reducing colorectal cancer incidence. *Gut* 48, 812-815.

Clifton, J.G., Li, X., Reutter, W., Hixson, D.C., Josic, D. (2007). Comparative proteomics of rat liver and Morris hepatoma 7777 plasma membranes. *J Chromatogr B Analyt Technol Biomed Life Sci*. 1-2, 293-301

Colon and rectum. (1997) In: American Joint Committee on Cancer: AJCC Cancer Staging Manual. Philadelphia: Lippincott-Raven Publishers, 5th ed :83.

Dall'Olio, F., and Trere, D. (1993). Expression of alpha 2,6-sialylated sugar chains in normal and neoplastic colon tissues. Detection by digoxigenin-conjugated Sambucus nigra agglutinin. *European Journal of Histochemistry* 37, 257 - 265.

D'Ardenne, A.J., Richman, P.I., Horton, M.A., McAulay A.E., Jordan, S. (1991). Coordinate expression of the alpha-6 integrin laminin receptor sub-unit and laminin in breast cancer. *J. Pathol.* 165, 213-220



Das, R.C., Heath, E.C. (1980). Dolichyldiphosphoryl oligosaccharide--protein oligosaccharyltransferase; solubilization, purification, and properties. *Proc Natl Acad Sci U S A.* 77(7), 3811-3815.

De Albuquerque Garcia Redondo, P., Nakamura, C.V., de Souza, W., Morgado-Diaz, J.A. (2004). Differential expression of sialic acid and N-acetylgalactosamine residues on the cell surface of intestinal epithelial cells according to normal or metastatic potential. *J Histochem Cytochem.* 52(5):629-40.

Dejana, E., Martin-Padura, I., Lauri, D., Bernasconi, S., Bani, M. R., Garofalo, A., Giavazzi, R., Magnani, J., Mantovani, A., Menard, S. (1992). Endothelial Leukocyte Adhesion Molecule-1 Dependent Adhesion of Colon Carcinoma Cells to Vascular Endothelium is Inhibited by an Antibody to Lewis Fucosylated Type I Carbohydrate Chain. *Laboratory Investigation* 66, 324-330.

Dekker, J., Strous, G.J. (1990). Covalent oligomerization of rat gastric mucin occurs in the rough endoplasmic reticulum, is N-glycosylation-dependent, and precedes initial O-glycosylation. *J Biol Chem.* 265(30):18116-22

Demetriou, M., Nabi, I.R., Coppolino, M., Dedhar, S., Dennis, J.W. (1995). Reduced contact-inhibition and substratum adhesion in epithelial cells expressing GlcNAc-transferase V. *J Cell Biol.* 130(2):383-392.

Dennis, J. W. (1986). Different metastatic phenotypes in two genetic classes of WGA-resistant tumor cell mutants. *Cancer Research* 46, 4594 - 4600.

Dennis, J. W., Laferte, S., Waghorne, C., Breitman, M. L., Kerbel, R. S. (1987).  $\beta$ 1-6 Branching of Asn-Linked Oligosaccharides is Directly Associated with Metastasis. *Science* 236, 236-239.

Dennis, J.W., Granovsky, M., Warren, C.E. (1999). Glycoprotein glycosylation and cancer progression. *Biochim Biophys Acta.* 1473(1), 21-34.



Dennis, J.W., Laferte, S. (1989). Oncodevelopmental expression of--GlcNAc beta 1-6Man alpha 1-6Man beta 1--branched asparagine-linked oligosaccharides in murine tissues and human breast carcinomas. *Cancer Res.* 49(4), 945-950.

Dennis, J.W., Laferte, S., Yagel, S., Breitman, M.L. (1989). Asparagine-linked oligosaccharides associated with metastatic cancer. *Cancer Cells* 1(3), 87-92.

DiPersio, C.M., Hovalala-Dilke, K.M., Jaenisch, R., Kreidberg, J.A., Hynes, R.O. (1997) alpha3 beta1 Integrin is required for normal development of the epidermal basement membrane. *J Cell Biol* 137(3), 729-742.

Distler, P., Holt, P.R. (1997). Are right- and left-sided colon neoplasms distinct tumors? *Dig Dis.* 15(4-5), 302-311.

Doll, R., Peto, R. (1981). The causes of cancer: quantitative estimates of avoidable risks of cancer in the United States today. *J Natl Cancer Inst.* 66(6):1191-308.

Dube, S., Heyen, F., Jenicek, M. (1997). Adjuvant chemotherapy in colorectal carcinoma: results of a meta-analysis. *Dis Colon Rectum* 40(1), 35-41.

Duff, E.K., Clarke, A.R. (1998). Smad4 (DPC4) - a potent tumor suppressor? *Brit. J. Cancer* 78, 1615-1619.

Dukes, C.E. (1932). The classification of cancer of the rectum. *J Pathol* 35,323-332.

Dwek, M. V., Ross, H. A., Streets, A. J., Brooks, S. A., Adam, E., Titcomb, A., Woodside, J. V., Schumacher, U., Leathem, A. J. (2001). *Helix pomatia* agglutinin lectin-binding oligosaccharides of aggressive breast cancer. *Int J Cancer* 95, 79-85.

Dwek, M.V., Brooks, S.A. (2004). Harnessing changes in cellular glycosylation in new cancer treatment strategies. *Curr Cancer Drug Targets* 4(5), 425-42.



Dwek, M.V., Ross, H.A., Streets, A.J., Brooks, S.A., Adam, E., Titcomb, A., Woodside, J.V., Schumacher, U., Leathem, A.J. (2001). Helix pomatia agglutinin lectin-binding oligosaccharides of aggressive breast cancer. *Int J Cancer*. 20; 95(2),79-85.

Eggens, I., Fenderson, B., Toyokuni, T., Dean, B., Stroud, M., Hakomori, S. (1989). Specific interaction between Le<sup>x</sup> and Le<sup>x</sup> determinants. A possible basis for cell recognition in preimplantation embryos and in embryonal carcinoma cells. *J Biol Chem*, 264, 9476–9484.

Evan G., Littlewood, T. (1998). A matter of life and cell death. *Science* 281, 1317–1322.

Fearon, E.R., Vogelstein, B. (1990). A genetic model for colorectal tumorigenesis. *Cell*. 61(5), 759-767.

Fedi, P., Tronick, S.R., Aaronson, S.A. (1997). Growth factors. In: J.F. Holland, R.C. Bast, D.L. Morton, E. Frei, D.W. Kufe and R.R. Weichselbaum, Editors, *Cancer Medicine*, Williams and Wilkins, Baltimore, MD 41–64

Feizi, T. (1985). Demonstration by monoclonal antibodies that carbohydrate structures of glycoproteins and glycolipids are onco-developmental antigens. *Nature* 314, 53-57.

Feizi, T., Childs, R.A. (1987). Carbohydrates as antigenic determinants of glycoproteins. *Biochem J*. 245(1):1-11.

Fenlon, S., Ellis, I. O., Bell, J., Todd, J. H., Elston, C. W., Blamey, R. W. (1987). *Helix pomatia* and *Ulex europeus* lectin binding in human breast carcinoma. *Journal of Pathology* 152, 169-176.

Fernandes, B., Sagman, U., Auger, M., Demetrio, M., Dennis, J.W. (1991). Beta 1-6 branched oligosaccharides as a marker of tumor progression in human breast and colon neoplasia. *Cancer Res*. 51(2), 718-723.



Fidler, I.J. (1977). Bucana C. Mechanism of tumor cell resistance to lysis by syngeneic lymphocytes. *Cancer Res.* 37(11),3945-3956.

Fidler, I.J. (1991). Cancer metastasis. *Br Med Bull.* 47(1), 157-177.

Fidler, I.J. (1994). Therapy of cancer metastasis by systemic activation of macrophages. *Adv Pharmacol.* 30, 271-326.

Fidler, I.J. (1995). Critical factors in the biology of human cancer metastasis. *Am Surg.* 61(12), 1065-1066.

Fidler, I.J. (1995). Modulation of the organ microenvironment for treatment of cancer metastasis. *J Natl Cancer Inst.* 87(21), 1588-1592.

Fidler, I.J., Balch, C.M. (1987). The biology of cancer metastasis and implications for therapy. *Curr Probl Surg.* 24(3), 129-209.

Fidler, I.J., Hart, I.R. (1982). Biological diversity in metastatic neoplasms: origins and implications. *Science.* 217(4564), 998-1003.

Fodde, R., Kuipers, J., Rosenberg, C. (2001). Mutations in the APC tumour suppressor gene cause chromosomal instability. *Nature Cell Biology* 3, 433–438.

Fogh, J., editor. (1975). Human tumor cells in vitro. 43: New York: Plenum Press; pp. 115-159

Folkman, J. (1997). Tumor angiogenesis. In: J.F. Holland, R.C. Bast, D.L. Morton, E. Frei, D.W. Kufe and R.R. Weichselbaum, Editors, *Cancer Medicine*, Williams and Wilkins, Baltimore, MD 181–204.



Folkman, J. (1986). How is blood vessel growth regulated in normal and neoplastic tissue? G.H.A. Clowes memorial Award lecture. *Cancer Res.* 46(2), 467-473.

Folkman, J., Klagsbrun, M. (1987). Angiogenic factors. *Science* 235(4787), 442-447.

Franco, A., Sikalidis, A.K., Solis Herruzo, J.A. (2005). Colorectal cancer: influence of diet and lifestyle factors. *Rev Esp Enferm Dig.* 97 (6):432-448

Franz, H. (1988). The ricin story. *Adv. Lectin Res.*, 1, 10–25.

Friedrichs, K., Ruiz, P., Franke, F., Gille, I., Terpe, H.J., Imhof, B.A. (1995). High expression level of alpha 6 integrin in human breast carcinoma is correlated with reduced survival. *Cancer Res.* 55(4), 901-906.

Fukushima, K., Yamashita, K. (2001). Interleukin-2 carbohydrate recognition modulates CTLL-2 cell proliferation. *J Biol Chem.* 276(10):7351-6

Fukutomi, T., Hirohashi, S., Tsuda, H., Nanasawa, T., Yamamoto, H., Itabashi, M., Shimosato, Y. (1991). The prognostic value of tumor-associated carbohydrate structures correlated with gene amplifications in human breast carcinomas. *Jpn J Surg.* 21(5), 499-507.

Fukutomi, T., Itabashi, M., Tsugane, S., Yamamoto, H., Nanasawa, T., Hirota, T. (1989). Prognostic contributions of Helix pomatia and carcinoembryonic antigen staining using histochemical techniques in breast carcinomas. *Jpn J Clin Oncol.* 19(2), 127-134.

Fuster, M.M., Brown, J.R., Wang, L., Esko J.D. (2003). A Disaccharide Precursor of Sialyl Lewis X Inhibits Metastatic Potential of Tumor Cells *Cancer Research* 63, 2775-2781.

Galea, M. H., Ellis, I. O., Bell, J., Elston, C. W., and Blamey, R. W. (1991). Prediction of lymph node involvement in breast cancer. *Lancet* 338, 392-393.



Gasic, G.J. (1984). Role of plasma, platelets, and endothelial cells in tumor metastasis. *Cancer Metastasis Rev.* 3(2), 99-114.

Gervaz, P., Bouzourene, H., Cerottini, J.P., Chaubert, P., Benhattar, J., Secic, M., Wexner, S., Givel, J.C., Belin, B. (2001). Dukes B colorectal cancer: distinct genetic categories and clinical outcome based on proximal or distal tumor location. *Dis Colon Rectum* 44, 364-372.

Gilcrease, M.Z. (2007). Integrin signaling in epithelial cells. *Cancer Lett.* 247(1), 1-25.

Giordano, T.P., Henderson, L., Landgren, O., Chiao, E.Y., Kramer, J.R., El-Serag, H., Engels, E.A. (2007). Risk of Non-Hodkin Lymphoma and Lymphoproliferative Precursor Diseases among 146,394 Hepatitis C Virus Infected U.S. Veterans, *Journal of the American Medical Association* 297 (18).

Giovannucci, E., Stampfer, M.J., Colditz, G., Rimm, E.B., Willett, W.C. (1992). Relationship of diet to risk of colorectal adenoma in men. *J Natl Cancer Inst* 84, 91-8.

Goodison, S., Urquidi, V., Tarin, D. (1999). CD44 cell adhesion molecules. *Mol Pathol.* 52(4), 189-196.

Gordon, J. I., Schmidt, G. M., Roth, K. A. (1992). Studies of intestinal stem cells using normal, chimeric, and transgenic mice. *FASEBJ.* 6, 3039-3050.

Gorg, A., Obermaier, C., Boguth, G., Weiss, W. (1999). Recent developments in two-dimensional gel electrophoresis with immobilized pH gradients: wide pH gradients up to pH 12, longer separation distances and simplified procedures. *Electrophoresis* 20(4-5), 712-717.



Guinez C., Lemoine J., Michalski J.C., Lefebvre T. (2004). 70-kDa-heat shock protein presents an adjustable lectinic activity towards O-linked N-acetylglucosamine. *Biochem Biophys Res Commun.* 319(1), 21-26.

Gunthert, U., Hofmann, M., Rudy, W., Reber, S., Zoller, M., Haussmann, I., Matzku, S., Wenzel, A., Ponta, H., Herrlich, P. (1991). A new variant of glycoprotein CD44 confers metastatic potential to rat carcinoma cells. *Cell* 65(1), 13-24.

Gusterson, B.A., for Group TILBCS (1993). Prognostic value of *Helix pomatia* in breast cancer. *Br J Cancer* 68, 146–150.

Hakomori, S. (1989). Aberrant glycosylation in tumors and tumor-associated carbohydrate antigens. *Adv Cancer Res* 52, 257–331.

Hakomori, S. (1999). Antigen structure and genetic basis of histo-blood groups A, B and O: their changes associated with human cancer. *Biochim Biophys Acta* 1473 (1): 247-266

Halling, K.C., French, A.J., McDonnell, S.K., Burgart, L.J., Schaid, D.J., Peterson, B.J., Moon-Tasson, L., Mahoney, M.R., Sargent, D.J., O'Connell, M.J., Witzig, T.E., Farr, G.H. Jr., Goldberg, R.M., Thibodeau, S.N. (1999). Microsatellite instability and 8p allelic imbalance in stage B2 and C colorectal cancers. *J Natl Cancer Inst.* 15, 1295-303.

Hammarstrom, S., Kabat, E. (1971). Studies on specificity and binding properties of the blood group A reactive hemagglutinin from *Helix pomatia*. *Biochemistry* 10, 1684-1692.

Hammarstrom, S., Kabat, E.A. (1969). Purification and characterization of a blood-group A reactive hemagglutinin from the snail *Helix pomatia* and a study of its combining site. *Biochemistry* 8(7), 2696-2705.



Hammarstrom, S., Westoo, A., Bjork, I. (1972). Subunit structure of Helix pomatia A hemagglutinin. *Scand J Immunol.* 1(4), 295-309.

Hanahan, D., Folkman, J. (1996). Patterns and emerging mechanisms of the angiogenic switch during tumorigenesis. *Cell* 86, 353–364

Hanahan, D., Weinberg, R.A. (2000). The hallmarks of cancer. *Cell* 100(1), 57-70.

Hanski, C., Drechsler, K., Hanisch, F.-G., Sheehan, J., Manske, M., Ogorek, D., Klusmann, E., Hanski, M.-L., Blank, M., Xing, P.-X., McKenzie, I.F.C, Devine, P. L., Riecken, E.-O. (1993). Altered glycosylation of the MUC-1 protein core contributes to the colon carcinoma-associated increase of mucin bound sialyl-Lewis expression. *Cancer Res.* 53, 4082-4088.

Harder, A., Wildgruber, R., Nawrocki, A. (1999). Cell protein solubilization procedures *Electrophoresis*, 20, 826-829.

Harewood, G.C., Lieberman, D.A. (2003). Prevalence of advanced neoplasia at screening colonoscopy in men in private practice versus academic and Veterans Affairs medical centers. *Am J Gastroenterol* 98, 2312-2316.

Harper, D.M., Franco, E.L., Wheeler, C., Ferris, D.G., Jenkins, D., Schuind, A., Zahaf, T., Innis, B., Naud, P., De Carvalho, N.S., Roteli-Martins, C.M., Teixeira, J., Blatter, M.M., Korn, A.P., Quint, W., Dubin, G., GlaxoSmithKline HPV Vaccine Study Group. (2004). Efficacy of a bivalent L1 virus-like particle vaccine in prevention of infection with human papillomavirus types 16 and 18 in young women: a randomised controlled trial. *Lancet* 364(9447), 1757-1765.

Harris, C.C. (1996). p53 tumor suppressor gene: from the basic research laboratory to the clinic—an abridged historical perspective. *Carcinogenesis* 17, 1187–1198.



Haydon, A.M., Macinnis, R.J., English, D.R., Giles, G.G. (2006). Effect of physical activity and body size on survival after diagnosis with colorectal cancer. *Gut* 55(1):62-67.

Hehlhans, S., Haase, M., Cordes, N. (2007). Signalling via integrins: implications for cell survival and anticancer strategies. *Biochim Biophys Acta*. 1775(1), 163-180.

Heldin, C.H., Miyazono, K., Ten Dijke, P. (1997). TGF- $\beta$  signalling from cell membrane to nucleus through SMAD proteins. *Nature (Lond.)* , 390, 465-471.

Hewitt, R.E., McMarlin, A. Kleiner, D., Wersto, R., Martin, P., Tsokos, M., Stamp, G.W., Stetler-Stevenson, W.G. (2000). Validation of a model of colon cancer progression. *J Pathol.* 4, 446-454.

Hoff, S.D., Matsushita, Y., Ota, D.M., Cleary, K.R., Yamori, T., Hakomori, S., Irimura, T. (1989). Increased expression of sialyl-dimeric LeX antigen in liver metastases of human colorectal carcinoma. *Cancer Res.* 49(24 Pt 1), 6883-6888.

Honn, K.V., Tang, D.G., Crissman, J.D. (1992). Platelets and cancer metastasis: a causal relationship? *Cancer Metastasis Rev.* 11(3-4), 325-351.

Howe, G.R., Aronson, K.J., Benito, E., Castelleto, R., Cornee, J., Duffy, S., Gallagher, R.P., Iscovich, J.M., Deng-ao, J., Kaaks, R., Kune, G.A., Kune, S., Lee, H.P., Lee, M., Miller, A.B., Peters, R.K., Potter, J.D., Ribolin E., Slattery, M.L., Trichopoulos, D., Tuyns, A., Tzonou, A., Watson, L.F., Whittemore, A.S., Shu, Z., et al. (1997). The relationship between dietary fat intake and risk of colorectal cancer: evidence from the combined analysis of 13 case-control studies. *Cancer Causes Control.* 8(2), 215-228.

Hubbard, S. C., Ivatt, R. J. (1981). Synthesis and processing of asparagine-linked oligosaccharides. *Ann. Rev. Biochem.* 50, 555-583.

Hynes, R. O. (1987). Integrins: a family of cell surface receptors. *Cell* 48: 549-554.



Hynes, R.O. (1992). Integrins: versatility, modulation, and signaling in cell adhesion. *Cell* 69(1), 11-25.

Hynes, R.O. (2002). Integrins: bidirectional, allosteric signaling machines. *Cell* 110(6), 673-687

Hynes, R.O., Zhao, Q. (2000). The evolution of cell adhesion. *J Cell Biol.* 150(2), 89-96.

Iacopetta, B., (2002). Are there two sides to colorectal cancer? *Int J Cancer.* 10; 101(5), 403-408.

IARC Working Group on the Evaluation of Cancer-Preventive Agents. Weight control and physical activity (2002). Handbooks of cancer prevention, vol. 6. Lyon: IARC Press.

Ichikawa, D., Handa, K., Hakomori, S. (1998). Histo-blood group A/B antigen deletion/reduction vs. continuous expression in human tumor cells as correlated with their malignancy. *Int J Cancer.* 2, 284-289.

Idikio, H. A., and Manickavel, V. (1991). Lewis Blood Group Antigens (a and b) in Human Breast Tissues. *Cancer* 68, 1303-1308.

Idikio, H. A., and Manickavel, V. (1993). A, B, H, and Lewis-a and Lewis-b blood group antigens in human breast cancer: correlation with steroid hormone receptor and disease status. *J Cancer Res Clin Oncol.* 119(8):486-92.

Ikeda, Y., Mori, M., Adachi, Y., Matsuchima, T., Sugimachi, K. (1994). Prognostic value of the histochemical expression of *Helix pomatia* agglutinin in advanced colorectal cancer. *Dis Colon Rectum* 37, 181-184.

Ito, H., Hiraiwa, N., Sawada-Kasugai, M., Akamatsu, S., Tachikawa, T., Kasai, Y., Akiyama, S., Ito, K., Takagi, H., Kannagi, R. (1997). Altered mRNA expression of specific molecular species of fucosyl- and sialyl-transferases in human colorectal cancer tissues. *Int J Cancer* 71, 556-564.



Itzkowitz, S. H., Yuan, M., Montgomery, C. K., Kjeldsen, T., Takahashi, H. K., Bigbee, W. L., Kim, Y. S. (1989). Expression of Tn, sialosyl-Tn and T antigens in human colon cancer. *Cancer Research* 49, 197-204.

Itzkowitz, S.H., Yuan, M., Fukushi, Y., Palekar, A., Phelps, P.C., Shamsuddin, A.M., Trump, B.F., Hakomori, S., Kim, Y.S. (1986). Lewisx- and sialylated Lewisx-related antigen expression in human malignant and non-malignant colonic tissues. *Cancer Res.* 46(5), 2627-2632.

Itzkowitz, S.H., Yuan, M., Fukushi, Y., Palekar, A., Phelps, P.C., Shamsuddin, A.M., Trump, B.F., Hakomori, S., Kim, Y.S. (1986). Lewisx- and sialylated Lewisx-related antigen expression in human malignant and nonmalignant colonic tissues. *Cancer Res.* 46(5), 2627-2632.

Iwakawa, K., Ueda, N., Murao, S., Kobayashi, N. (1996). Altered carbohydrate composition in colorectal adenomas and carcinomas: histochemical characterization of N-acetylgalactosamine, L-fucose, and o-acetylated sialic acid. *J Gastroenterol* 31, 24–32.

Jones, P.A., DeClerck, Y.A. (1980). Destruction of extracellular matrices containing glycoproteins, elastin, and collagen by metastatic human tumor cells. *Cancer Res.* 40(9), 3222-3227.

Juliano, R. L., Varner, J. A. (1983). Adhesion molecules in cancer: the role of integrins. *Current Opinions in Cell Biology* 5, 812-818.

Takeji, Y., Maehara, Y., Tsujitani, S., Baba, H., Ohno, S., Watanabe, A., Sugimachi, K. (1994). *Helix pomatia* agglutinin binding activity and lymph node metastasis in patients with gastric cancer. *Semin Surg Oncol.* 10(2), 130-134.



Takeji, Y., Tsujitani, S., Mori, M., Maehara, Y., Sugimachi, K. (1991). *Helix pomatia* agglutinin binding activity is a predictor of survival time for patients with gastric carcinoma. *Cancer* 68:2438–2442.

Kawai, T., Suzuki, M., Torikata, C., Suzuki, Y. (1991). Expression of blood group-related antigens and *Helix pomatia* agglutinin in malignant pleural mesothelioma and pulmonary adenocarcinoma. *Hum Pathol.* 22(2), 118-124.

Kemmer, W., Morgenthaler, J., Brossmer, R. (1992). Alterations in the cell surface carbohydrate composition of a human colon carcinoma cell line affect adhesion to extracellular matrix components. *Biochimie* 74,117–122.

Kemp, Z., Thirlwell, C., Sieber, O., Silver, A., Tomlinson, I. (2004). An update on the genetics of colorectal cancer. *Hum Mol Genet.* 13 (2),177-185.

Kerrigan, M.J., Hall, A.C. (2005). Stimulation of regulatory volume decrease (RVD) by isolated bovine articular chondrocytes following F-actin disruption using latrunculin B. *Biorheology* 42(4), 283-93.

Kilpatrick, D.C. (2002). Animal lectins: a historical introduction and overview. *Biochim Biophys Acta.* 1572(2-3), 187-197.

Kim, Y.S., Isaacs, R. (1975). Glycoprotein metabolism in inflammatory and neoplastic diseases of the human colon. *Cancer Res* 35,2092–2097.

Kim, Y.S. (1992). Altered glycosylation of mucin glycoproteins in colonic neoplasia. *J Cell Biochem Suppl.* 16G, 91-96.

Kim, Y.J., Borsig, L., Varki, N.M., Varki, A. (1998). P-selectin deficiency attenuates tumor growth and metastasis. *Proc Natl Acad Sci U S A.* 95(16), 9325-9330.



Kim, H.R., Wheeler, M.A., Wilson, C.M., Iida, J., Eng, D., Simpson, M.A., McCarthy, J.B., Bullard, K.M. (2004). Hyaluronan facilitates invasion of colon carcinoma cells in vitro via interaction with CD44. *Cancer Res.* 64(13):4569-76.

King, M.-J. (1994). Blood group antigens on human erythrocytes - distribution, structure and possible functions. *Biochimica et Biophysica Acta* 1197, 15-44.

Kinzler, K.W., Vogelstein, B. (1996). Lessons from hereditary colorectal cancer. *Cell* 87, 159–170.

Klein, G., Klein, E. (1985). Evolution of tumours and the impact of molecular oncology. *Nature* 315(6016), 190-195.

Knudson, A.G. Jr. (1989). Hereditary cancers disclose a class of cancer genes. *Cancer* 63: 1888-1891.

Kobata, A., and Takasaki, S. (1992). Structure and biosynthesis of cell surface carbohydrates. In *Cell surface carbohydrates and cell development.*, M. Fukuda, ed. (London: CRC Press), pp. 2-24.

Koch, A.E., Halloran, M.M., Haskell, C.J., Shah, M.R., Polverini, P.J. (1995). Angiogenesis mediated by soluble forms of E-selectin and vascular cell adhesion molecule-1. *Nature* 376(6540), 517-519.

Kohler, W., Prokop, O., Kuhnemund, O. (1983). Routine identification of group-C streptococci by means of an agglutinin (protectin) from the albumen gland of the edible snail, *Helix pomatia*. *J. Med. Microbiol.* 6, 127-130.

Konno, A., Hoshino, Y., Terashima, S., Motoki, R., Kawaguchi, T. (2002). Carbohydrate expression profile of colorectal cancer cells is relevant to metastatic patterns and prognosis. *Clin Exp Med* 19, 61–70.



Kornfeld, R., Kornfeld, S. (1985). Assembly of asparagine-linked oligosaccharides. *Annu Rev Biochem.* 54, 631-664.

Koutsky, L.A., Ault, K.A., Wheeler, C.M., Brown, D.R., Barr, E., Alvarez, F.B., Chiacchierini, L.M., Jansen, K.U. (2002). Proof of Principle Study Investigators. A controlled trial of a human papillomavirus type 16 vaccine. *N Engl J Med.* 347(21), 1645-1651.

Kovarova, H., Stulik, J., Hochstrasser, D.F., Bures, J., Melichar, B., Jandik, P. (1994). Two-dimensional electrophoretic study of normal colon mucosa and colorectal cancer. *Appl Theor Electrophor.* 4(3), 103-106.

Kudo, T., Ikehara, Y., Togayachi, A., Morozumi, K., Watanabe, M., Nakamura, M., Nishihara, S., Narimatsu, H. (1998). Up-regulation of a set of glycosyltransferase genes in human colorectal cancer. *Lab Investig* 78, 797–811.

Kuphal, S., Bauer, R., Bosserhoff, A.K. (2005). Integrin signaling in malignant melanoma. *Cancer Metastasis Rev.* 24(2), 195-222.

Laemmli, U.K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*, 227, 680-685.

Landsteiner, K. (1900). Zur Kenntnis des antifermentativen, lytischen und agglutinierenden Wirkungen des Blutserums und der Lymphe. *Zbl. Bakt.* 27, 357–363.

Lane, D.P. (1992). Cancer. p53, guardian of the genome. *Nature.* 358(6381),15-16.

Lasky, L.A. (1992). Selectins: interpreters of cell-specific carbohydrate information during inflammation. *Science.* 258(5084), 964-969.

Lauffenburger, D.A., Horwitz, A.F. (1996). Cell migration: a physically integrated molecular process. *Cell* 84(3), 359-369



Lauri, D., Needham, L., and Dejana, E. (1991). Tumor cell adhesion to the endothelium. In *Vascular endothelium: Interactions with circulating cells.*, J. I. Gordon, ed. (Oxford: Elsevier), pp. 111-128.

Le Blond, C. P., Cheng, H. (1976). Identification of stem cells in the small intestine of the mouse. In *Stem cells of renewing cell populations* (ed. A. B. Cairnie, P. K. Lala & D. G. Osmond), pp. 7-31. New York: Academic.

Le Marchand L. (2002). Meat intake, metabolic genes and colorectal cancer. *IARC Sci. Publ.*, 156, 481-485.

Leathem, A., Dokal, I., Atkins, N. (1983). Lectin binding to normal and malignant breast tissue. *Diagnostic Histopathology* 6, 171-180.

Leathem, A.J., Brooks, S.A. (1987). Predictive value of lectin binding on breast-cancer recurrence and survival. *Lancet* 9;1(8541),1054-1056.

Leathem, A.J., Dokal, I., Atkins, N. (1983). Lectin binding to normal and malignant breast tissue. *Diagn Histopathol.* 6(3-4), 171-180.

Lee, J.O., Rieu, P., Arnaout, M.A., Liddington, R. (1995). Crystal structure of the A domain from the alpha subunit of integrin CR3 (CD11b/CD18). *Cell* 80(4),631-638.

Lehner, I., Niehof, M., Borlak, J. (2003). An optimized method for the isolation and identification of membrane proteins. *Electrophoresis* 24, 1795-808.

Leibovitz, A., Stinson, J.C., McCombs, W.B, 3rd, McCoy, C.E., Mazur, K.C., Mabry, N.D. (1976). Classification of human colorectal adenocarcinoma cell lines. *Cancer Res.* 36(12), 4562-4569.



Le Pendu, J., Marionneau, S., Cailleau-Thomas, A., Rocher, J., Le Moullac-Vaidye, B., Clément, M. (2001). ABH and Lewis histo-blood group antigens in cancer. *APMIS*. 109(1):9-31

Lescar, J., Sanchez, J.F., Audfray, A., Coll, J.L., Breton, C., Mitchell, E.P., Imberty, A. (2007). Structural basis for recognition of breast and colon cancer epitopes Tn antigen and Forssman disaccharide by Helix pomatia lectin. *Glycobiology*. In press.

Lesley, J., English, N., Perschl, A., Gregoroff, J., Hyman, R. (1995). Variant cell lines selected for alterations in the function of the hyaluronan receptor CD44 show differences in glycosylation. *J Exp Med*. 182(2), 431-437.

Levi, G., Tarrab-Hazdai, R., Teichberg, V.I. (1983). Prevention and therapy with electrolectin of experimental autoimmune myasthenia gravis in rabbits. *Eur. J. Immunol*. 13, 500–507.

Levine, A.J., Momand, J., Finlay, C.A. (1991). The p53 tumour suppressor gene. *Nature* 351(6326), 453-456.

Lewis, J.D., Ng, K., Hung, K.E., Bilker, W.B., Berlin, J.A., Brensinger, C., Rustgi, A.K. (2003). Detection of proximal adenomatous polyps with screening sigmoidoscopy: a systematic review and meta-analysis of screening colonoscopy. *Arch Intern Med* 163, 413-420.

Liang, L., Qu, L., Ding, Y.(2007). Protein and mRNA characterization in human colorectal carcinoma cell lines with different metastatic potentials. *Cancer Invest*. 25(6):427-34.

Liotta, L.A. (1992). Cancer cell invasion and metastasis. *Sci Am*. 266(2), 54-59, 62-63.

Lis, H., and Sharon, N. (1993). Protein glycosylation. Structural and functional aspects. *Eur J Biochem* 218, 1-27.



Loftus, J.C., O'Toole, T.E., Plow, E.F., Glass, A., Frelinger, A.L. 3rd, Ginsberg, M.H. (1990). A beta 3 integrin mutation abolishes ligand binding and alters divalent cation-dependent conformation. *Science* 249(4971), 915-918.

Low, M. G., Ferguson, M. A. J., Futerman, A. H., Silman, I. (1986). Covalently attached phosphatidylinositol as a hydrophobic anchor for membrane proteins. *Trends in Biological Sciences* 11, 212-215.

Lowry, O.H., Rosebrough, N.J., Farr, A.L., Randall, R.J. (1951). Protein measurement with the Folin phenol reagent. *J Biol Chem.* 193(1),265-275.

Lynch, H.T., Smyrk, T.C., Watson, P., Lanspa, S.J., Lynch, J.F., Lynch, P.M., Cavalieri, R.J., Boland, C.R. (1993). Genetics, natural history, tumor spectrum, and pathology of hereditary nonpolyposis colorectal cancer: an updated review. *Gastroenterology* 104, 1535-1549.

Lynch, H.T., Watson, P., Lanspa, S.J., Marcus, J., Smyrk, T., Fitzgibbons, R.J. Jr., Kriegler, M., Lynch, J.F. (1988). Natural history of colorectal cancer in hereditary nonpolyposis colorectal cancer (Lynch syndromes I and II). *Dis Colon Rectum* 31, 439-444.

Mandel, J.S., Bond, J.H., Church, T.R., Snover, D.C., Bradley, G.M., Schuman, L.M., Ederer, F. (1993). Reducing mortality from colorectal cancer by screening for fecal occult blood. Minnesota Colon Cancer Control Study. *N Engl J Med* 328, 1365-1367.

Manne, U., Weiss, H.L., Grizzle, W.E. (2000). Racial differences in the prognostic usefulness of MUC1 and MUC2 in colorectal adenocarcinomas. *Clin Cancer Res* 6, 4017-4025.



Marionneau, S., Cailleau-Thomas, A., Rocher, J., Le Moullac-Vaidye, B., Ruvoën, N., Clément, M., Le Pendu, J. (2001). ABH and Lewis histo-blood group antigens, a model for the meaning of oligosaccharide diversity in the face of a changing world. *Biochimie*. 83(7):565-73.

Marth, J. D. (1996). Complexity of O-linked oligosaccharide biosynthesis engendered by multiple polypeptide N-acetylgalactosaminyltransferases. *Glycobiology* 6, 701 - 705.

Matsumoto, H., Muramatsu, H., Shimotakahara, T., Yanagi, M., Nisnijima, H., Mitani, N., Baba, J.C., Muramatsu, T., Shimazu, H. (1993). Correlation of expression of ABH blood group carbohydrate antigens with metastatic potential in human lung carcinomas. *Cancer* 72, 75-81.

Mercurio, A.M. (1990). Laminin: multiple forms, multiple receptors. *Curr Opin Cell Biol.* 2(5), 845-849.

Mercurio, A.M., Rabinovitz, I., Shaw, L.M. (2001). The alpha 6 beta 4 integrin and epithelial cell migration. *Curr Opin Cell Biol.* 13(5), 541-545.

Merida, I., Pratt, J. C., Gaulton, G. N. (1990). Regulation of interleukin 2-dependent growth responses by glycoposphatidylinositol molecules. *Proceedings of the National Academy of Sciences USA* 87, 9421-9425.

Miller, N., Vile, R. G., Hart, I. R. (1996). Selectin ligands on human melanoma cells. *Glycoconjugate Journal* 13, 33-43.

Mitchell, B.S., Brooks, S.A., Leathem, A.J., Schumacher, U. (1998). Do HPA and PHA-L have the same binding pattern in metastasizing human breast and colon cancers? *Cancer Lett.* 16;123(1):113-119.



Mitchell, B.S., Schumacher, U. (1999). The use of lectin *Helix Pomatia* agglutinin (HPA) as a prognostic indicator as a tool in cancer research. *Histol Histopathol* 14,217–226.

Mitchell, B.S., Vernon, K., Schumacher, U., Habil, M. (1995). Ultrastructural localization of *Helix pomatia* agglutinin-binding sites in human breast cancer cell lines and characterization of HPA-binding glycoproteins by Western blotting. *Ultrastruct Pathol* 19:51–59.

Mizejewski, G.J. (1999). Role of integrins in cancer: survey of expression patterns, *Proc. Soc. Exp. Biol. Med.* 222, 124–138.

Molloy, M.P. (2000). Two-dimensional electrophoresis of membrane proteins using immobilized pH gradients. *Anal Biochem.* 280(1), 1-10.

Morgan, W.T. and Watkins, W.M. (2000). Unraveling the biochemical basis of blood group ABO and Lewis antigenic specificity. *Glycoconj. J.*, 17, 501–530.

Moses, H.L., Yang, E.Y., Pietenpol, J.A. (1990). TGF- $\beta$  stimulation and inhibition of cell proliferation: new mechanistic insights. *Cell* 63, 245–247.

Muller, U., Wang, D., Denda, S., Meneses, J.J., Pedersen, R.A., Reichardt, L.F. (1997). Integrin  $\alpha 8 \beta 1$  is critically important for epithelial-mesenchymal interactions during kidney morphogenesis. *Cell* 88(5), 603-613.

Mustac, E., Melato, M., Sasso, F., Valkovic, T., Bottin, C., Jonjic, N. (1996). The lectin-binding sites for peanut agglutinin in invasive breast ductal carcinomas and their role as prognostic factors. *J. Cancer Res. Clin. Oncol.* 122, 693-697



Nakamori, S., Kameyama, M., Imaoka, S., Furukawa, H., Ishikawa, O., Sasaki, Y., Kabuto, T., Iwanaga, T., Matsushita, Y., Irimura, T. (1993). Increased expression of sialyl Lewisx antigen correlates with poor survival in patients with colorectal carcinoma: clinicopathological and immunohistochemical study. *Cancer Res.* 53(15):3632-3637.

Nakamori, S., Kameyama, M., Imaoka, S., Furukawa, H., Ishikawa, O., Sasaki, Y., Izumi, Y., Irimura, T. (1997). Involvement of carbohydrate antigen sialyl Lewis(x) in colorectal cancer metastasis. *Dis. Colon Rectum*, 40, 420-431.

Nangia-Makker, P., Conklin, J., Hogan, V., Raz, A. (2002). Carbohydrate-binding proteins in cancer, and their ligands as therapeutic agents. *Trends Mol Med.* 8(4), 187-192.

Natali, P.G., Nicotra, M.R., Botti, C., Mottolese, M., Bigotti, A., Segatto, O. (1992). Changes in expression of alpha 6/beta 4 integrin heterodimer in primary and metastatic breast cancer. *Br J Cancer* 66(2), 318-322.

(NICE), N.I.C.E. (2005), The use of bevacizumab and cetuximab for the treatment of metastatic colorectal cancer. Final protocol. 353–361.

Nicholson, F.B., Barro, J.L., Atkin, W., Lilford, R., Patnick, J., Williams, C.B., Pignone, M., Steele, R., Kamm, M.A. (2005). Review article: Population screening for colorectal cancer. *Aliment Pharmacol Ther.* 22(11-12), 1069-1077.

Nicholson, G. L. (1982). Cell surface properties of metastatic tumor cells. In *Tumour invasion and metastasis.*, L. A. Liotta and I. R. Hart, eds. (The Hague: Martinus Nijhoff Publishers), 57-79.

Nicolson, G.L. (1988 a). Cancer metastasis: tumor cell and host organ properties important in metastasis to specific secondary sites. *Biochim Biophys Acta.* 948(2), 175-224.



- Nicolson, G.L. (1988 b). Differential organ tissue adhesion, invasion, and growth properties of metastatic rat mammary adenocarcinoma cells. *Breast Cancer Res Treat.* 12(2), 167-176.
- Nishihara, S., Hiraga, T., Ikehara, Y., Kudo, T., Iwasaki, H., Morozumi, K., Akamatsu, S., Tachikawa, T. (1999). Molecular mechanisms of expression of Lewis  $\beta$  antigen and other type I Lewis antigens in human colorectal cancer. *Glycobiology* 9, 607–616.
- Noguchi, M., Thomas, M., Kitagawa, H., Kinoshita, K., Kinami, S., Takamura, H., Miyazaki, I., Mizukami, Y. (1993a). DNA Ploidy and helix pomatia lectin as predictors of regional lymph node metastases and prognostic factors in breast cancer. *Breast Cancer Research and Treatment.* 26, 67-75.
- Noguchi, M., Thomas, M., Kitagawa, H., Kinoshita, K., Ohta, N., Nagamori, M., Miyazaki, I. (1993b). Further analysis of predictive value of *Helix pomatia* lectin binding to primary breast cancer for axillary and internal mammary lymph node metastases. *British Journal of Cancer* 67(6), 1368-1371.
- Nowell, P.C. (1986). Mechanisms of tumor progression. *Cancer Res:* 46(5), 2203-2207.
- O' Farrell, P.H. (1975). High resolution two-dimensional electrophoresis of proteins. *J Biol Chem*, 250, 4007-4021.
- O'Connell, J.B., Maggard, M.A., Ko, C.Y. (2004). Colon cancer survival rates with the new American Joint Committee on Cancer Sixth Edition staging. *J Natl Cancer Inst.* 96, 1420-1425.
- Offner, H., Celnik, B., Bringman, T.S., Casentini-Borocz, D., Nedwin, C.E., Vandenbark, A.A. (1990). Recombinant human  $\beta$ -galactoside binding lectin suppresses clinical and histological signs of experimental autoimmune encephalomyelitis. *J. Neuroimmunol.* 28, 177–184.



Ono, M., Sakamoto, M., Ino, Y., Moriya, Y., Sugihara, K., Muto, T., Hirohashi, S. (1996). Cancer cell morphology at the invasive front and expression of cell adhesion-related carbohydrate in the primary lesion of patients with colorectal carcinoma with liver metastasis. *Cancer* 78(6), 1179-1186.

Orntoft, T.F., Meldgaard, P., Pedersen, B., Wolf, H. (1996). The blood group ABO gene transcript is down-regulated in human bladder tumors and growth-stimulated urothelial cell lines. *Cancer Res.* 56(5), 1031-1036.

Orntoft, V. F., Mores, N. P. O., Eriksen, G., Jacobsen, N. O., Poulsen, H. S. (1985). Comparative immunoperoxidase demonstration of T-antigens in human colorectal carcinomas and morphologically abnormal mucosa. *Cancer Research* 45, 447 – 452.

Paget, S. (1889). The distribution of secondary growths in cancer of the breast. *Lancet* 1, 571–573.

Pappin, D.J., Hojrup, P., Bleasby, A.J. (1993). Rapid identification of proteins by peptide-mass fingerprinting. *Curr Biol.* 3(6), 327-332.

Parkin, D.M., Muir, C.S., Whelan, S.L., Gao, Y.T, Ferlay, J., Powell, J. (1992). Cancer incidence in five continents. *IARC Sci Publ* (120), 45-173. Oxford: Oxford University Press.

Patchett, R.A., Kelly, A.F., Kroll, R.G. (1991). The adsorption of bacteria to immobilized lectins. *J Appl Bacteriol.* 71(3), 277-284.

Paulson, J. C., Colley, K. J. (1989). Glycosyltransferases. *J. Biol. Chem.* 264, 17615-17618.

Perkins, D.N., Pappin, D.J., Creasy, D.M., Cottrell, J.S. (1999). Probability-based protein identification by searching sequence databases using mass spectrometry data. *Electrophoresis.* 20(18):3551-67.



Petretti, T., Kemmner, W., Schulze, B., Schlag, P.M. (2000). Altered mRNA expression of glycosyltransferases in human colorectal carcinomas and liver metastases. *Gut* 46, 359–366.

Pierce, M., Arango, J. (1986). Rous sarcoma virus-transformed baby hamster kidney cells express higher levels of asparagine-linked tri- and tetraantennary glycopeptides containing [GlcNAc-beta (1,6)Man-alpha (1,6)Man] and poly-N-acetyllactosamine sequences than baby hamster kidney cells. *J Biol Chem.* 261(23), 10772-10777.

Pignatelli, M., Stamp, G. (1995). Integrins in tumour development and spread. *Cancer Surv.* 24, 113-127.

Piller, V., Piller, F., Cartron, J. (1990). Comparison of the carbohydrate-binding specificities of seven N-acetyl-D-galactosamine-recognizing lectins. *Eur J Biochem.* 191, 461-466.

Poste, G., Fidler, I.J. (1980). The pathogenesis of cancer metastasis. *Nature* 283(5743), 139-146.

Powell, S.M., Zilz, N., Beazer-Barclay, Y. (1992). APC mutations occur early during colorectal tumorigenesis. *Nature* 359, 235–237.

Prasad, S., Soldatenkov, V., Srinivasarao, G., Dritschilo, A. (1999). Intermediate filament proteins during carcinogenesis and apoptosis. *Int J Oncol* 14, 563-570.

Prokopishyn, N.L., Puzon-McLaughlin, W., Takada, Y., Laferte, S. (1999). Integrin alpha3beta1 expressed by human colon cancer cells is a major carrier of oncodevelopmental carbohydrate epitopes. *J Cell Biochem.* 72(2):189-209.

Pwynder, E.L. (1975). The epidemiology of large bowel cancer. *Cancer Res.* 35, 3388-3394.



Qian, F., Hanahan, D., Weissman, I.L. (2001). L-selectin can facilitate metastasis to lymph nodes in a transgenic mouse model of carcinogenesis. *Proc Natl Acad Sci U S A*. 98(7), 3976-3981.

Quinn, M.J., Babb, P.J., Brock, A., Kirby, L. Jones, J. (2001). Cancer Trends in England and Wales 1950–1999. Studies on Medical and Population Subjects No. 66. London: The Stationery Office.

Rabilloud, T., Adessi, C., Giraudel, A., Lunardi, J. (1997). Improvement of the solubilization of proteins in two-dimensional electrophoresis with immobilized pH gradients. *Electrophoresis* 18, 307-316.

Rabilloud, T. (1998). Use of thiourea to increase the solubility of membrane two dimensional electrophoresis. *Electrophoresis* 19, 758-760.

Rabilloud, T., Blisnick, T., Heller, M., Luche, S., Aebersold, R., Lunardi, J., Braun-Breton, C. (1999). Analysis of membrane proteins by two-dimensional electrophoresis: comparison of the proteins extracted from normal or *Plasmodium falciparum*-infected erythrocyte ghosts. *Electrophoresis*. 18, 3603-3610.

Rabilloud, T. (2003). Membrane proteins ride shotgun. *Nat Biotechnol*. 5, 508-510.

Rabinovitz, I., Nagle, R.B., Cress, A.E. (1995). Integrin alpha 6 expression in human prostate carcinoma cells is associated with a migratory and invasive phenotype in vitro and in vivo. *Clin Exp Metastasis*. 13(6):481-91

Rabinovitz, I., Mercurio, A.M. (1996). The integrin alpha 6 beta 4 and the biology of carcinoma. *Biochem Cell Biol*. 74(6), 811-821.



Rabinovitz, I., Toker, A., Mercurio, A.M. (1999). Protein kinase C-dependent mobilization of the  $\alpha 6 \beta 4$  integrin from hemidesmosomes and its association with actin-rich cell protrusions drive the chemotactic migration of carcinoma cells. *J Cell Biol.* 146(5), 1147-1160.

Ray, J.M., Stetler-Stevenson, W.G. (1994). The role of matrix metalloproteases and their inhibitors in tumour invasion, metastasis and angiogenesis. *Eur Respir J.* 7(11):2062-2072.

Reeder, J.A., Gotley, D.C., Walsh, M.D., Fawcett, J., Antalis, T.M. (1998). Expression of antisense CD44 variant 6 inhibits colorectal tumor metastasis and tumor growth in a wound environment. *Cancer Res.* 58(16), 3719-3726.

Remani, P., Nair, R.A., Sreelekha, T.T., Madhavan, J., Vijayakumar, T., Nair, M.K. (2000). Altered expression of jack fruit lectin specific glycoconjugates in benign and malignant human colorectum. *J Exp Clin Cancer Res* 19, 519–523.

Reymond, M.A., Sanchez, J.C., Hughes, G.J., Gunther, K., Riese, J., Tortola, S., Peinado, M.A., Kirchner, T., Hohenberger, W., Hochstrasser, D.F., Kockerling, F. Standardized characterization of gene expression in human colorectal epithelium by two-dimensional electrophoresis. *Electrophoresis* 18(15), 2842-2848.

Rhodes, J.M., Black, R.R., Savage, A. (1986). Glycoprotein abnormalities in colonic carcinomata, adenomata, and hyperplastic polyps shown by lectin peroxidase histochemistry. *J Clin Pathol.* 39(12), 1331-1334.

Rothberg, P.G., Spandorfer, J.M., Erisman, M.D., Staroscik, R.N., Sears, H.F., Petersen, R.O., Astrin, S.M. (1985). Evidence that c-myc expression defines two genetically distinct forms of colorectal adenocarcinoma. *Br J Cancer* 52: 629-632.

Ruoslahti, E., Giancotti, F.G. (1989). Integrins and tumor cell dissemination. *Cancer Cells* 1(4), 119-126.



Ruoslahti, E., Pierschbacher, M.D. (1987). New perspectives in cell adhesion: RGD and integrins. *Science* 238(4826), 491-497.

Sabarth, N., Lamer, S., Zimny-Arndt, U., Jungblut, P.R., Meyer, T.F., Bumann, D. (2002). Identification of surface proteins of *Helicobacter pylori* by selective biotinylation, affinity purification, and two-dimensional gel electrophoresis. *J Biol Chem.* 31, 27896-27902.

Saitoh, O., Wang, W.C., Lotan, R., Fukuda, M. (1992). Differential glycosylation and cell surface expression of lysosomal membrane glycoproteins in sublines of a human colon cancer exhibiting distinct metastatic potentials. *J Biol Chem.* 267(8):5700-5711.

Sams, J.S., Lynch, H.T., Burt, R.W., Lanspa, S.J., Boland, C.R. (1990). Abnormalities of lectin histochemistry in familial polyposis coli and hereditary nonpolyposis colorectal cancers. *Cancer* 66:502–508.

Saint-Guirons, J., Zeqiraj, E., Schumacher, U., Greenwell, P., Dwek, M. (2007). Proteome analysis of metastatic colorectal cancer recognised by the lectin *Helix pomatia* agglutinin. *Proteomics*, in press.

Sanchez, J.F., Lescar, J., Chazalet, V., Audfray, A., Gagnon, J., Alvarez, R., Breton, C., Imberty, A., Mitchell, E.P. (2006). Biochemical and structural analysis of *Helix pomatia* agglutinin. A hexameric lectin with a novel fold. *J Biol Chem.* 281(29), 20171-20180.

Santoni, V., Molloy, M., Rabilloud, T. (2000). Membrane proteins and proteomics: un amour impossible? *Electrophoresis.* 21(6), 1054-1070.

Santucci, L., Fiorucci, S., Cammilleri, F., Servillo, G., Federici, B., Morelli, A. (2000). Galectin-1 exerts immunomodulatory and protective effects on Concanavalin A-induced hepatitis in mice. *Hepatology* 31, 399–406.



Schacter, H. (1994). Molecular cloning of glycosyltransferase genes. In *Molecular Glycobiology*, M. Fukuda and O. Hindsgaul, eds. (New York: Oxford University Press), pp. 88-162.

Schmidt, G. H., Wilkinson, M. M., Ponder, B. A. J. (1985). Cell migration pathway in the intestinal epithelium: an in situ marker system using mouse aggregation chimeras. *Cell* 40, 425-429.

Schumacher, U., Adam, E. (1997). Lectin histochemical HPA-binding pattern of human breast and colon cancers is associated with metastases formation in severe combined immunodeficient mice. *Histochem J.* 29, 677-84.

Schumacher, U., Adam, E., Brooks, S.A., Leathem, A.J. (1995). Lectin-binding properties of human breast cancer cell lines and human milk with particular reference to *Helix pomatia* agglutinin. *J Histochem Cytochem* 43:275–281

Schumacher, U., Higgs, D., Loizidou, M., Pickering, R., Leathem, A., Taylor, I. (1994a). *Helix pomatia* binding is a useful prognostic indicator in colorectal carcinoma. *Cancer* 74, 3104–3107.

Schumacher, U., Adam, E., Flavell, D.J., Boehm, D., Brooks, S.A., Leathem, A.J. (1994b). Glycosylation patterns of the human colon cancer cell line HT-29 detected by *Helix pomatia* agglutinin and other lectins in culture, in primary tumours and metastases in SCID mice. *Clin. Exp. Metast.* 12, 398–404.

Schumacher, U., Brooks, S., Mester, J. (2005). The lectin *Helix pomatia* agglutinin as a marker of metastases--clinical and experimental studies. *Anticancer Res*, 25.(3A), 1829-30.

Seiter, S., Arch, R., Reber, S., Komitowski, D., Hofmann, M., Ponta, H., Herrlich, P., Matzku, S., Zoller, M. (1993). Prevention of tumor metastasis formation by anti-variant CD44. *J Exp Med.* 177(2):443-455.



Shang, J., Qiu, R., Wang, J., Liu, J., Zhou, R., Ding, H., Yang, S., Zhang, S., Jin, C. (1999). Molecular cloning and expression of Galbeta1,3GalNAc alpha2, 3-sialyltransferase from human fetal liver. *Eur J Biochem.* 265(2), 580-588..

Sgantzos, M.N., Galani, V., Arvanitis, L.D., Charchanti, A., Psathas, P., Nakou, M., Havaki, S., Kallioras, V., Marinos, E., Vamvakopoulos, N.C., Kittas, C. (2007). Expression of the O-linked N-acetylglucosamine containing epitope H in normal myometrium and uterine smooth muscle cell tumors. *Pathol Res Pract.* 203(1):31-37.

Shattil, S.J., Kashiwagi, H., Pampori, N. (1998). Integrin signaling: the platelet paradigm. *Blood* 91(8), 2645-2657.

Shay, J.W., Bacchetti, S. (1997). A survey of telomerase activity in human cancer. *Eur. J. Cancer* 33, 787-791.

Shaw, L.M., Chao, C., Wewer, U.M., Mercurio, A.M. (1996). Function of the integrin alpha 6 beta 1 in metastatic breast carcinoma cells assessed by expression of a dominant-negative receptor. *Cancer Res.* 56(5), 959-963.

Sheppard, D. (2003). Functions of pulmonary epithelial integrins: from development to disease. *Physiol Rev.* 83(3), 673-686

Shiraishi, T., Atsumi, S., and Yatani, R. (1992). Comparative study of prostatic carcinoma bone metastasis among Japanese in Japan and Japanese Americans and whites in Hawaii. *Advances in Experimental Medicine and Biology* 324, 7-16.

Shiraishi, T., Atsumi, S., Yatani, R. (1992). Comparative study of prostatic carcinoma bone metastasis among Japanese in Japan and Japanese Americans and whites in Hawaii. *Adv Exp Med Biol.* 324, 7-16.



- Singh, R.K., Gutman, M., Bucana, C.D., Sanchez, R., Llansa, N., Fidler, I.J. (1995). Interferons alpha and beta down-regulate the expression of basic fibroblast growth factor in human carcinomas. *Proc. Natl. Acad. Sci. USA* 92, 4562–4566.
- Slamon, D.J., Clark, G.M., Wong, S.G., Levin, W.J., Ullrich, A., McGuire, W.L. (1987). Human breast cancer: correlation of relapse and survival with amplification of the HER-2/*neu* oncogene. *Science* 235, 177–182.
- Slifkin, M., Cumbie, R. (1989). Rapid detection of herpes simplex virus with fluorescein-labeled *Helix pomatia* lectin. *J Clin Microbiol.* 27(5), 1036-1039.
- Smets, L.A., Van Beek, W.P. (1984). Carbohydrates of the tumor cell surface. *Biochim Biophys Acta.* 738(4),237-249.
- Sonnenberg, A., Linders, C.J., Daams, J.H., Kennel, S.J. (1990). The alpha 6 beta 1 (VLA-6) and alpha 6 beta 4 protein complexes: tissue distribution and biochemical properties. *J Cell Sci.* 96 (2), 207-217.
- Sporn, M.B. (1996). The war on cancer. *Lancet* 347(9012), 1377-1381.
- Springer, G. F. (1984). T and Tn, general carcinoma auto-antigens. *Science* 224, 1198 – 1206.
- Springer, G. F. (1988). Tn epitope density predicts aggressiveness of primary breast carcinoma. *Proc. Annu. Meet. Am. Assoc. Cancer Res.* 29, A785.
- Springer, G. F. (1989). Tn Epitope (N-acetyl-D-galactosamine-O-serine/threonine) Density in Primary Breast Carcinoma: a Functional Predictor of Aggressiveness. *Molecular Immunology* 26, 1-5.



Springer, G.F., Desai, P.R., Banatwala, I. (1975). Blood group MN antigens and precursors in normal and malignant human breast glandular tissue. *J Natl Cancer Inst.* 54(2), 335-339.

Springer, G.F., Desai, P.R., Robinson, M.K., Tegtmeyer, H., Scanlon, E.F. (1986). The fundamental and diagnostic role of T and Tn antigens in breast carcinoma at the earliest histologic stage and throughout. *Prog Clin Biol Res.* 204, 47-70.

Springer, G.F., Taylor, C.R., Howard, D.R., Tegtmeyer, H., Desai, P.R., Murthy, S.M., Felder, B., Scanlon, E.F. (1985). Tn, a carcinoma-associated antigen, reacts with anti-Tn of normal human sera. *Cancer* 55(3),561-569.

Springer, T. A. (1990). Adhesion receptors of the immune system. *Nature* 346, 425-434.

Springer, T. A., Lasky, L. A. (1991). Sticky sugars for selectins. *Nature* 349, 196 - 197.

Springer, T.A. (1997). Folding of the N-terminal, ligand-binding region of integrin alpha-subunits into a beta-propeller domain. *Proc Natl Acad Sci U S A.* 94(1), 65-72.

Steel, L.F., Trotter, M.G., Nakajima, P.B., Mattu, T.S., Gonye, G., Block, T. (2003). Efficient and specific removal of albumin from human serum samples. *Mol Cell Proteomics.* 2(4), 262-270.

Stemmermann, G.N., Nomura, A.M., Heilbrun, L.K. (1984). Dietary fat and the risk of colorectal cancer. *Cancer Res.* 44, 4633-4637.

Stillmark, H. (1888). Ueber Ricin, ein giftiges Ferment aus den Samen von *Ricinus communis* L. und einigen anderen Euphorbiaceen. Inaugural Dissertation, Dorpat

Stockert, R.J., Morell, A.G., and Scheinberg, I.H. (1974) Mammalian hepatic lectin. *Science*, 186, 365–366.



Sy, M.S., Guo, Y.J., Stamenkovic, I. (1991). Distinct effects of two CD44 isoforms on tumor growth in vivo. *J Exp Med.* 174(4), 859-866.

Takada, A., Ohmori, K., Yoneda, T., Tsuyuoka, K., Hasegawa, A., Kiso, M., Kannagi, R. (1993). Contribution of carbohydrate antigens Sialyl Lewis A and Sialyl Lewis X to adhesion of human cancer cells to vascular endothelium. *Cancer Research* 53, 354-361.

Takahashi, H., Oyaizu, T., Fujita, Y., Tsubura, A. (1994). Lectin-binding profiles in MNNG-induced shrew esophageal carcinomas. *Anticancer Res.* 14(4A), 1569-1572.

Tan, S., Seow, T.K., Liang, R.C., Koh, S., Lee, C.P., Chung, M.C., Hooi, S.C. (2002). Proteome analysis of butyrate-treated human colon cancer cells (HT-29). *Int J Cancer.* 98(4):523-31.

Tang, D.G., Honn, K.V.(1994). Adhesion molecules and tumour metastasis: an update. *Invasion metastasis* 14, 109-122.

Taylor, C.W., Anbazhagan, R., Jayatilake, H., Adams, A., Gusterson, B.A., Price, K., Gelber, R.D., Goldhirsch, A. (1991). *Helix pomatia* in breast cancer. *Lancet* 338, 580-581.

Thomas, M., Noguchi, M., Fonesca, L., Kitagawa, H., Kinoshita, K., Miyazaki, I. (1993). Prognostic Significance of *Helix pomatia* Lectin and the c-erbB-2 oncoprotein in human breast cancer. *Br. J. Cancer* 68, 621-626.

Thomas, M., Noguchi, M., Fonseca, L., Kitagawa, H., Kinoshita, K., Miyazaki, I. (1993). Prognostic significance of *Helix pomatia* lectin and c-erbB-2 oncoprotein in human breast cancer. *Br J Cancer.* 68(3), 621-626.

Toms, J. R.(ed) (2004). CancerStats Monograph 2004. Cancer Research UK: London.

Trichopoulos, D., Li, F.P., Hunter, D.J. (1996). What causes cancer? *Sci Am.* 275(3), 80-87.



Tuckwell, D., Calderwood, D.A., Green, L.J., Humphries, M.J. (1995). Integrin alpha 2 I-domain is a binding site for collagens. *J Cell Sci.* 108 (4), 1629-1637.

Uhlenbruck, G., Prokop, O. (1966). An agglutinin from *Helix pomatia*, which reacts with terminal N-acetyl-D-galactosamine. *Vox Sang.* 11(4), 519-520.

van den Eijnden, D.H., Koenderman, A.H., Schiphorst, W.E. (1988). Biosynthesis of blood group i-active polygalactosaminoglycans. Partial purification and properties of an UDP-GlcNAc:N-acetylgalactosaminide beta 1----3-N-acetylglucosaminyltransferase from Novikoff tumor cell ascites fluid. *J Biol Chem.* 263(25), 12461-12471.

van Kuik, J.A., van Halbeek, H., Kamerling, J.P., Vliegthart, J.F. (1985). Primary structure of the low-molecular-weight carbohydrate chains of *Helix pomatia* alpha-hemocyanin. Xylose as a constituent of N-linked oligosaccharides in an animal glycoprotein. *J Biol Chem.* 260(26):13984-8.

van Munster, I.P., Nagengast, F.M. (1993). The role of carbohydrate fermentation in colon cancer prevention. *Scand J Gastroenterol Suppl* 200, 80-86.

Varki, A. (1993). Biological roles of oligosaccharides: all of the theories are correct. *Glycobiology* 3(2), 97-130.

Varki, A. and Marth, J. (1995). Oligosaccharides in vertebrate development. *Semin. Dev. Biol.*, 6, 127-138.

Vasen, H.F., Wijnen, J.T., Menko, F.H., Kleibeuker, J.H., Taal, B.G., Griffioen, G., Nagengast, F.M., Meijers-Heijboer, E.H., Bertario, L., Varesco, L., Bisgaard, M.L., Mohr, J., Fodde, R., Khan, P.M. (1996). Cancer risk in families with hereditary nonpolyposis colorectal cancer diagnosed by mutation analysis. *Gastroenterology* 110, 1020-1027.



Virtanen, I. (1990). *Helix pomatia* agglutinin binds specifically to the Golgi apparatus in cultured human fibroblasts and reveals two Golgi apparatus-specific glycoproteins. *Histochemistry* 94(4), 397-401.

Vleminckx, K., Vackat, L., Mateel, M., Fiers, W., and Van Roy, F. V. (1991). Genetic manipulation of E-Cadherin expression by epithelial tumour cells reveals an invasion suppressor role. *Cell* 66, 107-119.

Vogel, B.E., Tarone, G., Giancotti, F.G., Gailit, J., Ruoslahti, E. (1990). A novel fibronectin receptor with an unexpected subunit composition (alpha v beta 1). *J Biol Chem.* 265(11), 5934-5937.

Vogelstein, B., Fearon, E.R., Hamilton, S.R., Kern, S.E., Preisinger, A.C., Leppert, M., Nakamura, Y., White, R., Smits, A.M., Bos, J.L. (1988). Genetic alterations during colorectal-tumor development. *N Engl J Med.* 319(9), 525-532.

Vogelstein, B., Fearon, E.R., Kern, S.E., Hamilton, S.R., Preisinger, A.C., Nakamura, Y., White, R. (1989). Allelotype of colorectal carcinomas. *Science.* 244(4901), 207-211.

Volpert, O.V., Dameron, K.M., Bouck, N. (1997). Sequential development of an angiogenic phenotype by human fibroblasts progressing to tumorigenicity. *Oncogene* 14, 1495–1502.

Vretblad, P., Hjorth, R. , Laas, T. (1979). The isolectins of *Helix pomatia*. Separation by isoelectric focusing and preliminary characterization. *Biochem. et Biophys. Acta* 579, 52-61.

Walgren, J.L., Vincent, T.S., Schey, K.L., Buse, M.G. (2003). High glucose and insulin promote O-GlcNAc modification of proteins, including alpha-tubulin. *Am J Physiol Endocrinol Metab.* 284(2):E424-434.



Walker, A.R., Burkitt, D.P. (1976). Colonic cancer: hypotheses of causation, dietary prophylaxis, and future research. *Am J Dig Dis* 21, 910-7.

Walker, R.A. (1993). *Helix pomatia* and prognosis of breast cancer. *Br J Cancer* 68(3):453-4.

Weinberg, R.A. (1995). The retinoblastoma protein and cell cycle control. *Cell* 81, 323–330.

Wilkins, M.R., Gasteiger, E., Sanchez, J.C., Bairoch, A., Hochstrasser, D.F. (1998). Two-dimensional gel electrophoresis for proteome projects: the effects of protein hydrophobicity and copy number. *Electrophoresis*. 8-9, 1501-1505.

Willett, W.C. (1995). Diet, nutrition and avoidable cancer. *Environ Health Perspect* 103 (8), 165-170.

Willett, W.C., Stampfer, M.J., Colditz, G.A., Rosner, B.A., Speizer, F.E. (1990). Relation of meat, fat, and fiber intake to the risk of colon cancer in a prospective study among women. *N Engl J Med*. 323(24), 1664-1672.

Winawer, S.J., Stewart, E.T., Zauber, A.G., Bond, J.H., Ansel, H., Waye, J.D., Hall, D., Hamlin, J.A., Schapiro, M., O'Brien, M.J., Sternberg, S.S., Gottlieb, L.S. (2000). A comparison of colonoscopy and double-contrast barium enema for surveillance after polypectomy. National Polyp Study Work Group. *N Engl J Med* 342, 1766-1772.

Wright, N. A., Irwin, M. (1982). The kinetics of villus cell populations in the mouse small intestine: normal villi-the steady state requirement. *CellTissue Kinet*. 15, 595-609.

Wu, C.C., Yates, J.R. 3rd. (2003). The application of mass spectrometry to membrane proteomics. *Nat Biotechnol*. 3, 262-267.



Wu, D.H., Liu, L., Chen, L.H., Ding, Y.Q. (2004). KAI1 gene expression in colonic carcinoma and its clinical significances. *World J Gastroenterol.* 1;10(15):2245-9.

Xiong, J.P., Stehle, T., Diefenbach, B., Zhang, R., Dunker, R., Scott, D.L., Joachimiak, A., Goodman, S.L., Arnaout, M.A. (2001). Crystal structure of the extracellular segment of integrin alpha V beta3. *Science.* 294(5541), 339-345.

Yagel, S., Feinmesser, R., Waghorne, C., Lala, P.K., Breitman, M.L., Dennis, J.W. (1989). Evidence that beta 1-6 branched Asn-linked oligosaccharides on metastatic tumor cells facilitate invasion of basement membranes. *Int J Cancer.* 44(4), 685-690.

Yamamoto, H., Irie, A., Fukushima, Y., Ohnishi, T., Arita, N., Hayakawa, T., Sekiguchi, K. (1996). Abrogation of lung metastasis of human fibrosarcoma cells by ribozyme-mediated suppression of integrin alpha6 subunit expression. *Int J Cancer.* 65(4), 519-524.

Yamashita, K., Ohkura, T., Tachibana, Y., Takasaki, S., Kobata, A. (1984). Comparative study of the oligosaccharides released from baby hamster kidney cells and their polyoma transformant by hydrazinolysis. *J Biol Chem.* 259(17), 10834-10840.

Yamashita, Y., Chung, Y. S., Horie, R., Kannagi, R., Sowa, M. (1995). Alterations in gastric mucin with malignant transformation: novel pathway for mucin synthesis. *Journal of the National Cancer Institute* 87, 441-445.

Yang, J. M., Byrd, J. C., Siddiki, B. B., Chung, Y. S., Okuno, M., Sowa, M., Kim, Y. S., Matta, K. L., Brockhausen, I. (1994). Alterations of O-glycan biosynthesis in human colon cancer tissues. *Glycobiology* 4, 873-884.

Yeatman, T.J., Nicolson, G.L. (1993). Molecular basis of tumor progression: mechanisms of organ-specific tumor metastasis. *Semin Surg Oncol.* 9(3), 256-63.



Yonezawa, S., Byrd, J.C., Dahiya, R., Ho, J.J., Gum, J.R., Griffiths, B., Swallow, D.M., Kim, Y.S. (1991). Differential mucin gene expression in human pancreatic and colon cancer cells. *Biochem J.* 276 ( Pt 3), 599-605.

Yoshida, Y., Okamura, T., Shirakusa, T. (1993). An immunohistochemical study of *Helix pomatia* agglutinin binding on carcinomas of the esophagus. *Surg Gynecol Obstet.* 177(3), 299-302.

Yoshida, Y., Okamura, T., Yano, K., Ezaki, T. (1994). Silver stained nucleolar organizer region proteins and *Helix pomatia* agglutinin immunostaining in esophageal carcinoma: correlated prognostic factors. *J Surg Oncol.* 56(2), 116-121.

Yousefi, S., Higgins, E., Daoling, Z., Pollex-Kruger, A., Hindsgaul, O., Dennis, J.W. (1991). Increased UDP-GlcNAc:Gal beta 1-3GalNAc-R (GlcNAc to GalNAc) beta-1, 6-N-acetylglucosaminyltransferase activity in metastatic murine tumor cell lines. Control of polylactosamine synthesis. *J Biol Chem.* 266(3), 1772-1782.

Zhang, W., Zhou, G., Zhao, Y., White, M.A., Zhao, Y. (2003). Affinity enrichment of plasma membrane for proteomics analysis. *Electrophoresis.* 16, 2855-2863.

Zhao, L., Liu, L., Wang, S., Zhang, Y.F., Yu, L., Ding, Y.Q. (2007). Differential proteomic analysis of human colorectal carcinoma cell lines metastasis-associated proteins. *J Cancer Res Clin Oncol* 133(10):771-82

Zubay, Biochemistry (3rd ed. (1993) Wm. C. Brown Publishers, Dubuque.



# **PUBLICATION**







# **PUBLICATION**



## RESEARCH ARTICLE

# Proteome analysis of metastatic colorectal cancer cells recognized by the lectin *Helix pomatia* agglutinin (HPA)

Julien Saint-Guirons<sup>1</sup>, Elton Zeqiraj<sup>1</sup>, Udo Schumacher<sup>2</sup>, Pamela Greenwell<sup>1</sup> and Miriam Dwek<sup>1</sup>

<sup>1</sup> School of Biosciences, University of Westminster, London, UK

<sup>2</sup> Institute for Anatomy, University Hospital Hamburg-Eppendorf, Hamburg, Germany

The lectin from *Helix pomatia* (HPA) binds to adenocarcinomas with a metastatic phenotype but the glycoconjugates of cancer cells that bind to the lectin have yet to be characterized in detail. We used a model of metastatic (HT29) and nonmetastatic (SW480) human colorectal cancer cells and a proteomic approach to identify HPA binding glycoproteins. Cell membrane proteins purified by HPA affinity chromatography, were separated by 2-DE and analyzed by MS. Competitive inhibition experiments with *N*-acetylgalactosamine, *N*-acetylglucosamine, and sialic acid confirmed that HPA binding was *via* a glycan-mediated interaction. Western blot analysis showed that HPA binds to proteins not recognized by an antibody against blood group A epitope. The proteomic study showed the main HPA binding partners include integrin  $\alpha v/\alpha 6$  and annexin A2/A4. These proteins were found complexed with microfilament proteins  $\alpha$  and  $\beta$  tubulin, actin, and cytokeratins 8 and 18. HPA also bound to Hsp70, Hsp90, TRAP-1, and tumor rejection factor 1. This study revealed that the prognostic utility of HPA lies in its ability to bind simultaneously to many glycoproteins involved in cell migration and signaling, in addition, the proteins recognized by HPA are glycosylated with structures distinct from the blood group A epitope.

Received: May 4, 2007  
Revised: July 24, 2007  
Accepted: August 6, 2007

**Keywords:**

Cancer / Glycoproteome / HPA / Lectin / Metastatic

## 1 Introduction

Cancer mortality is a major problem in the West and a growing problem in developing nations. One of the main causes of patient mortality is an inability to identify and selectively target metastatic tumors. Although progress has been made in unraveling the molecular and cellular changes that occur during metastasis, there remains a need to identify cell membrane molecules as targets for drug discovery programs. Most biomarkers are glycopro-

teins and a vast body of evidence has chronicled that changes in protein glycosylation correlate with metastasis formation [1].

The carbohydrate binding protein (lectin) from the Roman snail *Helix pomatia* (HPA) was first reported to identify metastatic breast cancer two decades ago [2] and further studies have confirmed its utility for detecting metastatic solid tumors, including those of the colon, stomach, and esophagus [3]. Approximately 80% of metastatic tumors contain HPA binding epitopes [4] and an association between HPA binding to cancer cells and metastasis *in vivo* has been described [5]. Despite the evidence of its utility HPA does not yet have a role in clinical decision-making for any tumor type, and the glycoproteins of HPA binding cancer cells have similarly never been defined.

It was initially hypothesized that HPA simply detects cancer cells overexpressing blood group A substance but this was discounted [6]. Extraction of cancer-associated HPA-binding glycans revealed that they contain both *N*-

**Correspondence:** Dr. Miriam Dwek, School of Biosciences, University of Westminster, 115 New Cavendish Street, London W1W 6UW, UK

**E-mail:** m.v.dwek@westminster.ac.uk

**Fax:** +44-0-20 7911 5076

**Abbreviations:** GalNAc, *N*-acetylgalactosamine; HPA, *Helix pomatia* agglutinin



acetylgalactosamine (GalNAc) as well as sialic acid [7] and the predictive power of HPA was lost when cancer tissue sections were pretreated with neuraminidase [8]. A seemingly diverse range of glycan structures bind to HPA including GalNAc $\alpha$ 1-3GalNAc, GalNAc $\alpha$ -Ser/Thr (Tn antigen), and the Forsmann antigen [9, 10]. With the exception of the Tn antigen, the relevance to cancer of these antigens and the proteins to which they are attached has not been established.

We set out to identify HPA binding glycoproteins in metastatic cancer. A proteomic approach using HPA affinity chromatography and 2-DE allowed us to discern the HPA binding proteins in a model of cancer metastasis. The data that we have obtained has enabled us to make significant inferences regarding the basis for HPA recognition of metastatic cancer cells.

## 2 Experimental procedures

### 2.1 Chemicals

All chemicals were obtained from Sigma–Aldrich, Poole, UK, and Milli-Q grade water was used unless otherwise stated.

### 2.2 HT29 and SW480 cell culture

Human colorectal cancer cell lines SW480 and HT29 were maintained in DMEM supplemented with 10% v/v FCS and 0.1% v/v gentamycin. The cells were grown to near confluence, washed twice in PBS and mechanically removed from the surface of the tissue culture flask using a sterile plastic cell scraper. The cells were lysed by sonication and used for protein extraction using the ‘centrifugal protein extraction method’ described by Lehner *et al.* [11].

### 2.3 Confocal microscopy

Cells were seeded into 12-well plates or 7.5 cm<sup>2</sup> Petri dishes and maintained as above for 24 h until 80% confluent. The cells were processed using a modification of the HPA binding protocol [12]: cells were fixed, trypsinized and blocked as described then incubated in the dark with HPA-TRITC (Sigma, UK) at 10  $\mu$ g/ml for 60 min in lectin buffer. The cells were treated with 100  $\mu$ g/mL DNase-free ribonuclease A for 20 min at 37°C and the nuclei counterstained using 1  $\mu$ M To-Pro-3 in PBS for 20 min (Invitrogen, UK). Images were acquired by sequential scanning using a Leica TCS SP2 confocal microscope with a x63 ceramic dipping objective. Images were collected at 1024  $\times$  1024 pixel resolution with a scanning speed of 400 Hz and line average of 2. For 3-D images z-stacks with increments of 1  $\mu$ m were scanned. A 543 nm laser was used for the excitation of the HPA-TRITC (intensity 100%) and 633 nm laser for To-Pro-3 (intensity 35%), emission was recorded over the bandwidth of 553–

630 nm, HPA-TRITC, and 650–720 nm for To-Pro-3. The specificity of lectin binding was investigated by preincubating the lectin with GalNAc for 30 min at a concentrations of 25–250 mM.

### 2.4 HPA-affinity chromatography

A 1 mL HPA affinity chromatography column was prepared using a HiTrap NHS-activated Sepharose column according to the manufacturer’s recommendations (GE Healthcare), 5 mg of HPA was coupled to the column. HT29 (0.5 mg) or SW480 membrane proteins were loaded onto the column in either 20 mM Tris, pH 7.4, 0.5 M NaCl, (for the 1-DE analysis) or in lectin buffer containing 0.1% w/v CHAPS (for 2-DE analysis). The column was washed with five column volumes of loading buffer and HPA-binding proteins eluted using loading buffer containing 250 mM GlcNAc.

### 2.5 Experimental design/1-D and 2-DE

Membrane proteins were prepared on at least three separate occasions from HT29 and SW480 cells according to the conditions shown above. Twenty micrograms of each membrane proteins preparation was separated by 1-D SDS-PAGE, again, on at least three separate occasions [13]. Either a 10 or 12% acrylamide gel and run at 150 V for 1 h 15 min.

2-DE experiments for each cell line, HT29 or SW480, used 7 cm IPG strips of pH 3–10L according to [14] but modified as follows: 90  $\mu$ g of membrane proteins were prepared in 7 M urea, 2 M thiourea, 4% w/v CHAPS, 2% v/v carrier ampholytes, 1% w/v DTT, the strip was rehydrated in this buffer at room temperature overnight and the IEF performed at 20°C with 300 V for 30 min; 600 V for 30 min followed by 3500 V for 2 h 45 min. Focused strips were processed as described [14] and run in the second dimension. In all procedures proteins were visualized using either Sypro Ruby (Invitrogen) or by fixing the gel in 50% methanol, 10% acetic acid for 30 min and staining with 0.025% w/v Coomassie Blue R-250 in 10% acetic acid for 1 h, followed by destaining in 10% acetic acid for 2 h. Two replicates of the cell membrane preparations from HT29 and SW480 were run on the 2-DE system.

### 2.6 Analysis of 2-DE data

The 2-DE gel images were obtained using either, for Sypro Ruby stained gels, a dual-laser Typhoon scanner (GE Healthcare) or, for Coomassie stained gels, transillumination with white light and UVP Biochemi Image Capture System. Images in.TIFF format were imported into Phoretix 2-D Advanced Software (Non-Linear Dynamics) and processed by background subtraction, spot detection, landmarking, and overlay analysis.



## 2.7 Western blotting

Gels were transferred to NC membranes using a wet blotting system for 1.5 h at 100 V in transfer buffer (25 mM Tris, 192 mM glycine, 20% v/v methanol). The HPA blots were developed by blocking with 2% w/v BSA in TBS/Tween 0.05% v/v (TBS/T) at 4°C overnight, the membrane was incubated with 5 µg/mL biotinylated HPA for 2 h and 2 µg/mL HRP conjugated streptavidin (Pierce, UK) for 1 h. The detection system was diaminobenzidine (DAB) prepared in TBS/H<sub>2</sub>O<sub>2</sub>. A negative control, in which the lectin was omitted, was included in the analysis. For the antibody group A analysis, gels and nitrocellulose membranes were prepared as detailed above and incubated in antibody group A antibody (Ortho-Reagent, Johnson and Johnson, 1 in 5 dilution) for 2 h, peroxidase labeled goat antimouse antibodies (1 in 10 000 dilution) for 2 h and developed using the West Signal Dura reagent (Pierce). The chemiluminescent signal was captured onto X-ray film with an exposure time ranging from 30 s to 2 min.

## 2.8 Inhibition work

The blots were prepared as above but the lectin was preincubated with GalNAc, GlcNAc, or sialic acid for a period of 30 min prior to application on the blot. The monosaccharides were used in the range 50–250 mM.

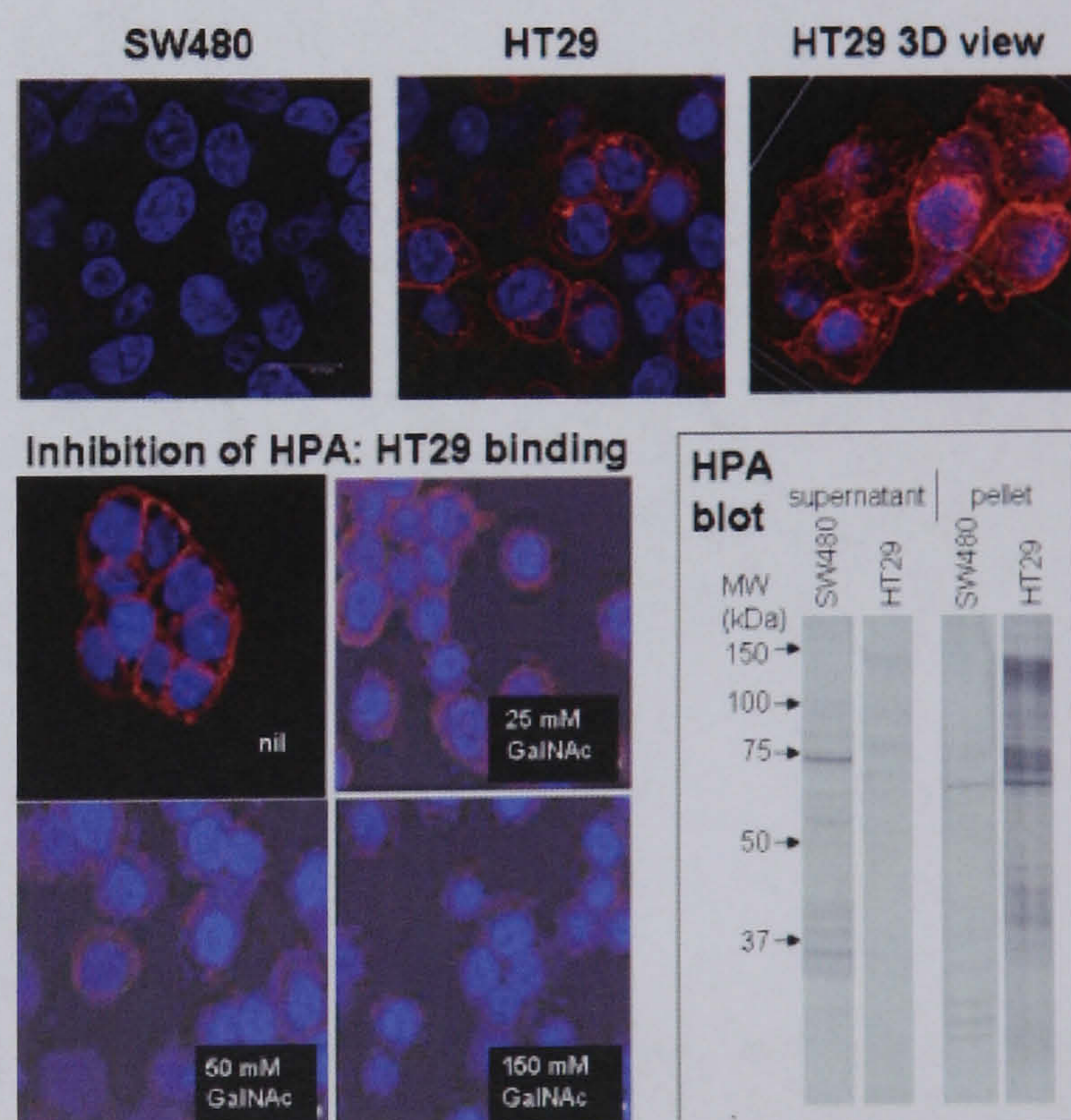
## 2.9 MS analysis for protein identification

Protein identification was carried out by commercial arrangement with Dr. Jerry Thomas, Department of Biology, University of York, using a MALDI-TOF/TOF 4700 analyzer (Applied Biosystems). In brief, 2-DE protein spots stained with CBB were excised from the gel. The proteins were reduced using DTT and S-carbamidomethylation was performed using iodoacetamide prior to the tryptic digest. The gels pieces were washed three times in 50% v/v ACN/25 mM ammonium bicarbonate and air dried before rehydration in 10 µL of 0.02 µg/µL sequencing-grade, modified porcine trypsin (Promega). Digestion was performed overnight at 37°C. A 0.5 µL aliquot of each tryptic digest and 0.5 µL of a solution of CHCA (Sigma) in 50% aqueous (v/v) ACN containing 0.1% TFA v/v, was applied to the MALDI target plate. Mass spectra were obtained in reflection mode with an accelerating voltage of 20 kV. The peptide mass fingerprint generated was compared to the masses of all theoretical tryptic peptides generated *in silico* by the MASCOT search program [15]. CID MS/MS was also performed to corroborate the significant matches from the MALDI/MS.

## 3 Results

### 3.1 HPA binding to HT29 and SW480 cells

We used the human colorectal cell lines SW480 and HT29 as an *in vitro* model of metastasis. These represent the extremes of HPA binding: SW480 is HPA negative and HT29 shows intense HPA binding. Subcutaneous implantation of SW480 and HT29 cell pellets between the scapulae of SCID mice shows SW480 to be nonmetastatic and HT29 to be metastatic [16]. Confocal microscopy confirmed that the HPA binding pattern to these cell lines was the same as previously observed (Fig. 1), this analysis is particularly important as glycosylation changes can occur as a result of *in vitro* cell culture. The confocal analysis also enabled detailed localization of HPA binding; HPA bound weakly to SW480 with diffuse cytoplasmic staining pattern and no detectable cell membrane staining, in contrast, HT29 showed intense membrane and granular cytoplasmic staining as described elsewhere [16]. The lectin binding to the HT29 cells was almost entirely inhibited when HPA was preincubated with GalNAc (at concentrations of 50 mM or above) prior to probing the cancer cells (Fig. 1).



**Figure 1.** Confocal images showing binding of TRITC-labeled HPA (red) to SW480 and HT29 cells. The nuclei were counterstained with ToPro-3 (blue). HT29 showed intense lectin binding on the cell surface with some granular intracellular staining. There is almost no membrane or intracellular binding of HPA to SW480. The binding of HPA to HT29 cells was inhibited when the lectin was preincubated with GalNAc at concentrations of 50 mM or above. Inset panel shows HPA-Western blot analysis of 20 µg proteins prepared by ultracentrifugation. The pellet fraction of HT29 shows intense HPA binding confirming enrichment of membrane proteins using this system.



### 3.2 HPA interaction with HT29 and SW480 membrane proteins

HPA binding membrane glycoproteins from HT29 and SW480 cells were obtained by HPA affinity chromatography and analyzed by SDS-PAGE. This approach revealed four weakly staining protein bands in SW480 and 16 discrete Coomassie staining protein bands in HT29 (Fig. 2, panel A). Some proteins were represented in both cell lines whereas others did not appear in SW480 and are assumed to be either specific to, or more highly represented in, HT29. The data supports the hypothesis that the development of a metastatic phenotype is accompanied by alterations in glycosylation on a range of proteins, in this case detected using the lectin HPA.

### 3.3 Inhibition studies

HT29 membrane proteins were separated by SDS-PAGE, blotted to nitrocellulose and probed with HPA-biotin. Again 16 protein bands were observed (Fig. 2, panel B) although some of the protein bands only faintly visible in the CBB stained gel were the most dominant HPA binding proteins on the Western blot and we assume that these are heavily glycosylated proteins [17]. A negative control in which HPA-biotin was omitted was run and the results of this analysis concurred with previous reports of nonspecific binding of streptavidin to endogenous cellular proteins (Fig. 2, panel B) [18].

To determine the monosaccharide specificity of HPA we preincubated the lectin for 30 min in a buffer containing between 50 and 250 mM GalNAc, GlcNAc, or sialic acid before probing the Western blots, the data for the 250 mM solutions are shown (Fig. 2, panel B). GalNAc, GlcNAc, and sialic acid all inhibited binding of HPA to the predominant polypeptide of 130 kDa as well as to the 80 kDa species. GalNAc was a slightly more effective inhibitor than GlcNAc but, intriguingly, incubation with sialic acid at this con-

centration abrogated almost all HPA binding; as well as some of the nonspecific streptavidin-binding (Fig. 2, panel B). These results confirm that the interaction between HPA and the cancer cell glycoproteins is *via* a lectin-mediated interaction that can be inhibited using GalNAc, GlcNAc, and/or sialic acid.

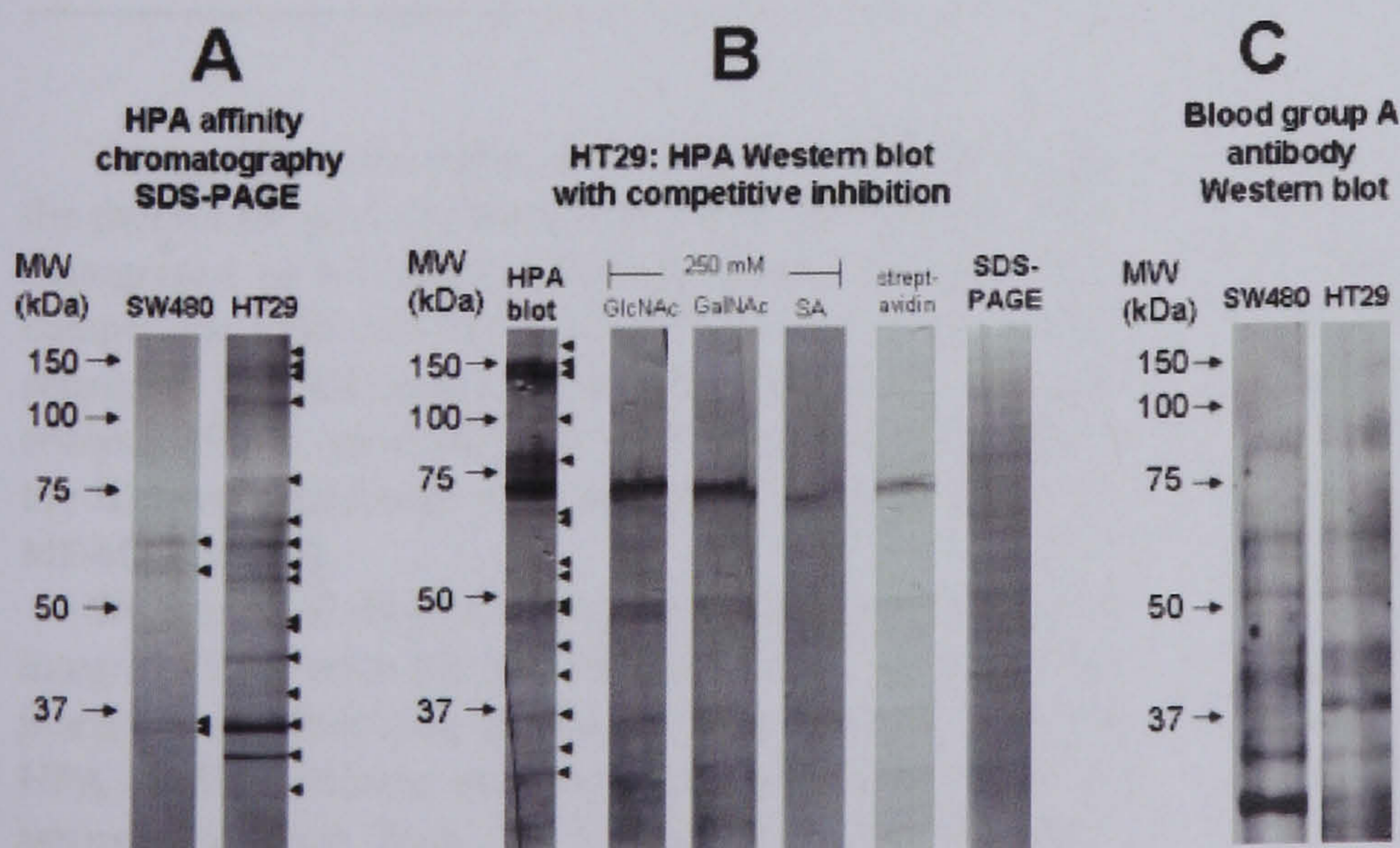
### 3.4 Antiblood group A antibody

SW480 and HT29 membrane proteins were separated by SDS-PAGE, blotted to nitrocellulose and probed with an antihuman blood group A antibody. The predominant 130 kDa protein species observed to bind HPA in HT29 did not interact with the antibody (Fig. 2C). In contrast, a range of lower molecular weight protein species (from 26 to 80 kDa) bound to the antibody in SW480 and HT29 but these proteins either did not, or only weakly, bound to HPA. In both HT29 and SW480 the same amount of protein (20 µg) was loaded onto the gel and transferred to nitrocellulose, nevertheless, SW480 membrane proteins bound more intensely to the antibody compared with HT29 membrane proteins, suggesting that the HPA binding epitope in HT29 cells encompasses glycoconjugates containing glycans other than the blood group A antigen.

The HPA binding proteins from SW480 and HT29 separated by 1-dimensional and 2-dimensional SDS-PAGE are shown in Table 1.

### 3.5 Proteome analysis of HPA interaction with HT29

To obtain an overview of the HPA binding proteins on HT29 cells, membrane proteins were separated by 2-DE and stained with Sypro Ruby. A representative gel is shown in Fig. 3, panel A. The reproducibility of the system was investigated using replicate gels as described in the materials and methods and the boxed areas shown in Fig. 3 (panel C, I, II, and III) illustrate the robustness of the system for both the high and low abundance proteins.



**Figure 2.** 1-D SDS-PAGE separation of SW480 and HT29 membrane glycoproteins (20 µg loading throughout). Panel A: affinity purified HPA binding proteins from SW480 and HT29 stained with CBB. Panel B: Western blot of total HT29 membrane proteins incubated with HPA-biotin, visualized using streptavidin-HRP and DAB/H<sub>2</sub>O<sub>2</sub>. Competitive inhibition of HPA preincubated with 250 mM GlcNAc, GalNAc, or sialic acid (SA), SDS-PAGE analysis of HT29 proteins stained with CBB. Panel C: Western blot of SW480 and HT29 membrane proteins probed with antibody group A antibodies, visualized using West Signal Dura chemiluminescence system.



**Table 1.** Relative intensity and molecular weight of protein species identified by SDS-PAGE and Western blot analysis, shown in Fig. 2

Relative MW (kDa)	RF value	SDS-PAGE analysis of HPA affinity chromatography		HPA Western blot HT29 cells				SDS-PAGE analysis of HT29 membrane proteins	Blood group A antibody	
		HT29	SW480	HPA alone	GalNAc (250 mM)	GlcNAc (250 mM)	Sialic acid (250 mM)		HT29	SW480
141	0.04	8.64		4.29			4.29	1.67		
133	0.06	12.24							3.60	
108	0.08	12.90		0.53			0.53	2.97		
89	0.13	2.26		0.53			0.53	5.33		7.26
78	0.18	4.18		6.78	6.22	1.33	6.78	2.81	2.92	
77	0.20							1.15		
73	0.24			16.07	1.70	11.51	16.07	2.55	7.59	7.24
68	0.27			2.54	1.36	2.27	2.54			
67	0.28	4.75		0.35	0.29	0.56	0.35	4.01		
62	0.31			0.11	0.16	0.28	0.11	4.04		
62	0.34			2.85	2.50	1.70	2.85			
62	0.35							0.94		
62	0.36	6.28						2.13		
59	0.39							2.60		
58	0.41	2.00	17.63			18.67		1.20	11.40	8.31
58	0.44							8.29	15.78	
57	0.46		16.11	5.13	3.44		5.13			2.14
55	0.48	6.50		8.46	3.90		8.46	4.65		
49	0.53				13.67	1.65	26.88	1.37	6.13	7.90
48	0.56	3.51		27.85	13.00	18.63	0.98	3.06		1.91
47	0.57	5.68						4.98		
43	0.63	5.67						5.72	10.25	4.63
40	0.66							11.40		
40	0.67							0.67	12.25	5.84
39	0.70	4.82						2.11		16.46
36	0.76		24.55	13.24	19.16	27.85	13.24	22.28	8.61	2.99
35	0.77	11.18	41.72							
35	0.78									2.44
34	0.80									
33	0.82	5.29		10.04					5.30	11.68
32	0.84			10.41				0.66		
31	0.86			0.62	33.47	6.48	0.62			
30	0.87			10.66			10.66			
29	0.89	4.10		10.78	1.14			3.36		
28	0.93								8.73	21.20
27	0.97	8.64							7.44	

To investigate the HPA binding proteins of HT29 cells, the membrane proteins were purified by HPA affinity chromatography in a buffer containing CHAPS and eluted by competition with 0.25 M GlcNAc. The eluted proteins were separated by 2-DE and stained with CBB to facilitate MS analysis (Fig. 3, panel B). The protein species that showed the highest abundance were subjected to MALDI-MS and MS-MS (Table 2).

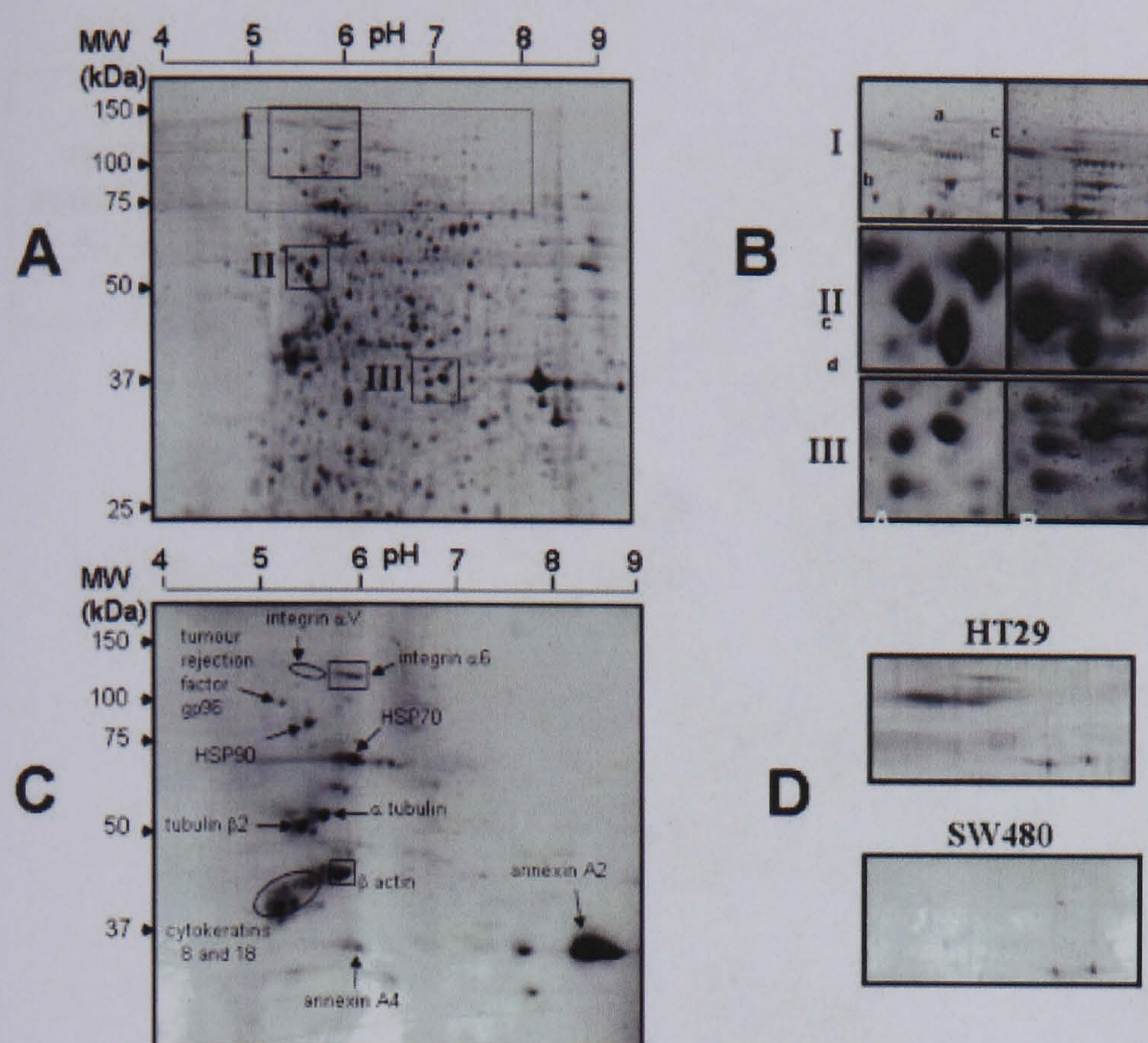
We identified the HPA binding proteins as integrin  $\alpha 6$ , integrin  $\alpha V$ , annexin A2, and annexin A4. We also identified  $\beta$ -actin,  $\alpha$  and  $\beta$ tubulin, cytokeratin 8, cytokeratin 18 in the HPA binding fraction and these cytoplasmic proteins are assumed to elute from the HPA-affinity chromatography

column as protein complexes with the cell membrane proteins. The presence of Hsp96, Hsp90, and Hsp70 as well as TNFR-1 (although with a weaker MASCOT score) suggests that HPA also recognizes proteins involved in antiapoptotic pathways (Hsp70, Hsp90).

#### 4 Discussion

In this study we used a well characterized model of human colorectal cancer cell lines with defined *in vivo* behaviors and lectin binding properties to discern the glycoproteins that bind to the lectin HPA. In the first instance we found that





**Figure 3.** 2-DE separation of HT29 membrane glycoproteins (90 $\mu$ g). Panel A: HT29 membrane proteins stained with Sypro Ruby. Panel B: zoom on boxed areas indicated as I, II, and III, from panel A. Two replicates are shown for each area I, II, and III and illustrate the reproducibility of the 2-DE separation system. Panel C: HT29 membrane proteins prefractionated by HPA-affinity chromatography, stained with CBB. The proteins identified by MS analysis are shown with arrows on the 2-DE gel. Panel D: Western blot of 2-DE separated membrane proteins from HT29 and SW480, the blots were probed with HPA. The area of the 2-DE Western blot corresponds to the boxed area indicated by the dashed line in panel A.

**Table 2.** MS analysis of HT29 membrane proteins, prefractionated by HPA affinity chromatography and separated by 2-DE

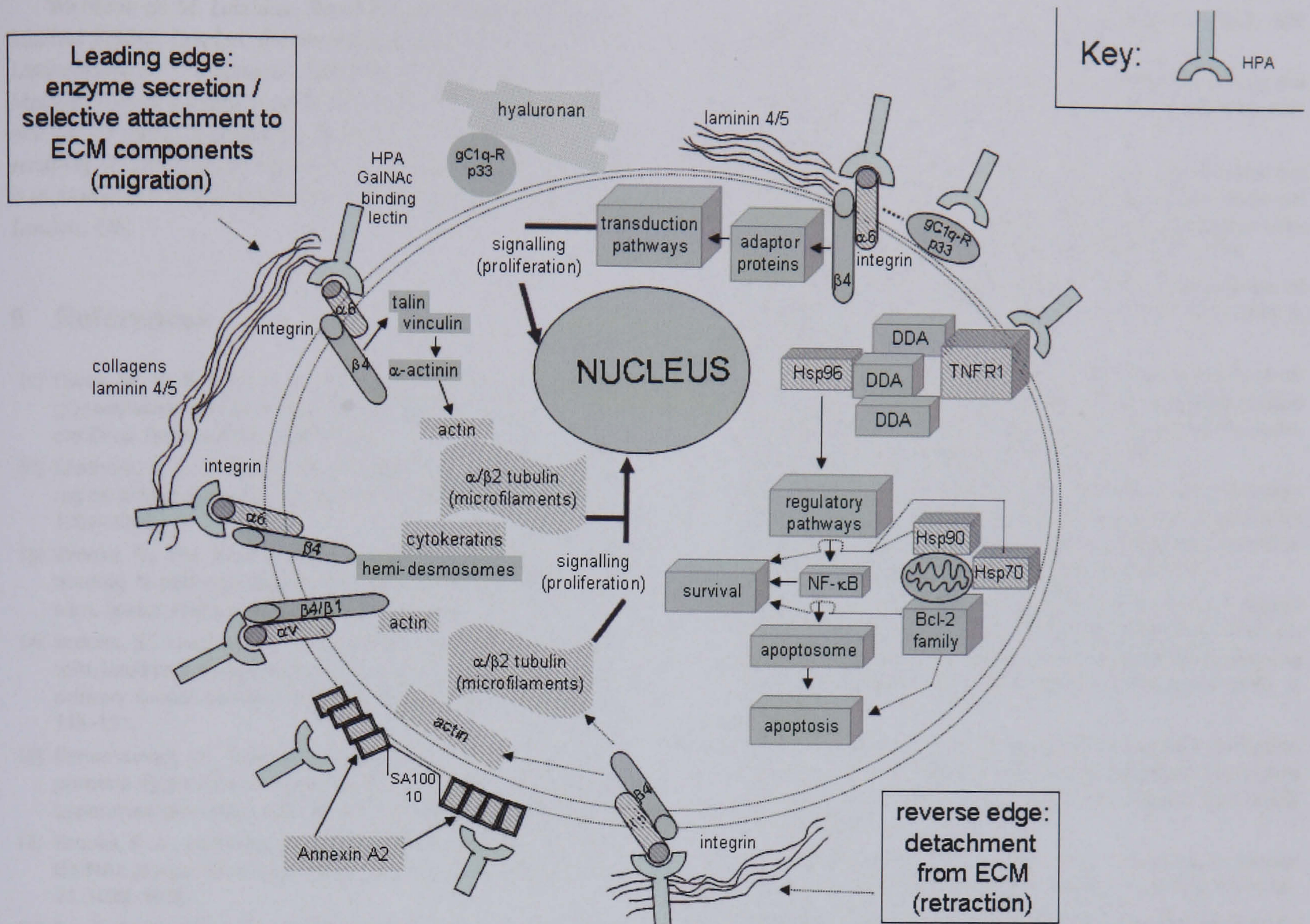
Protein name	Accession number	MASCOT score	Nominal mass (kDa)	Predicted pI	Sequence coverage (%)
Integrin alpha 6	gi 5726563	389	120.6	6.44	19
Integrin alpha 6	gi 5726563	599	120.6	6.35	25
Cytokeratin 8	gi 181573	758	53.3	5.5	45
Cytokeratin 8	gi 181573	758	53.3	5.5	43
Integrin alpha V	gi 4504763	431	117	5.45	14
Tumor rejection antigen gp 96	gi 4507677	166	92.7	4.76	9
Heat shock (90 kDa) protein	gi 306891	348	83.5	4.97	20
Cytokeratin 18	gi 30311	127	47.3	5.27	19
Cytokeratin 18	gi 30311	127	47.3	5.27	15
Annexin A2 isoform 2	gi 4757756	1010	38.8	7.57	62
Guanine nucleotide-binding protein beta 2 subunit	gi 20357529	470	38	5.6	36
Heat shock protein 8 isoform 1	gi 5729877344	344	71	5.37	14
Alpha tubulin	gi 340021662	662	50.8	4.94	30
Tubulin beta 2	gi 20809886419	419	50.2	4.76	22
Beta actin	gi 4501885	511	42	5.29	26
Annexin A4	gi 1703319	563	36	5.9	32

The table shows the protein identity, accession number, MASCOT score, nominal mass, predicted pI, and sequence coverage following MS analysis.

high-resolution confocal microscopy in conjunction with 3-D modeling allowed location of the HPA binding epitopes to the surface of the metastatic cancer cell line (HT29). Membrane proteins were enriched by high-speed centrifugation followed by solubilization in the presence of CHAPs and subsequent affinity purification on an HPA affinity chroma-

tography column. In this manner, sufficient HPA binding membrane proteins were obtained to allow analysis using 1-D and 2-D SDS-PAGE. The comparative analysis showed considerable differences between the HPA binding protein species in the two cell lines. Inhibition experiments showed that the interaction between HPA and the proteins was





**Figure 4.** Schematic diagram showing HT29 HPA binding proteins. The proteins identified in this study are shown in the hatched boxes and the putative binding partners are shown in the solid boxes.

indeed *via* a lectin-like interaction that could be inhibited with both GalNAc and GlcNAc. Somewhat surprisingly, sialic acid, also inhibited interaction of the lectin with the glycoproteins and this supports the idea that HPA recognizes a sialylated epitope [7, 8]. Indeed, metastatic cancer cells which bind HPA adhere to activated endothelia, presumably *via* the sialic acid recognizing cell adhesion molecules Selectins [19]. Analysis using an antibody against blood group A has shown that the glycan epitopes that are recognized by HPA includes the blood group antigens but also a range of other protein species, presumably exhibiting different glycosylation, the detailed analysis of these is being undertaken in our laboratories.

We used proteomic analysis to unravel the glycoproteins recognized by HPA in the metastatic HT29 cancer cells. The proteins that eluted in the HPA binding affinity chromatography fraction included molecules involved in cell adhesion and migration (integrin  $\alpha 6$ , integrin  $\alpha V$ , annexins) [20] remodeling (filament proteins including  $\alpha$  tubulin,  $\beta$  tubulin, cytokeratins, actin) [21] and antiapoptotic pathways (Hsp-70, Hsp-90, Hsp-96, and TNFR-1) [22]. Some of the proteins

are present because they bind directly with HPA and others are found as complexes with HPA binding proteins, residing in the cytoplasm and therefore nonglycosylated or glycosylated with O-GlcNAc. In other systems, Hsp-70, actin, and tubulin have been shown to be modified by the attachment of O-GlcNAc (refs. [23–25], respectively); this may be the case in HT29 and HPA may recognize these proteins by virtue of O-GlcNAcylation but further work would be required to support this hypothesis. To piece together the results of the proteomic analysis, the proteins identified in this study and their role(s) as described extensively in the literature in relation to cancer metastasis, are shown schematically in Fig. 4.

Whilst many of these proteins have previously been described in cancer [21, 22, 26] we are not aware of any reagent – other than HPA – which will bind these proteins simultaneously. HPA may recognize these proteins by virtue of changes in the normal glycan repertoire and the data obtained using the antibody against blood group A supports this hypothesis. Further work is being undertaken to elucidate the way in which HPA interacts with the proteins identified in this study.



We thank Dr M. Loizidou, Royal Free and University College Medical School, London, for providing the cell lines and Dr A. Leathem and Dr J Chowaniec, Royal Free and University College Medical School, London and Dr M. Odell, University of Westminster for useful discussions. We thank Dr M. Kerrigan, University of Westminster, for help with the confocal microscopy. JSG is in receipt of a Scholarship from the University of Westminster, London, UK.

## 5 References

- [1] Dwek, M. V., Brooks, S. A., Harnessing changes in cellular glycosylation in new cancer treatment strategies. *Curr. Cancer Drug Targets* 2004, 4, 425–442.
- [2] Leathem, A. J., Brooks, S. A., Predictive value of lectin binding on breast-cancer recurrence and survival. *Lancet* 1987, 1, 1054–1056.
- [3] Brooks, S., The involvement of Helix pomatia lectin (HPA) binding N acetylgalactosamine glycans in cancer progression. *Histol. Histopathol.* 2000, 15, 143–158.
- [4] Brooks, S., Leathem, A., Expression of N-acetyl galactosaminylated and sialylated glycans by metastases arising from primary breast cancer. *Invasion Metastasis* 1998–1999, 18, 115–121.
- [5] Schumacher, U., Brooks, S., Mester, J., The lectin Helix pomatia agglutinin as a marker of metastases – clinical and experimental studies. *Anticancer Res.* 2005, 25, 1829–1830.
- [6] Brooks, S. A., Leathem, A. J. C., Altered expression of alpha-GalNAc glycoproteins by breast cancers. *Br. J. Cancer* 1995, 71, 1033–1038.
- [7] Dwek, M. V., Ross, H. A., Streets, A. J., Brooks, S. A. *et al.* Helix pomatia agglutinin lectin-binding oligosaccharides of aggressive breast cancer. *Int. J. Cancer* 2001, 95, 79–85.
- [8] Fenlon, S. *et al.*, Helix pomatia and Ulex europeus lectin binding in human breast carcinoma. *J. Pathol.* 1987, 152, 169–176.
- [9] Chen, Y. *et al.*, Use of sialylated or sulfated derivatives and acrylamide copolymers of Gal beta 1, 3 GalNAc alpha- and GalNAc alpha- to determine the specificities of blood group T- and Tn-specific lectins and the copolymers to measure anti-T and anti-Tn antibody levels in cancer patients. *Glycoconj. J.* 1995, 12, 55–62.
- [10] Piller, V., Piller, F., Cartron, J., Comparison of the carbohydrate-binding specificities of seven N-acetyl-D-galactosamine-recognizing lectins. *Eur. J. Biochem.* 1990, 191, 461–466.
- [11] Lehner, I., Niehof, M., Borlak, J., An optimized method for the isolation and identification of membrane proteins. *Electrophoresis* 2003, 24, 1795–1808.
- [12] Brooks, S., Wilkinson, D., Validation of a simple avidin-biotin detection method for Helix pomatia lectin (HPA) binding as a prognostic marker in cancer. *Acta Histochem.* 2003, 105, 205–212.
- [13] Laemmli, U. K., Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 1970, 227, 680.
- [14] Gorg, A. *et al.*, Recent developments in two-dimensional gel electrophoresis with immobilized pH gradients: Wide pH gradients up to pH 12, longer separation distances and simplified procedures. *Electrophoresis* 1999, 20, 712–717.
- [15] Pappin, D., Hojrup, P., Bleasby, A., Rapid identification of proteins by peptide mass fingerprinting. *Curr. Biol.* 1993, 3, 327–332.
- [16] Schumacher, U., Adam, E., Lectin histochemical HPA-binding pattern of human breast and colon cancers is associated with metastases formation in severe combined immunodeficient mice. *Histochem. J.* 1997, 29, 677–684.
- [17] Fountoulakis, M., Juranville, J., Manneberg, M., Comparison of the CBB, bicinchoninic and Lowry quantitation assays, using non-glycosylated and glycosylated proteins. *J. Biochem. Biophys. Methods* 1992, 24, 265–274.
- [18] Banks, R. E. *et al.*, Use of a sensitive EnVision +-based detection system for Western blotting: Avoidance of streptavidin binding to endogenous biotin and biotin-containing proteins in kidney and other tissues. *Proteomics* 1993, 3, 558–561.
- [19] Valentiner, U. *et al.*, HPA binding and metastasis formation of human breast cancer cell lines transplanted into severe combined immunodeficient (scid) mice. *Cancer Lett.* 2005, 219, 233–242.
- [20] Juliano, R. L., Varner, J. A., Adhesion molecules in cancer: The role of integrins. *Curr. Opin. Cell Biol.* 1983, 5, 812–818.
- [21] Prasad, S. *et al.*, Intermediate filament proteins during carcinogenesis and apoptosis (review). *Int. J. Oncol.* 1999, 14, 563–570.
- [22] Ciocca, D., Calderwood, S., Heat shock proteins in cancer: Diagnostic, prognostic, predictive, and treatment implications. *Cell Stress Chaperones* 2005, 10, 86–103.
- [23] Guinez, C., Lemoine, J., Michalski, J. C., Lefebvre, T., 70-kDa-heat shock protein presents an adjustable lectinic activity towards O-linked N-acetylglucosamine. *Biochem. Biophys. Res. Commun.* 2004, 319, 21–26.
- [24] Sgantzos M. N. *et al.*, Expression of the O-linked N-acetylglucosamine containing epitope H in normal myometrium and uterine smooth muscle cell tumors. *Pathol. Res. Pract.* 2007, 203, 31–37.
- [25] Walgren J. L., *et al.*, High glucose and insulin promote O-GlcNAc modification of proteins, including alpha-tubulin. *Am. J. Physiol. Endocrinol. Metab.* 2003, 284, E424–E434.
- [26] Prokopishyn, N. *et al.*, Integrin alpha3beta1 expressed by human colon cancer cells is a major carrier of oncogene-developmental carbohydrate epitopes. *J. Cell. Biochem.* 1999, 72, 189–209.