CAUSES AND CONSEQUENCES OF ALTERED *O*-GLYCOSYLATION IN COLON EPITHELIAL CELLS

Thesis submitted in accordance with the requirements of the University of Liverpool for the degree of Doctor in Philosophy by

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August 2008

UNIVERSITY OF LIVERPOOL

DECLARATION

All the techniques and experiments performed and described in this thesis were undertaken by myself as a PhD student between October 2004 and October 2007, with the exception of the flow cytometry which was carried out by Miss Monica Barclay, the confocal microscopy was carried out with the assistance of Dr Spiller and mass spectrometry by Dr Deborah Ward and Professor Anne Dell.

Neither this thesis nor any part of it has been submitted in support of an application of another degree or qualification of this or any other University or other institute of learning.

Benjamin Armstrong Tam 2008

Dedicated to my family, Ailie and Palms

ACKNOWLEDGEMENTS

I would like to express my sincere thanks to Professor Jonathan Rhodes and Dr Lu-Gang Yu for their continued guidance and support and whose energy and ideas made this work possible. I would also like to thank the entire Gastoenterology research unit for the enjoyable time I spent in the department and for the contributions and daily advice in the laboratory. Special mention must go to Dr Barry Campbell for his expertise in glycobiology and help with the bacteria/Golgi experiments and Dr Sree Subramanian who first introduced me to laboratory techniques and practices. I must also thank Dr Deborah Ward in the School of Biosciences for her advice and time to help me with the mass spectrometry of samples and Professor Anne Dell at Imperial College London for the mass spectrometry of the Orp150 samples. My thanks also go to Dr David Spiller in the School of Biosciences who advised and supervised my time on the confocal microscope.

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ABBREVIATIONS

α2,3ST1	α-2,3-sialyltransferase
ACN	Acetonitrile
ABL	*Agaricus bisporus* mushroom lectin
Ago	Argonaute
Ambic	Ammonium Bicarbonate
Asn	asparagines
C2GnT1	core 2 N-acetylglucosaminyltransferase
CD	Crohn's disease
Core 1 GalT	Core 1 ß1-3 glycosyltransferase
CRC	colorectal cancer
Dcr-2	Dicer enzyme
DMSO	Dimethylsulphoxide
DTT	Dithiothreitol
ER	endoplasmic reticulum
FCS	Fetal calf serum
FITC	Flurescein isothiocynate
GalNAc	N-acetylgalactosamine
GalNAcT	GalNAc transferase
GalT	glycosyltransferase
GDI	glycosyl phosphatidyl inositol
HSP	hexosamine biosynthetic pathway
IBB	importin-β binding domain
IBD	inflammatory bowel disease
IL-8	inter leukin 8
kDa	kilodalton
MAPK	mitogen- activated protein kinase
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
NFκB	nuclear factor kappa B pathway
NPC	nuclear pore complex
O-GlcNAcase	ß-N-acetylglucosaminidase
OGT	O-GlcNAc transferase
Orp150	oxygen regulated protein 150
OST	oligosaccharyltransferases
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PMA	phorbol 12-myristate 13-acetate
PNA	peanut lectin
dsRNA	double stranded RNA
mRNA	messenger RNA
siRNA	silencing RNA
RNAi	RNA interference
RISC	siRNA-induced silencing complex
RLC	RISC loading complex
SD	standard deveation
SDS	sodium dodecyl sulphate
Ser	serine
S-Tn	Sialyl-Tn antigen

T antigen	Galß1-3GalNAcα-Ser/Thr disaccharide/ Thomsen-Freidenreich antigen
Tn antigen	Galß1-3GalNAcα-Ser/Thr disaccharide
TCβ1	T-cell receptor beta chain
Thr	Threonine
TEMED	Tetra-methyl-1,2-diamino-ethane
UC	ulcerative colitis
UPR	Unfolded protein response
V-ATPase	vacuolar ATPases
VEGF	vascular endothelial growth factor
VVA	*Vicia villosa* (hairy vetch seeds) Tn binding lectin
WAS	Wiscott Aldrich syndrome
WGA	Wheat germ agglutin

ABSTRACT

Changes of cellular glycosylation, such as the increased expression of the oncofetal Thomsen-Freidenreich antigen (galactoseß1-3*N*-acetylgalactosamineα-,TF), are common in cancer and pre-cancerous conditions. The glycosylation changes observed in colon cancer can be induced by prevention of the normal acidification of the Golgi in colonocytes probably through fragmentation of the Golgi apparatus. This might in turn be triggered by bacterial-epithelial interactions or by consequential inflammation. It seems highly likely that the altered mucosal glycosylation seen in the inflamed, pre-malignant and malignant epithelium leads to recruitment of 'non-pathogenic' bacteria. It would also result in increased recruitment of dietary TF-binding lectins. The edible mushroom (*Agaricus bisporus)* lectin (ABL), that binds TF and sialyl-TF, has an anti-proliferative effect that relates to its internalisation and inhibition of NLS-dependent nuclear protein import mediated via its interaction with an N-terminally truncated form of the stress glycoprotein Orp150. This thesis sets out to characterise Orp150 glycosylation and investigate further the role of Orp150 in nuclear protein import, and to observe whether bacteria alter mucosal glycosylation by inducing a disorganisation of the Golgi apparatus.

In this study, immunoblotting of protein extracts from HT29 colon cancer cells showed the presence of two Orp150 isoforms of which the lower molecular weight isoform is stress-inducible by glucose starvation; selective membrane permeabilisation with digitonin showed release of a constitutive Orp150 isoform in the released cytoplasm. Lectin affinity purification of protein extracts from a glucose-starved cell preparation with Jacalin yielded selective extraction of the constitutive Orp150 form suggesting differential glycosylation of the two Orp150 isoforms. siRNAOrp150 treatment of HT29 cells resulted in 63±5% reduction of Orp150 expression and subsequently 61% reduction (% nuclear fluorescence, without heat stress 46±4, after heat stress 58±3, siOrp150 + heat stress 51±3, $P<0.0001$) of the nuclear translocation of Hsp70 in response to heat stress, thus confirming a role for Orp150 in nuclear localisation sequence (NLS)-dependent nuclear protein import.

The functional importance of sialyl-TF on Orp150 was investigated by utilising known *O*-glycosylation inhibitors: 5-CDP, benzyl-GalNAc, TNFα and bafilomycin or siRNAGalT treatments to alter TF expression. The consequent glycosylation alterations were not seen to alter NLS-dependent nuclear protein import. A number of other possible cytoplasmic proteins expressing sialyl TF were identified through ABL lectin affinity purification and mass spectrometry. Further investigation demonstrated the possibility of Hsp90 also expressing sialyl-TF.

Bacterial supernatant from *E.coli* HM427 isolated from a Crohn's disease patient was shown to cause fragmentation of the Golgi in HT-29 MTX goblet cells (differentiated colon cancer cells) after 10 days incubation, increasing the average number of Golgi fragments per nuclei from 2.7±0.5 to 4.5±0.6 ($p<0.01$), as observed by immunohistochemical staining with a *cis*-Golgi structural protein Giantin. The supernatant also increased TF expression as measured by PNA binding density from 1400±300 ADU in control to 2300±200 ADU ($p<0.01$) after 10 days incubation.

This is one of only 2 reports so far of a cytoplasmic protein expressing sialyl-TF and could be of significant importance in the understanding *O*-glycosylation pathways. The finding in this study of Golgi fragmentation in response to bacterial supernatant is an exciting phenomenon which could be an important component in the complex interactions between bacteria and the host epithelium and consequent aberrant glycosylation.

CHAPTER 1

INTRODUCTION

Glycans are abundant in nature and the field of glycobiology, which has grown rapidly in the past 20 years due to advances in technology has not only given insights into the vast number of enzymes and proteins that are devoted to the mechanism of glycosylation, but also the emerging biological role of these glycans. It is now known that glycans are important in a large and diverse range of functions and this is highlighted by the growing number of human diseases that are the result of defective glycan assembly. Aberrant epithelial glycosylation is a common consequence of disease and this has been clearly demonstrated in colon cancer with the increased expression of certain truncated glycans.

This thesis examines the importance of glycosylation in human colon epithelial cells with particular attention to the oncofetal Thomsen-Freidenreich antigen. This antigen in its sialylated form has been demonstrated to be expressed on a cytoplasmic protein which has been implicated in protein transport into the nucleus. This thesis will investigate the function of this antigen and investigate whether other proteins present in the cytoplasm also express the TF antigen. The Golgi apparatus is at the centre of the glycosylation process and this thesis will finally investigate relationship between the structure of the Golgi and aberrant glycosylation.

1.1 Glycoproteins

Glycoproteins are proteins that have carbohydrate covalently attached to their peptide portion. They are widely distributed throughout animal, bacterial and plant kingdoms and are involved in a broad range of processes and functions. More than half of all proteins are glycosylated in eukaryote biological systems (Van den Steen et al.

1998). The predominant type of posttranslational modification which involves carbohydrates is the most heavily studied *N*-glycosylation, followed by *O*-glycosylation.

1.2 *N*-glycosylation

N-glycans are initiated by a series of stepwise enzyme-catalysed reactions in various localised cellular compartments. This begins with the transfer of a lipid linked oligosaccharide precursor $Glc_3Man_9GlcNAc_2$ –P-P-Dol to the endoplasmic reticulum (ER) (Kornfeld et al. 1985). The formation of complex carbohydrate structures is initiated by the addition of 2 core GlcNAc monosaccharides proceeded by 5 mannose residues for which the donors are UDP-GlcNAc and GDP-Man (Abeijon et al. 1992). The $Man_5GlcNAc_2$-Dol then flips across the lipid bilayer to become positioned in the ER lumen where a further 4 mannoses and 3 glucose residues are added to give rise to a complex oligosaccharide that can be transferred from the lipid precursor to the nascent polypeptide chain by oligosaccharyltransferase (OST), this process is described in figure 1.1.

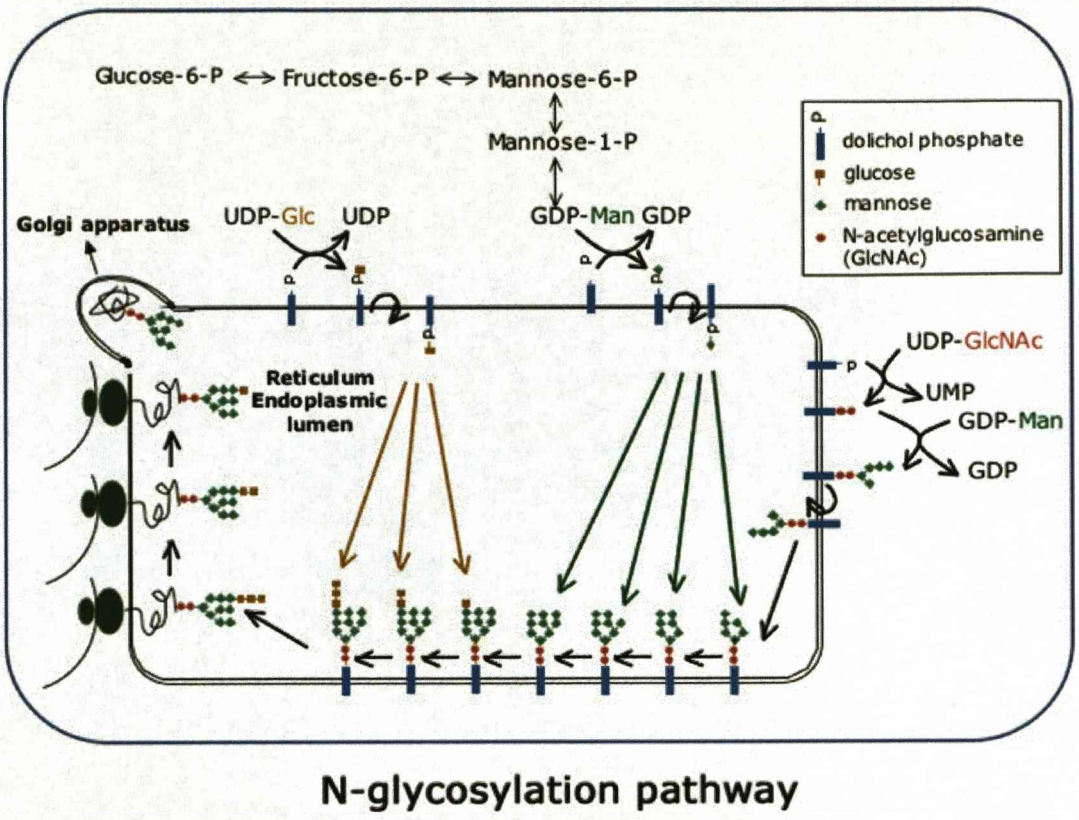


Figure 1.1: The *N*-glycosylation pathway (Adapted from www.icp.ucl.ac.be)

Attachment occurs at the site Asn-X-Thr/Ser, where X is any amino acid except proline. Modification of the attached oligosaccharide then occurs with glucosidases I and II acting first on the protein-linked oligosaccharide precursor to remove all three glucoses sequentially. These glycosidases are present in the lumen of the ER with glucosidase I acting specifically on the single α1-2-linked terminal glucose. The three glucose residues are cleaved sequentially, in a process conserved in eukaryotes and now known to be crucial in protein folding. The *N*-glycans then become available to enzymes in the Golgi and ER which are responsible for the formation of three main oligosaccharide types, as shown in figure 1.2.

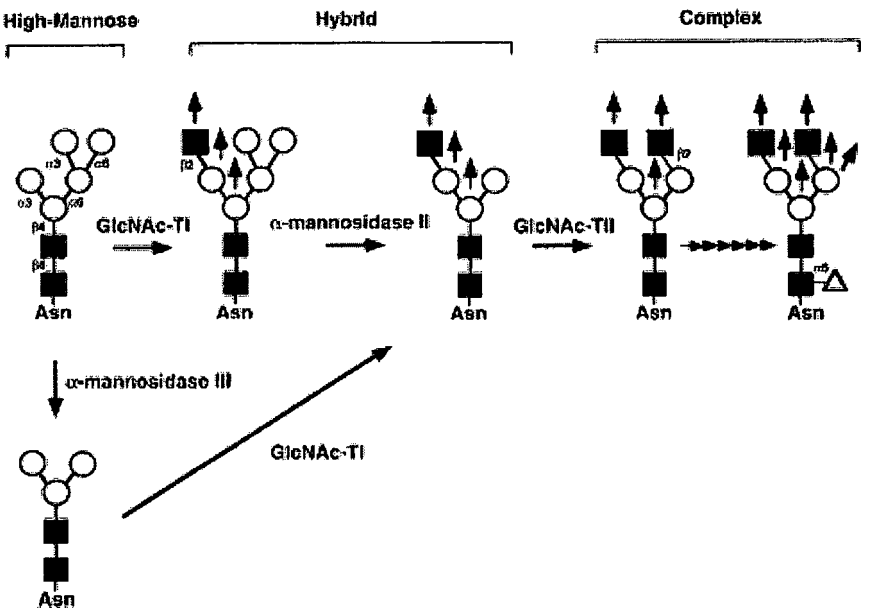


Figure 1.2: Diversification of *N*-glycans

(Adapted from Essentials of Glycobiology, Varki et al 1999)

The structures share the same pentasaccaride core but differ in the type and sequence of sugars in the branches which are altered by the action of mannosidases or glycosyltransferases. The high mannose type has only mannose sugars on the branches, hybrid types have GlcNAc substituted on one branch with mannose on the other, while complex types have both branches substituted with GlcNAc. These differing core structures bestow specificity, allowing only certain enzymes to act upon them and thus leading to the enormous amount of diversity in *N*-glycans. Protein may also be glycosylated differently at other sites, this is termed microheterogeneity.

1.3 *O*-glycosylation

1.3.1 Mucin type *O*-glycosylation: addition of GalNAc

The initial step in *O*-glycosylation is simpler than that for *N*-glycosylation although the glycans produced probably have greater variation in linkage and

substitution; it is a posttranslational modification in which the monosaccharide GalNAc is added from a nucleotide donor, UDP-GalNAc, to the serine or threonine residues of the fully folded and assembled protein, catalysed by a polypeptide GalNAc transferase (GalNAcT) [Figure 1.3]. This distinguishes it from other types of glycosylation and also other forms of *O*-glycosylation such as *O*-GlcNAc (Hart 1997), *O*-mannose and *O*-fucose (Harris et al. 1993). A family of GalNAcTs are responsible for the addition of GalNAc to the serine and threonine residues. Initially in 1993, Hagen and colleagues cloned the cDNA of bovine GalNAcT (Hagen et al. 1993), then it was shown that other GalNAcTs existed (Sorensen et al. 1995, White et al. 1995), GalNAcTs continue to be identified (Bennett et al. 1996, Clausen et al. 1996) and to date up to 20 members have been identified (Ten Hagen et al. 2003)

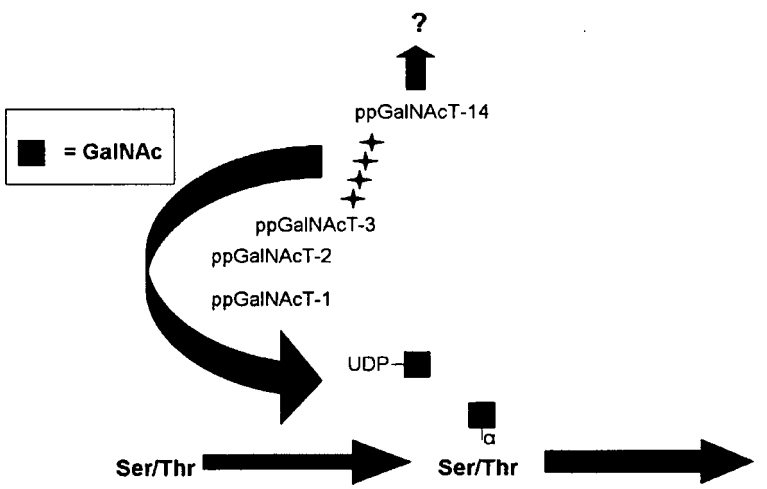


Figure 1.3: Initiation of *O*-glycosylation by a family of GalNAcTs facilitating the addition of GalNAc to serine and threonine residues of folded protein.

It has been shown that the various GalNAcTs are expressed in a tissue-specific manner which changes during development (Roth et al. 1994, Young et al. 2003). An understanding of the reason for the large number and tissue specificities of these GalNAcTs will give an insight in to the functions of *O*-glycans.

The transfer of GalNAc is thought to occur in the Golgi apparatus (Rottger et al. 1998, Storrie et al. 1998), specific regions of the endoplasmic reticulum or an inter ER-Golgi compartment (ERGIC) (Bieberich et al. 2000, de Graffenried et al. 2004, Young 2004). Subsequently, the GalNAc residue is often acted upon by specific transferases in a stepwise fashion to yield several core structures; to date 8 core structures have been identified (Hounsell et al. 1996). The core structures can then be further modified by acetylation, fucosylation, sialylation, sulphation or polylactosamine-extension.

1.3.2 Core structures and T antigen

Core structures are formed after the action of the GalNAcTs; many *O*-glycans contain the Core 1 structure by the addition of galactose in a ß1-3 linkage to GalNAc, which is catalysed by the glycosyltransferase: Core 1 ß1-3 glycosyltransferase (Core 1 GalT). Core 1 GalT has also been shown to be differentially expressed in tissue types. Core 2 *O*-glycans are formed by the addition of GlcNAc to the GalNAc in a ß1-6 linkage and may be elongated in a mono or biantennary form. A further 6 Core structures have been identified, these can be elongated further by competitive action of specific core GalNAcTs (Schachter et al. 1989) [Figure 1.4].

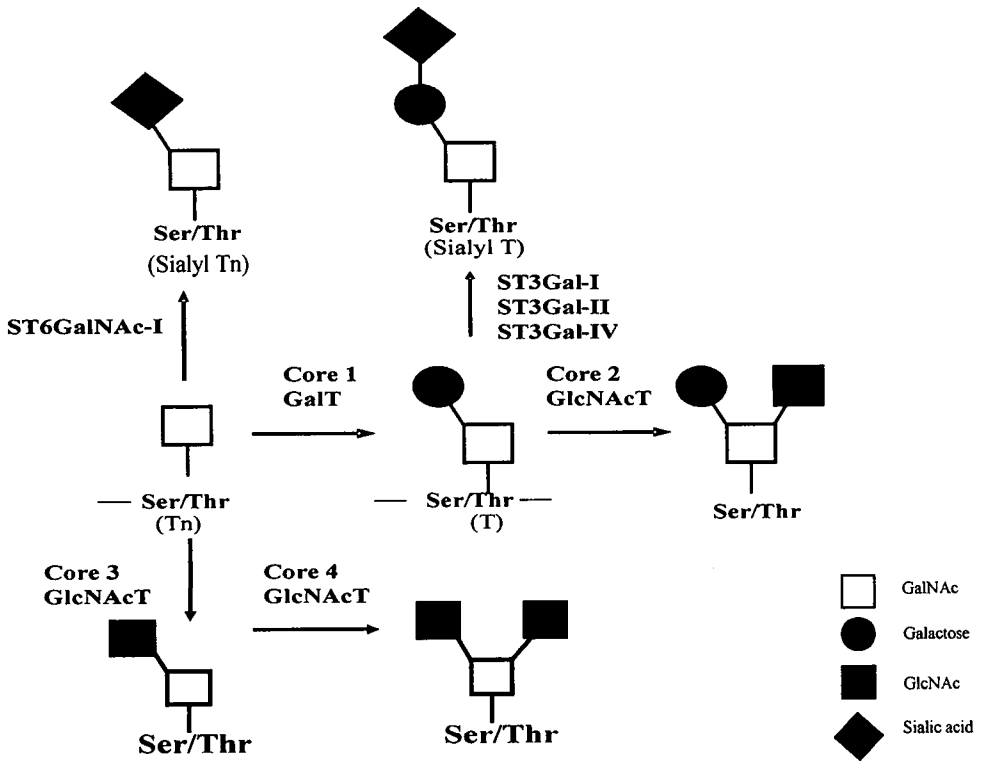


Figure 1.4: Core structures and TF antigens.

O-glycans can also be modified and terminated by sialic acid. The sialyltransferases that are responsible can act after the transfer of GalNAc to the serine /threonine or on the Core 1 structure. The addition of sialic acid restricts further elongation of the structure. The GalNAcα-Ser/Thr structure is called the Tn antigen and the Galß1-3GalNAcα-Ser/Thr disaccharide is called the T or Thomsen-Friedenreich (TF) antigen (Hanisch et al. 1997). These structures are rarely seen under normal circumstances, as they are the acceptors for the glycosyl and sialyl transferases located in the specific areas of the Golgi and ER. They act as oncofetal antigens, which are epitopes expressed only in embryonic tissues and tumour cells. They have been demonstrated to be expressed in a number of cancers including breast and colon (Campbell et al. 1995, Kumar et al. 2005). A list of blood group and related antigens

on *O*-linked cores is presented in table 1.1 and mucin type *O*-glycans in cancer in table 1.2.

1.3.3 Mucins

Mucins are a family of heavily *O*-glycosylated proteins with apparent molecular weights of about 10^7Da. They have distinct peptide domains of functional importance that are Ser/Thr/Pro-rich and contain a variable number of tandem repeat (VNTR) domains that are heavily *O*-glycosylated (Hollingsworth et al. 2004). Mucins form a physical barrier over surface epithelial cells conveying protection, lubrication and transport functions. Mucins are often resistant to proteases, especially in the heavily *O*-glycosylated regions. The most likely reason is that attached carbohydrate blocks access to the peptide core as the same sequences are susceptible in the absence of the carbohydrate (Kozarsky et al. 1988). Currently there are 20 genes that encode mucin type proteins (Ali et al. 2007) that are expressed by epithelial cells in the gastrointestinal, tracheobronchial and reproductive tracts. Their biophysical properties are due to the extensive *O*-glycosylation of the serine- and threonine-rich tandem repeat domains and each mucin appears to have its own unique repeat sequence. The increased expression of MUC1 has been demonstrated in many carcinomas (Denda-Nagai et al. 2000). Salivary oral mucins have an important role in oral health by protecting the teeth by acting as protective barrier, but also have the ability to act as a microbial acceptor thus adding further protection (Moniaux et al. 2001). It has also been shown that MUC5B secreted in the saliva is a key molecule in the initial interaction between host and invading pathogens in the mouth (Thomsson et al. 2005).

Blood group and related antigens on O-linked cores	
TF(Thomsen Friedenreich antigen)	Galβ1-3GalNAcα1-Ser/Thr
Disialyl TF	NeuAcα2-3 Galβ1-3 GalNAcα1-Ser/Thr α2,6 NeuAc
Sialyl T n	NeuAcα2-6GalNAcαl-Ser/Thr
Tn	GalNAcα 1-Ser/Thr
Type 1 backbone	(Gal/β1-3GlcNAcβ1-3/6)n(Galβ1-3)GalNAcα1-Ser/Thr
Type 2 backbone	(Galβ1-4GlcNAcβl-3/6)n(Galβ1-3)GalNAcαl-Ser/Thr
Blood group H	Fucαl-2Galβ1-
Blood group A	GalNAcα1-3 Galβ1 Fucα1-2
Blood group B	Galα1-3 Galβ1 Fucα1-2
CAD (SD[a])	GalNAcβ1-4 Galβ1 NeuAcα2-6
Sialyl Lewis[a]	NeuAcα2-3Galβ1-3GlcNAcβ1- 1,4 Fucα
Lewis[a]	Galβ1-3GlcNAcβ1- 1,4 Fucα
Lewis[b]	Galβ1-3GlcNAcβ1- 1,2 1,4 Fucα Fucα
X antigen (SSEA-1, Le[x])	Galβ1-4GlcNAcβ1- 1,3 Fucα
Y antigen (Le[y])	Galβ1-4GlcNAcβ1- 1,2 1,3 Fucα Fucα

Table 1.1: Blood group and related antigens on *O*-linked cores (Hounsell et al. 1996)

1.3.4 Intracellular/nuclear glycosylation: *O*-GlcNAc

It was thought that *N*- and *O*-glycosylation mechanisms that take place in the Golgi and ER destined proteins for secretory pathways and that glycoproteins were all either restricted to the plasma membrane and extra cellular spaces or secreted (Haltiwanger et al. 1997). However, there is now a large amount of data to show that there are glycoproteins in the cytoplasm (Hart et al. 1989a, Hart et al. 1989b). The most studied cytoplasmic form of glycosylation is *O*-GlcNAc, which is β-N-acetylglucosamine *O*-linked to the hydroxyl groups of Ser/Thr (Torres et al. 1984). Over 50 proteins bearing this glycosylation have been identified and the vast majority are nuclear localised, but some reside in the cytoplasm. As for other *O*-glycans no consensus sequence has been identified, however most known *O*-GalNAc Ser/Thr sites have a proline residue within a few amino acids of the serine and threonine. The enzymes responsible for the addition of *O*-GlcNAc, *O*-GlcNAc transferase (OGT), and removal, β-N-acetylglucosaminidase (*O*-GlcNAcase) have been characterised (Iyer et al. 2003). The biological role of *O*-GlcNAc is still being uncovered, but so far it has been shown to be involved in a regulatory mechanism, which shares similarities with phosphorylation in terms of its impact on dynamic cycling and the proteins that are modified (Comer and Hart. 2000). The sugar nucleotide UDP-GlcNAc is the donor for *O*-GlcNAc addition and it is generated through the hexosamine biosynthetic pathway. An increased flux through this pathway has been shown to coincide with a resistance to insulin. A possible mechanism for this resistance lies with the *O*-GlcNAc modification, as it is dependent on the hexosamine biosynthetic pathway (Marshall et al. 1991).

Further studies on *O*-GlcNAc have confirmed that *O*-GlcNAc modification has a regulatory role:

- *O*-GlcNAc modification occurs at the same site as phosphorylation in c-myc. This modification has a role in protein stability (Chou et al. 1995)
- Insulin stimulates the biosynthesis of Sp1 and also regulates both its *O*-glycosylation and phosphorylation (Majumdar et al. 2004)
- Transcription factors and RNA polymerase II can be modified by *O*-GlcNAc to promote gene silencing (Yang et al. 2002)

1.4 Altered glycosylation in disease

Glycosylation is one of the most common posttranslational modifications of proteins. It is therefore not surprising that a common phenotypic change seen in cancer cells is altered glycosylation (Kim et al. 1996) (Kobata et al. 2005). Early interest in the pathophysiological role of glycosylation arose from experimental research showing the appearance of certain oligosaccharides during tumour progression. These truncated glycan structures were also seen in embryonic tissues so were termed oncofetal antigens. Tumour cells undergo activation and rapid growth and have the ability to adhere to different cell types (Varki, A. *et al.* 1999). These features are accompanied by altered glycosylation, some of which can be recognised by various lectins and antibodies. Alterations in glycosylation can arise in various forms (Kim et al. 1997), most are truncated versions of normally occurring oligosaccharides such as TF, Tn or Lewis structures in native and sialylated forms. These can occur due to the up regulation or down regulation of competing glycosyltransferases or changes in the elongation of the core oligosaccharide structures that give rise to more favourable acceptors for later glycotransferases (Corfield et al. 1995). A common glycosylation

change seen in cancer is the more frequent occurrence of highly branched heavily sialylated glycoproteins (Warren et al. 1978). This increase, often as (α2-6)-linked sialic acids, is frequently the reason for the increased binding of Wheat Germ lectin in tumour samples. The increased size of tumour cell glycopeptides has been shown to be due to the increased β1-6 branching of *N*-glycans which is the result of an increased expression of GlcNAc transferase V (Saito et al. 1995). Increased GlcNAc transferase V bestows cell lines an increased frequency of metastasis, this was highlighted by an experiment where transfection of GlcNAcT-V cDNA into a cell line caused tumorigenic behaviour by previously nontumorrigennic cells. In Wiscott Aldrich syndrome (WAS), patients demonstrate abnormal glycosylation of CD43, due to increased β1-6GlcNAcT activity resulting in more branched core 2 glycan or lower molecular weight CD43. This may be the cause of the development of the autoimmune disorders and progressive lymphopenia observed in WAS patients (Khan et al. 2007). Hypogalactosylation of salivary and gingival fluid immunoglobulin G has been observed in patients with advanced periodontitis (Stefanovic et al. 2006). Reelin is a protein essential for the correct cytoarchitectonic organization of the developing CNS. In Alzheimer's patients reelin was differently glycosylated depending on its localisation in the plasma or cerebrospinal fluid (Botella-Lopez et al. 2006). In cancer cells mucin type *O*-glycans are the major carriers of altered glycosylation and these alterations in cancer can have differing biological and pathological consequences.

1.4.1 Aberrant glycosylation in colonic disease

Glycosylation changes seen in colon cancer, adenomatous and metaplastic polyps and cases of chronic inflammation such as UC and CD are similar, typically showing truncated *O*-linked oligosaccharides, reduced sulphation, increased sialylation

and Lewis variants [Table 1.2]. The TF antigen has been demonstrated following its specific cleavage by *O*-glycanase from mucins extracted from colon cancer and UC mucosal samples (Campbell et al. 1995). In other studies the Tn antigen in its native and sialylated form (Brockhausen et al. 1998, Karlen et al. 1998) is seen in UC patients and in colonic adenomas (Itzkowitz et al. 1992). Sialyl-Tn (S-Tn) is also under investigation as a diagnostic marker in breast (Sewell et al. 2006) and gastric cancers (Nakagoe et al. 2001).

Mucin-type *O-glycans and alterations in cancer*

O-glycan	***Structure***	**Increased/ decreased in cancer***
Tn antigen	GalNAcα-Ser/Thr	↑
STn antigen	Sialylα2-6GalNAcα-Ser/Thr	↑
Core 1, T antigen	Galβ1-3GalNAcα-Ser/Thr	↑
Sialyl-T antigens	Sialylα2-3Galβ1-3GalNAcα-Ser/Thr	↑
	Sialylα2-6(Galβ1-3)GalNAcα-Ser/Thr	↑
Core 2	GlcNAcβ1-6(Galβ1-3)GalNAcα-Ser/Thr	↑↓
Core 3	GlcNAcβ1-3GalNAcα-Ser/Thr ↓	
Core 4	GlcNAcβ1-6(GlcNAcβ1-3)GalNAcα-Ser/Thr	↓
Type 1 chain	[GlcNAcβ1-3 Galβ1-3]*n*	↓
Type 2 chain	[GlcNAcβ1-3 Galβ1-4]*n*	↑
	poly-*N-acetylactosamines*	
Sialyl-Lewisa	Sialylα2-3Galβ1-3 (Fucα1-4)GlcNAcβ1-3Gal-	↑
SLex	Sialylα2-3Galβ1-4 (Fucα1-3)GlcNAcβ1-3Gal-	↑
Sialyl-dimeric Lewisx	Sialylα2-3Galβ1-4 (Fucα1-3)GlcNAcβ1-3 Galβ1-4 (Fucα1-3)GlcNAcβ1-3Gal-	↑

*The symbol ↑ denotes an increase in cancer,whereas the symbol ↓ denotes a decrease
in cancer. Fuc, fucose; Gal, galactose; GalNAc,*N-acetylgalactosamine; GlcNAc, N-acetylglucosamine; sialyl, sialic acid; SLex, Sialyl-Lewisx; STn, Sialyl-Tn.*

Table 1.2: Mucin type *O*-glycans in cancer. Adapted from (Brockhausen 2006)

Increased sialylation of mucins has been demonstrated in IBD colonic mucosal explants. The importance of sialic acids in cancer metastasis was highlighted when it was shown that they prevent attachment of the cell to fibronectin and collagen type IV (Dennis et al. 1982). Sialyl-Tn expression has been correlated with poor prognosis in

cancer patients; clones of the breast cell line T47D over expressing sialyl-Tn and the enzyme responsible for its synthesis have slower cell growth, but increased cell motility, a feature of malignancy, as they had decreased adhesion to matrix protein (Julien et al. 2005). The sialic acids in cancer states can also be modified, for example sialic acid 9-*O*-acetylation is decreased in colon cancer (Yamashita et al. 1995).

Further alterations seen in human malignant colonic tissues affect Lewis antigens (Kim et al. 1986) known to be ligands which interact with various lectins and antibodies. In normal colonic mucosa type 1 and 2 chain extensions are formed, in colonic adenocarcinomas there is an increase type 2 chains, which are the precursor for the increased activities of (α2-3)-sialyltransferases seen in colorectal cancer, resulting in higher expression of sialyl-Lewis^x (SLe^x) in particular sialyl-dimeric Lexis^x antigen is correlated to poor prognosis.

Colonic mucins in colitis can also show reduced sulphation (Brockhausen 2003, Brockhausen 2003) and UC mucosal samples show lower sulphate incorporation compared to IBD controls when cultured *in vitro* (Campbell et al. 2001). Mucins are able to inhibit cell-cell adhesion mediated by integrins and E-cadherin. A competitive inhibitor of *O*-glycosylation, benzyl-α-GalNAc, was shown to increase E-cadherin-mediated cell adhesion by decreasing the inhibitory effect of *O*-glycosylated dysadherin (Tsuiji et al. 2003). South Asians diagnosed with colitis show do not show this reduced mucin sulphation and it has been suggested that this might be the reason for the lower rates of cancer in this population as compared to Europe (Probert et al. 1995). There are reports of alteration of specific sub classes of mucin in IBD and UC, based on the fractionation by chromatography of mucin samples (Podolsky et al. 1983, Podolsky et al. 1984), close examination of the results however shows that the techniques used are probably just showing overall mucus depletion seen in human UC

(Raouf et al. 1991). Depleted mucus would give an altered interaction with luminal contents, perhaps an altered response to bacteria, which could lead to inflammation. The Cotton-top Tamarin, a New World monkey, also develops colitis which is indistinguishable from human UC (Boland et al. 1987). In this model there is also an increase in the TF antigen and mucin sub-class depletion similar to humans.

1.4.2 Mechanisms of altered glycosylation

In normal colonic tissues a large range of *O*-glycan structures is seen. However in colon cancer increased expression of the TF and Tn antigens are seen, both are markers of poorly differentiated adenocarcinomas and have been correlated with advanced cancer. It is not known whether these alterations in glycosylation arise because of an alteration in the donor carbohydrate availability, altered substrate protein sequence or alterations in the glycosyltransferase enzymes. It has been shown that an alteration in the relative activities of glycosyltransferases or alterations in the localisation of the glycosyltransferases can cause aberrant glycosylation, with some studies demonstrating that glycosyltransferase localisation is regulated by Golgi pH (Kellokumpu et al. 2002)

The increase in TF and Tn seen in colon cancer could be due to a change in glycotransferase levels (Brockhausen et al. 2001); a reduced level of Core 3 β1-3-*N*-acetylglucosamine (GlcNAc) is observed allowing the GalNAc peptide to be acted upon by the Core 1 and 2 enzymes thus providing a higher prevalence of TF antigens. In addition to this, the level of the transferase responsible for addition of sialic acid to this structure, is increased, leading to increased levels of sialyl-TF (Schneider et al. 2001). In normal colonic tissue there is a high mucin type M-enzyme (C2Gnt) activity, which alongside leucocyte-type L-enzyme (C1GnT) is responsible for the conversion

of the TF antigen to the Core 2 structure. In tumorigenic cells derived from human adenoma cells this C2GnT activity was diminished, but in some other human colon cancer cell lines such as HT-29 the activity was still high (Vavasseur et al. 1994, Vavasseur et al. 1995). Overall, however, it seems that C2GnT is upregulated relative to C1GnT in most colon cancer tissues. This shift in relative activity would seemingly result in a decrease of Core 4 structures and an overall increase in Core 2 structures which are the main bearers of SLe^x.

However, a detailed study of the relative expressions of glycosyl-, sialyl- and sulpho-transferases in colon cancer showed that although there are differences, these differences related poorly to the changes in the carbohydrate expression (Yang et al. 1994). A further mechanism by which alteration in glycosylation could occur could be due to an alteration distribution of the glycosyltransferases in the Golgi and the manner and order by which they are able to act on their substrate. Proteins in the Golgi are fully folded and so only the accessible Ser/Thr residues can be glycosylated. Many studies have investigated the localisation of specific glycosyltransferases, for example the enzymes that synthesise Core 1 and Core 2 structures are seen mainly in the *cis*-Golgi (Roth et al. 1994, Rottger et al. 1998). A logical assumption is that proteins travelling through the Golgi from the *cis*- to *trans*- Golgi in normal cells will encounter the glycosyltransferases in an order that allows the stepwise addition of glycans. In disease states the localisation of these glycosyltransferases could be disturbed resulting in incomplete or altered glycosylation (Egea et al. 1993)

Altered Golgi pH has been shown to cause altered glycosylation, possibly acting via a change in the localisation of the glycosyltransferases, or reducing the activity of the glycosyltransferases. The pH was significantly more alkaline in HT-29 and SW-48 colon cancer cell lines compared to control, and a 0.2 pH increase in pH

induced an increase in TF antigen expression in control cells (Rivinoja et al. 2006). Drug-induced alkalinisation of the Golgi by bafilomycin A_1 or monensin in goblet cell differentiated colonic cell line LS174T caused increased TF expression and mucin depletion as seen in colon cancer (Campbell et al. 2001). Furthermore an altered Golgi structure in colon cancer cells, visualised by Golgi structural protein Giantin, is shown alongside alteration in glycosylation [Figure 1.5] (Kellokumpu et al. 2002).

In another human goblet cell differentiated cell line, HT-29 MTX (Methotrexate-conditioned), Campbell and colleagues have shown that pro-inflammatory cytokine tumour necrosis factor alpha (TNFα) could induce disease-associated glycosylation patterns, with an increased expression of TF antigen and a reduction in mucosal sulphation (Campbell 2002). However, further investigation is required to understand the impact of cytokines on Golgi pH and the altered glycosylation seen. In cystic fibrosis opposite alterations in glycosylation are observed such as under-sialylation of glycans, this is due to the hyper acidification of the *trans*-Golgi as the result of the dysfunctional cystic fibrosis transmembrane conductance regulator (CFTR) gene product (Poschet et al. 2001). Alternative splicing has also been identified as being involved in an altered glycosylation profile. Notably the adhesion molecule CD44 shows expression of TF antigen on its higher molecular weight variants as compared to the standard CD44 from both normal and colon cancer tissues which do not express TF antigen (Singh et al. 2001). Higher molecular weight CD44 splice variants with TF expression are expressed in colitis and provide a link between cancer associated changes and splice variants. This and other reports, therefore, show that amino acid sequence of the CD44 plays a part in *O*-glycosylation but does not explain the change in glycosylation of secreted mucins (Campbell et al. 2001).

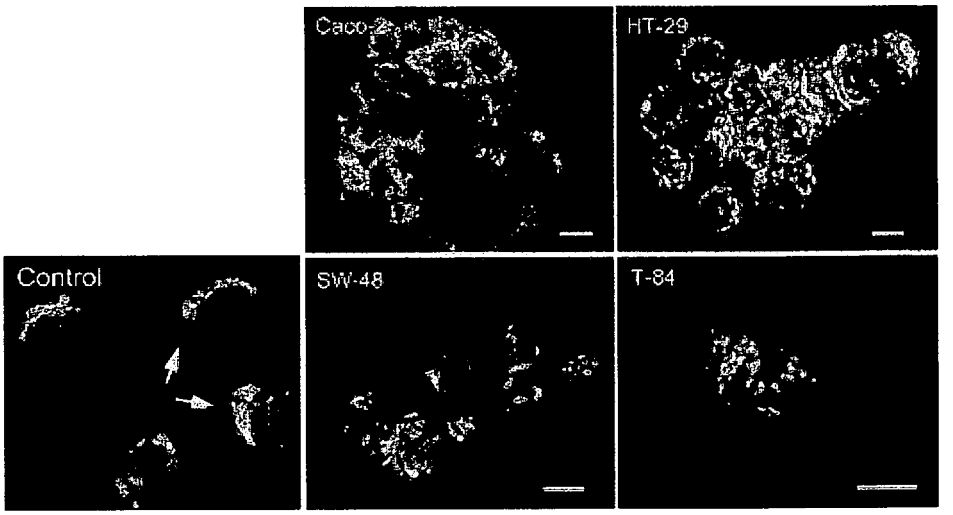


Figure 1.5: Structural organisation of the Golgi apparatus in colorectal cancer cell lines, visualised by anti-Giantin immunohistochemistry (Kellokumpu et al. 2002). The Golgi apparatus of colon cancer cells is fragmented and scattered around the nuclei and the cytoplasm compared to the control non-cancerous normal rat kidney (NRK) cell line. All the cell lines also were positive for the expression of the TF antigen.

1.5 Functional consequences of altered glycosylation

1.5.1 The colonic epithelium

The human colonic epithelium [Figure 1.6] is a physical barrier of cell membranes perpetrated with tight junctions, which is further protected by the secretion of mucin glycoproteins and trefoil peptides by goblet cells (Podolsky et al. 1984), and the integrity of which is paramount as the human intestine is colonised with between 500-1000 different bacterial species (Egert et al. 2006). The colonic epithelium as well as providing an effective barrier has also been demonstrated to be integral to the immune response and must be able to discriminate between resident flora and enteric pathogens.

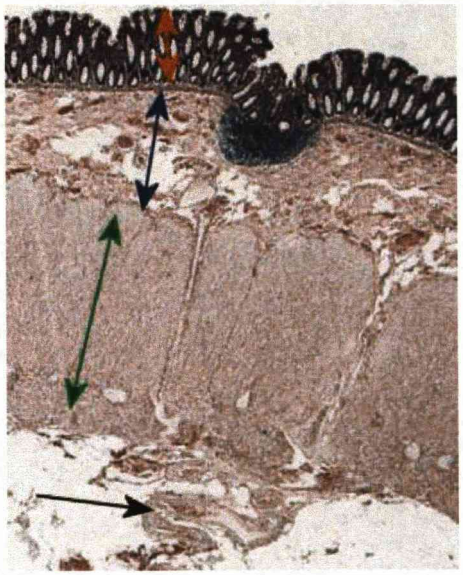


Figure 1.6: Human colonic epithelium (adapted from http://www3.umdnj.edu/histsweb). **Red arrow** – Mucosa, **Blue arrow** – Submucosa, **Green arrow** - Muscularis Externa, **Black arrow** - Taenia coli

1.5.2 Bacterial-epithelial interactions

In order for bacterial pathogens to colonise the colon and eventually cause disease it is usually necessary for them to successfully adhere to the epithelium. This initial ability to adhere will enable them to resist the fluid flow of the luminal contents and peristalsis of intestinal contraction (Lu et al. 2001) and once bound these bacteria can become resident in the gut. The interactions of bacteria and the epithelium are complex and involve interactions between the bacterial surface determinants and the host epithelium receptors. Pathogens share similar mechanisms of interactions with the host, but individual bacteria possess unique abilities to exploit the host processes. The glycocalyx is a layer of glycoconjugates expressed at the surface epithelium and these oligosaccharides can serve as receptors for microorganisms [Figure 1.7]. The

commensal bacteria of the gut have a role in the prevention of pathogenic bacteria adherence by steric hindrance and covering the expressed glycoconjugates. However, it has also been shown that indigenous microflora can influence the expression of glycoconjugates, by inducing the expression of fucosylated glycoconjugates on the host intestinal epithelium, which provides a lectin-like receptor for pathogens (Bry et al. 1996).

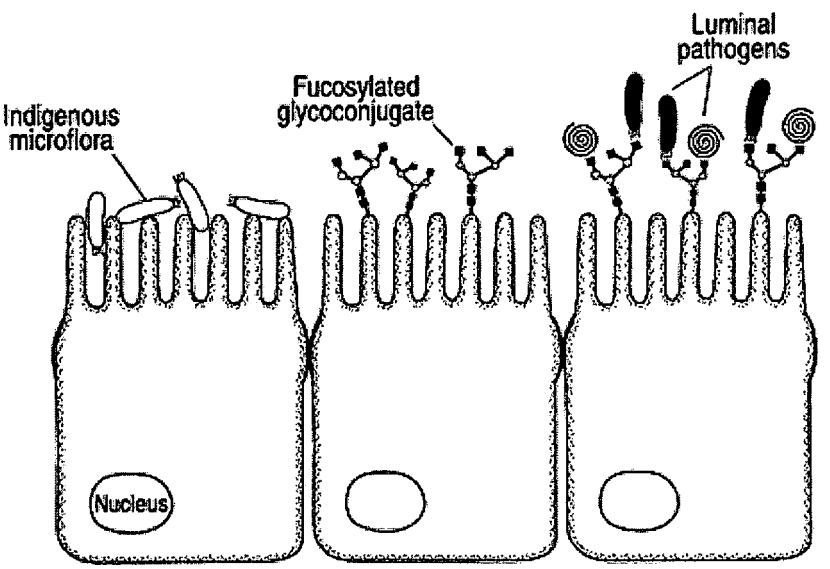


Figure 1.7: Crosstalk between intestinal bacteria and the host epithelium. Colonisation by indigenous microflora induces the expression of fucosylated glycoconjugates on the host intestinal epithelium. The expression of the glycoconjugates provides lectin-like receptors for the attachment of luminal pathogens and eventually confers susceptibility to pathogen colonisation and disease (Lu et al. 2001)

The functional importance of altered glycosylation such as the increased expression of oncofetal antigens and how they are related to the progression of disease are still under investigation and it is highly probable that they may lead to the recruitment of 'non-pathogenic' bacteria, which results in epithelial inflammation. It

has been demonstrated by Martin and colleagues that there are increased numbers of mucosa-associated bacteria, particularly adherent and invasive *E. coli*, in patients with Crohn's disease and colon cancer. These *E.coli,* which lack known pathogenicity genes, do not have to adhere to induce expression and release of the pro-inflammatory cytokine interleukin 8 (IL-8) (Martin et al. 2004). However many of these *E.coli* express haemagglutinins which allow them to adhere to intestinal epithelial cell lines and subsequently cause IL-8 release mediated by extracellular-regulated kinase (ERK) and mitogen-activated protein kinase (MAPK) and inhibited by mesalamine (Subramanian et al. 2008). It has also previously been demonstrated that the truncated glycans can act as ligands for microbial and plant lectins, which can lead to an increase in mucosal proliferation (Ryder et al. 1994). It is, therefore, of clear interest to investigate this complex interaction between altered glycosylation, inflammation and disease progression.

1.5.3 Bacteria and inflammation

There are around 500-1000 bacterial species that have been identified so far in the human gut, the colon being most populated with anaerobes with bacterial counts of $\sim 10^{14}$ per gram of luminal content (Egert et al. 2006). 16S RNA sequence analyses show that the dominant bacterial species in human faeces and the colon are low G+C content Gram positives (*Firmicutes)* and Gram negative *Bacteroidetes* (Backhed et al. 2005). A commensal or symbiotic relationship exists in humans with its colonic bacterial flora. Humans provide a nutrient rich environment and the bacteria play a role in developing the mucosal immune system, providing essential nutrients and prevent colonisation of pathogenic bacteria (Eckburg et al. 2005).

1.5.4 Normal bacterial flora as a prerequisite for inflammation

Recent studies have highlighted the balance in the relationship between the normal colonic bacterial flora and the host as it has been shown that the normal bacterial flora is a prerequisite for the development of inflammation and inflammation-related colorectal tumours. The emergence of genetically engineered mouse models which require the normal colonic flora to develop colorectal tumours and inflammation has helped greatly in furthering the understanding of this process. The T-cell receptor beta chain/ p53 (TCRβ/p53) double knockout mouse colitis model mimics the development of adenocarcinoma in UC with 70% of the mice developing adenocarcinomas of the ileocecum under normal housing conditions and no colonic adenocarcinomas in those housed in germ-free conditions (Kado et al. 2001). Similar observations have been made with interleukin-10 (IL-10) knockout (Balish et al. 2002) and Gpx1/Gpx2 double knockout mice (Chu et al. 2004). A well-characterised mouse model of inflammation is that of the IL-10 knockout. IL-10 is a cytokine affecting the growth and differentiation of many hemopoietic cells *in vitro* (Yang et al. 2006) and in particular is a suppressor of macrophage and T-cell functions. Under conventional housing conditions there is an increase in morbidity with all male mice and 50% of female mice dying at 4 months and significant inflammation is observed. The abnormal changes include thickening of the mucosa, disorganisation of the crypts, epithelial ulceration and accumulation of bacteria. When housed under germ-free conditions all mice survived up at least 8 months (Sellon et al. 1998). Other inflammation models that develop inflammation with the normal bacteria flora include, transforming growth factor β-1 (Tgfβ1/Rag2) (Engle et al. 1999), Smad3 (Zhu et al.

1998) and cytokeratin 8 gene knockouts (Habtezion et al. 2005), but these models have not been tested under germ-free conditions.

Numerous studies have utilised the 16S RNA sequence diversity to establish differences in the microbial flora in health disease states. A significant decrease in the proportion of bacteria belonging to the *Firmicutes* (Manichanh et al. 2006) and *Clostridium leptum* (Sokol et al. 2006) phylum have been reported in Crohn's disease and a reduction in *Clostridium coccoides* phylum in ulcerative colitis (Sokol et al. 2006). Examining the bacterial flora at a mucosal level shows differences in the bio mass film, with >60% of the biofilm mass attributed to *Bacteroides fragilis* in patients with IBD, 30% in self-limiting colitis and <15% in irritable bowel syndrome (IBS) (Swidsinski et al. 2005). However, a study by Prindiville *et al* (2004) showed no significant evidence that one specific organism is associated with IBD. At present it not possible to associate mucosal infection by a specific bacteria with IBD although there is evidence for some change in the microflora in disease, which is not secondary to the effect of inflammation i.e. changes that are not specifically associated with lesion sites.

1.5.5 Role of non pathogenic bacteria in inflammation

Evidence supporting a causal relationship between *Helicobacter pylori* infection and the pathogenesis of gastric carcinomas, stemmed from animal models and epidemiological studies, resulting in *H. pylori* being classified as a group 1 carcinogen in 1994 by the world health organisation (1994). This relationship triggered an interest in to the association between intraepithelial *E.coli* and colorectal cancer. It was found that the colonic mucosa of patients with colorectal carcinoma, but not normal colonic mucosa, has an increase in mucosa-associated *E.coli*, and that

E.coli was present in non cancerous mucosa distant to the tumour (Swidsinski et al. 1998). In IBD there is evidence for quantitative alterations in the mucosa-associated flora and the mucus-associated flora with evidence for a novel class of *E.coli* that lacks conventional pathogenicity genes (Darfeuille-Michaud et al. 1998). It has been shown that both the mucosa associated *E.coli* from CD and colon cancer samples can induce the release of pro-inflammatory cytokines and that they do not have to adhere to the epithelial membrane to cause an inflammatory response (Martin et al. 2004). The close association of the bacteria to the membrane is capable of inducing stress in the bacterial cell due to the oxygen-rich environment. This could cause the activation of the SOS operon protein in the bacteria (Broom et al. 1993). The degradation of this protein leads to the formation of *N*-formyl-methionyl-leucyl-phenylalanine (fMLP). This formylated chemotactic peptide is able to elicit IL-8 release from the host epithelial (Leiper et al. 2001). This supports the hypothesis that non-pathogenic *E.coli* may play a key role in the pathogenesis of colon cancer.

1.5.6 Mechanisms by which bacteria can cause inflammation

The mechanisms by which commensal bacteria without known pathogenicity genes cause an inflammatory response by the host are still under study. Altered glycosylation such as the increase in cell surface oncofetal antigens, could be responsible for the recruitment of bacteria with lectins specific for TF and sialyl Tn. It is known that the protozoan parasite responsible for amoebiasis, *Entamoeba histolytica*, possesses a TF binding lectin, which is crucial to its pathogenicity (Leroy et al. 1995). This recruitment of bacteria to the mucosa could then lead to an immune response and through various pathways result in inflammation.

Interleukin 8 (IL-8) is a pro-inflammatory cytokine which is produced as a result of a number of different pathways, and also implicated in intestinal inflammation (Crabtree et al. 1994). One of the pathways known to induce IL-8 is the nuclear transcription factor kappa B pathway (NFκB), which regulates the promoters of a variety of genes whose products are critical for inflammatory processes such as IL-8, IL-1B and Cox-2. Cox-2 is induced by IL-8 and causes inflammation through the production of prostaglandins, which inhibit apoptosis and promote cellular proliferation. Immune and inflammatory responses in the gut and other immune-competent tissues often involves NFκB. Proinflammatory stimuli activate NFκB through tightly regulated phosphorylation, ubiqutinisation and proteolysis of a physically associated class of inhibitor molecule, IκB (Carlotti et al. 2000). *Salmonella typhimurium* has already been shown to induce epithelial IL-8 expression via Ca^{2+} - mediated activation of the NFκB pathway (Gewirtz et al. 2000). Another mechanism by which these bacteria could stimulate a host response is through MAPKs. MAPK pathways are well conserved major signalling systems involved in the transduction of extracellular signals into cellular responses in many organisms including mammals (Kolch 2000). One of the best characterised MAPK pathways is the Ras/MAPK signal transduction pathway (also known as the ERK p44/42 pathway), which is responsive to signals from receptor tyrosine kinase, haematopoietic growth factor receptors and some G protein coupled receptors, which promote cell proliferation or differentiation; this pathway could be activated by IL-8 release stimulated by bacteria. It has also been shown that bacterial DNA can elicit IL-8 release from HT-29 colon cells through Toll-like receptor 9 (TLR-9) expressed on the cell surface via a MAPK-dependent, NFκB independent, pathway (Akhtar et al. 2003). Phorbol esters such as PMA (phorbol 12-myristate 13-acetate) function as tumour promoters and have been implicated in the

immune response. PMA activates PKC, which can mediate signalling through a MAPK pathway, which is either Ras-dependent or Ras-independent (Lee et al. 2002); this pathway could be involved in a host response to bacteria.

1.6 Inflammatory bowel disease and risk of colon cancer

Inflammatory bowel disease is a chronic inflammatory disorder separately known as ulcerative colitis (UC) and Crohn's disease (CD). Crohn's disease, also known as regional enteritis, is inflammation of the gastrointestinal tract characterised by transmural inflammation and skip lesions, most often affecting the distal ileum and caecum. Ulcerative colitis affects the colon and causes more superficial and non-granulomatous ulceration (Papadakis et al. 2000). Epidemiological studies have shown that IBD is a disease that predominantly affects Western society with as many as 1.4 million persons in the United States and 2.2 million persons in Europe affected. Differences in race and ethnicity seemingly are now narrowing and it is becoming clear that environmental factors are most influential in expression of the disease (Loftus 2004). The incidence of IBD was shown not to be more frequent in northern compared to southern Europe (Shivananda et al. 1996), however, most recent studies have included eastern European countries (Lakatos et al. 2006) and a have demonstrated that IBD incidence can be distributed on a developed-developing country gradient (Frangos 2007, Frangos 2007).

IBD can be categorised as an idiopathic inflammatory disease, as its etiology is unknown. Many factors such as genetic, infectious and immunological have all been implicated resulting in range of clinical problems for the patient and now there is strong evidence to suggest that chronic inflammation increases risk of colorectal cancer (CRC). The risk of developing CRC increases with duration (Itzkowitz 2002),

CRC is rarely seen until after 7 years of colitis, thereafter risk increases by 0.5-1% a year. The extent of the area affected by colitis is also a determining factor as risk of CRC increases with greater colonic surface area affected increases. The efficacy of treatment of IBD with anti-inflammatory drugs, especially 5-aminosalicylates in reducing colorectal dysplasia also points to the link between inflammation and cancer development (Itzkowitz et al. 2004). There are a number mechanisms understood which could lead to this chronic inflammation.

Research into the genetics of IBD patients have long shown that polymorphisms in the NOD2 (CARD 15) gene to be associated with the risk of developing CD (Cho et al. 2007). It encodes a protein selectively expressed in macrophages and it is plausible that this alteration may be the cause of the altered macrophage response. The relative risk of developing CD in homozygous or heterozygous genotypes is estimated to be between 10- and 40- fold greater than that of the general population (Hugot 2006). Although studies have shown it is not as simple as just alterations in the NOD2 gene alone and that a combination with as yet unknown are responsible for the increase in risk of developing CD (Hugot et al. 2007). There is also support for another genetic component to IBD and a prime candidate is the vitamin D3 receptor, which has an immunosuppressive capacity, a polymorphism in which could be related to the aberrant regulation of mucosal response (Csaszar et al. 2001). CD patients have also been shown to have increased incidence of homozygotes for the Taq1 polymorphisms compared to controls or UC patients (Simmons et al. 2000). How this relates to the pathogenesis of the disease is unclear, but it may also be linked to immune regulation. One remarkable hypothesis is that CD subjects may have been positively selected by having resistance to infectious diseases. This would reinforce evidence that polymorphic genes that have modest effects by

themselves could in combination with environmental factors play a role in the pathogenesis of the disease (Csaszar et al. 2001)

The evidence to suggest that cancer risk is due to inflammation and not a co-inherited phenomenon is detailed by a study in Sweden: 30,000 cases of IBD were investigated and it showed that there was no significantly increased risk of colorectal cancer in first degree relatives (Askling et al. 2001), however a family history of colorectal cancer did increase risk. This was also seen in a Danish study of 4,496 patients with IBD (Fonager et al. 1998) and in a more recent but smaller study of an Italian population (632 patients) (Riegler et al. 2006).

The present evidence, therefore, supports the hypothesis that colorectal cancer susceptibility is increased by extent and duration of inflammation. IBD associated cancer, therefore, is an excellent model for inflammation-associated cancer. Arguably the colon is in a mild state of continual inflammation as a result of the normal micro flora, it is also plausible that an altered host response to normal bacterial gut flora is responsible for the chronic inflammation seen in IBD [Figure 1.8]. This could be in part due to altered glycosylation allowing recruitment of dietary lectins and bacteria that are normally allowed to pass through the gut lumen.

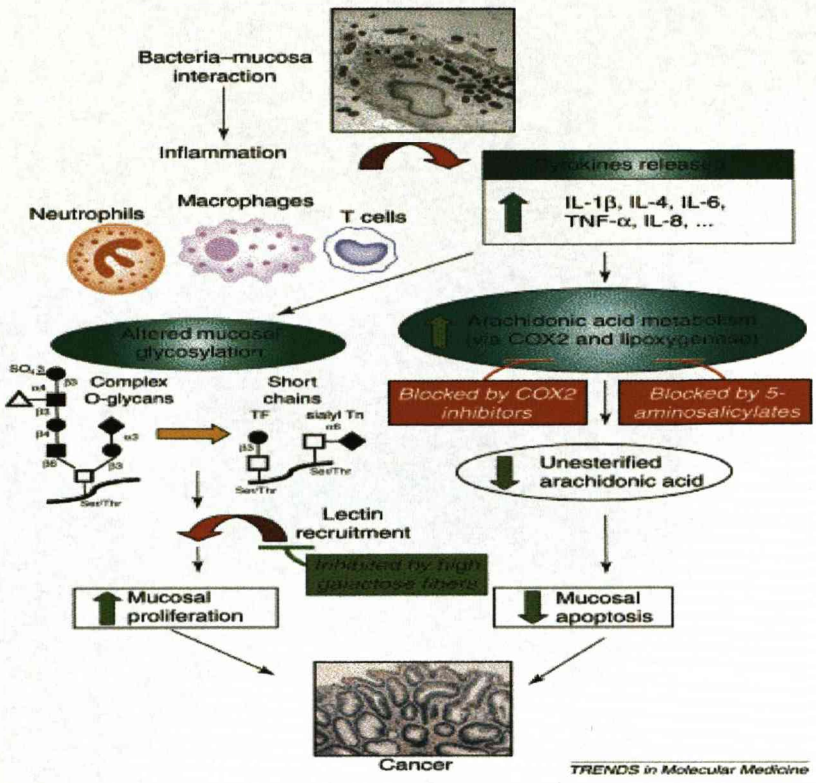


Figure 1.8: Mechanisms for colon cancer development as a consequence of altered glycosylation (Rhodes et al. 2002)

1.7 Lectins

1.7.1 Plant lectins

A lectin, from the Latin *lectus*: to gather or select (Boyd et al. 1954), by definition is a carbohydrate binding protein of non immune origin. Lectins occur frequently in nature and may be soluble or membrane bound. They are involved in a diverse array of biological processes, such as clearance of glycoproteins from the circulatory system (Ashwell et al. 1982), leucocyte recruitment to inflammatory sites (Melrose et al. 1998) and cancer metastasis (Gabius 1988). Classically lectins were deemed to have at least 2 sugar binding sites as lectins with only one binding site will not agglutinate or precipitate sugar-containing structures, however, it is now recognised that agglutination is not a defining characteristic of a lectin. Lectins have

the ability to interact with carbohydrates in a highly specific non-covalent fashion which is usually reversible. A lectin's specificity is normally defined by the monosaccharides and oligosaccharides that inhibit its activity (in some cases the inhibition of the agglutination properties). Work by Sharon has classified lectins into 5 groups according to the monosaccharide ligand to which the lectin has most affinity (Lis et al. 1998). The five groups being: mannose, galactose/*N*-acetylgalactosamine, *N*-acetylgalactosamine, fucose and *N*-acetylneuraminic acid. It is noted that usually lectin specificity is high, and that a lectin generally will bind specific sugar configurations e.g. will only bind glucose and not galactose.

Plant lectins have a tightly globular structure and are often resistant to digestion by mammalian enzymes allowing them to pass through the gut lumen were they remain active. The first pure plant lectin to be identified was concanavalin A (ConA) isolated from jack beans and its hemagglutination properties were described over seventy years ago (Sumner et al. 1935). The function of lectins in plants is unclear, but they are thought to have a role in immunity and cell-cell interactions (Van Damme et al. 2004). Microbial lectins often are adhesins that can be directly involved in the pathogenic effects of enteric bacteria. Direct evidence for the first animal lectin was observed when studying the turnover of glycoproteins in the blood. In order to attach a tritium label to the terminal sequence of sialyl-Gal-GlcNAc, the sialic acid had to be either be removed or altered by mild periodate oxidation. The glycoproteins terminated with sialic acid were retained in the blood for a number of days whereas the galatose terminated glycoproteins were rapidly cleared (Ashwell et al. 1982). This led to the discovery of the asialoglycoprotein receptor in the liver, responsible for recognition of β-linked galactose or GalNAc residues (Stockert 1995), and involved in the clearance of ageing desialylated glycoproteins from circulation. The further study

of animal lectins has shown that the primary amino acid sequence of a protein could be used to predict carbohydrate binding properties with the discovery of the hyaluronan-binding properties of CD44 and prediction that selectins would recognise carbohydrates (Varki 1998). The diverse roles and classification of animal lectins [Table 1.3].

Lectin family	**Typical saccharide ligands**	**Subcellular location**	**Examples of functions**
Calnexin	Glc_1Man_9	ER	Protein sorting in the endoplasmic reticulum.
M-type lectins	Man_8	ER	ER-associated degradation of glycoproteins.
L-type lectins	Various	ER, ERGIC, Golgi	Protein sorting in the endoplasmic reticulum.
P-type lectins	Man 6-phosphate, others	Secretory pathway	Protein sorting post-Golgi, glycoprotein trafficking, ER-associated degradation of glycoproteins, enzyme targeting.
C-type lectins	Various	Cell membrane, extracellular	Cell adhesion (selectins), glycoprotein clearance, innate immunity (collectins).
Galectins	β-Galactosides	Cytoplasm, extracellular	Glycan crosslinking in the extracellular matrix.
I-type lectins (siglecs)	Sialic acid	Cell membrane	Cell adhesion.
R-type lectins	Various	Golgi, Cell membrane	Enzyme targeting, glycoprotein hormone turnover.
F-box lectins	$GlcNAc_2$	Cytoplasm	Degradation of misfolded glycoproteins.
Ficolins	GlcNAc, GalNAc	Cell membrane, extracellular	Innate immunity.
Chitinase-like lectins	Chito-oligosaccharides	Extracellular	Collagen metabolism (YKL-40).
F-type lectins	Fuc-terminating oligosaccharides	Extracellular	Innate immunity.
Intelectins	Gal, galactofuranose, pentoses	Extracellular/cell membrane	Innate immunity. Fertilization and embryogenesis.

Table 1.3 Classification and functions of animal lectins

(Adapted from http://www.imperial.ac.uk/research/animallectins/ctld/lectins.html)

1.7.2 Plant lectins as molecular tools

Plant lectins can be utilised as molecular tools due to their specific recognition of carbohydrate structures; originally their ability to recognise specific carbohydrate determinants in human blood cells was utilised for blood typing. Their specificity for carbohydrate structures is also useful in lectin-blotting assays (equivalent to Western blotting) and histological staining, although some caution must be shown as some lectins display non-specific binding. Lectins can be utilised in the characterisation of animal cell glycoconjugates when the binding properties of the lectin have been characterised, which has become especially useful in the recognition of aberrant glycosylation in colonic disease. Purification of glycoconjugates is also possible if the lectin is immobilised, and a high purity of glycoconjugate can be isolated, especially when combined with HPLC (Merkle et al. 1987).

1.7.3 TF and Tn binding lectins

Since it has been shown that there is an increase in TF antigen expression in colonic disease, TF/Tn antigen binding lectins have been utilised to demonstrate this change in glycosylation. There are four well known dietary TF-binding lectins, all of which lack cytotoxicity and can be eaten raw.

The legume family of lectins is the largest, with around 100 members. They typically consist of two or four identical subunits of 25-30 kDa each with one binding site which have a tightly bound Ca^{2+} or Mn^{2+}. They are also characterised by a high content of β-sheets and a lack of α-helices (Sharon et al. 1990). The primary sequences of over 15 legume lectins have been established, with remarkable homologies. An important legume lectin is peanut agglutinin, isolated from *Arachis*

hypogaea, which exhibits an open quaternary structure where the homotetramer possesses neither 2 nor 4 fold symmetry as shown by X-ray crystallography. The PNA tetramer can be described as a dimer of a dimer with similarities to the lectin *Griffonia simplifolia* (Banerjee et al. 1994) The PNA lectin has high affinity for the TF antigen with 4 strong invariant hydrogen bonds, alongside numerous Van der Waals interactions. The boletaceae family of lectins includes six lectins of similar homological sequence. Of this family, the lectin from the edible mushroom *Agaricus bisporous* was one of the first isolated and noted for its erythrocyte agglutinating activities (Presant et al. 1972). It is able to reversibly bind the TF antigen and also its sialylated form. Jacalin is another TF antigen binding lectin, which is derived from the Jack fruit *Artocarpus integrifolia*, it can also bind human IgA. Structurally Jacalin consists of complex subunit of around 60 kDa, which recognises the terminal galactose of the TF antigen and also has some specificity to the *N*-acetylgalactosamine structure (Tachibana et al. 2006); it can bind in the presence or absence of sialic acid. The lectin obtained from *Amaranthus caudatus* (ACA), which is an important food source in South America is also capable of binding to TF and sialyl-TF (Yu et al. 2001). A commonly used Tn antigen binding lectin is isolated from *Vicia villosa* (VVA) the hairy vetch seed. VVA preferentially recognises α or β linked terminal N-acetylgalactosamine, especially a single α *N*-acetylgalactosamine.

1.8 Effects of lectins on proliferation of colonic epithelial cells

The incidence of colorectal cancer is about 28,000 cases per annum in the United Kingdom and it is the second most common cause of cancer-related deaths in the world (Evans et al. 2002). Much work on the understanding of the pathogenesis of the disease has focussed on diet. A protective role against colorectal cancer

development has long been associated with a high intake of cereal fibre. Dennis Burkitt conducted research in to the dietary habits and cancer incidence in several populations from developed and developing countries, which demonstrated that low dietary fibre intake could increase risk of colorectal cancer (Burkitt 1972). However this association has not been successfully demonstrated by a number of recent epidemiological studies (Alberts et al. 2000, Asano et al. 2002) and notably the nurses cohort study (Fuchs et al. 1999) also showed no protective effect from the consumption of high amounts of dietary fibre. However the protective effects may be due to non starch polysaccharides (NSP) not found in cereals, but certain vegetable and fruit with fibres high in galactose (Bingham 1990). It has been hypothesised that these galactose epitopes on non starch polysaccharides may inhibit the galactose binding lectins such as PNA, preventing their interaction with the colonic epithelium (Evans et al. 2002).

PNA lectin is one of the most commonly ingest dietary lectins with specificity for the TF antigen. PNA lectin has been shown to stimulate proliferation of HT-29 human colonic epithelium cells (Ryder et al. 1992), SW837 rectal adenocarcinoma and HCT-15 human colonic carcinoma cells (Kiss et al. 1997) and is able to remain active in the digestive tract, as PNA extracted from faeces is still able to agglutinate desialylated red blood cells (Ryder et al. 1992). Further studies to determine the effects of PNA on abnormal colonic epithelium which express the TF antigen, measured the crypt cell proliferation rate (CCPR). UC, CD and colonic polyps biopsy samples showed an average 25% greater CCPR upon stimulation with 25 μg/ml PNA compared to normal colonic biopsies. UC biopsies showed an almost doubled CCPR when stimulated with PNA (Ryder et al. 1994). PNA is also able to stimulate proliferation *in vivo* of rectal epithelium; patients with normal colonic mucosa who

ingested 100 g of peanuts a day for 5 days had a 41% increase in rectal mucosal proliferation (Ryder et al. 1998). Additional studies have indicated that high molecular weight variant CD44v6 which expresses TF and is expressed in inflamed and neoplastic colonic epithelia as a result of altered mRNA splicing, is bound by PNA lectin. Binding stimulated phosphorylation of the hepatocyte growth factor c-MET which is known to associate with CD44v6, followed by downstream activation of p44/p42 mitogen activated protein kinase (MAPK). The activation of c-MET and MAPK can be competitively inhibited by asialofetuin which is a TF-expressing glycoprotein and PNA proliferation effects are also diminished by addition of MAPK inhibitor PD98059. These effects were demonstrated in HT-29 and T84 colonic cancer cell lines but not CaCo-2 colonic cancer cells which do not express CD44v6 (Singh et al. 2006). This overall clearly demonstrates that PNA is able to interact with the TF antigen presented in the colonic epithelium stimulating cellular proliferation with its effects mediated through activation of MAPK.

A number of other lectins also stimulate proliferation in various neoplastic colonic cell lines. Wheat germ lectin (WGA), which binds *N*-acetylglucosamine and neuraminic acid, and Con A both stimulate proliferation at high concentrations in SW837, HCT-15, LoVo colonic cancer cells and *Griffonia simplicifolia* I-A4 (GSA) predominantly stimulated proliferation in LoVo colonic cancer cell lines (Kiss et al. 1997), LS174t and SW1116 colonic cancer cells (Chen et al. 1994). The broad bean lectin *Vicia faba* agglutinin (VFA) also stimulated proliferation of LS174t, SW1222 and HT-29 cells and also stimulated the differentiation of LS174t cells into gland-like structures (Jordinson et al. 1999). The lectin from *Amaranthus caudatus* (ACA) which recognises TF and sialyl-TF is able to cause dose-dependent stimulation of proliferation in HT-29 cells (Yu et al. 2001). Taken together, these reports give direct

evidence that edible lectins are able to have profound effects on human colonic epithelial cell lines. The mechanisms for the functional consequences of these effects are still under investigation but this type of interaction is likely to be of significant importance in the relationship between diet and gastro-intestinal proliferation leading to intestinal cancer, However, apart from the PNA these lectins have not been studied *in vivo*.

The mushroom lectin, ABL, and lectin from the jackfruit, JAC, have the opposite effect on HT-29 colonic epithelium cells by reversibly inhibiting proliferation with no apparent toxicity to the cells (Yu et al. 1993, Yu et al. 2001). JAC at a concentration of 20 μg/ml caused a 46% reversible inhibition of HT-29 cells with this activity inhibited by incubation with TF expressing glycoproteins (Yu et al. 2001). ABL immobilised on agarose, which is therefore not internalised, showed no effect on cellular proliferation. Electron microscopy of gold-conjugated ABL revealed that ABL was internalised to the endocytotic vesicles and multivescular bodies. This was also confirmed by confocal microscopy of fluorescein isothiocynate-conjugated ABL which showed perinuclear accumulation and also inhibited proliferation, which signified a possible interference with nuclear protein import. This was confirmed when ABL prevented heat shock protein 70 translocation from the cytoplasm to the nucleus upon heat stress. Further to this, in digitonin-permeabilised HT-29 cells, nuclear uptake of a NLS-peptide conjugated to BSA was inhibited by pre-incubation with ABL, indicating that ABL specifically blocks NLS-dependent nuclear protein import (Yu et al. 1999). Pulse labelling experiments following the location of the ABL has highlighted the nuclear localisation and reversibility of the ABL binding and hence reversibility of its anti- proliferative effect (Yu et al. 2000). The determination of a cytoplasmic ligand for ABL was achieved through ABL lectin affinity

chromatography of digitonin-released cytosolic proteins. The major glycoprotein purified expressed sialyl-TF, which was selected for *N*-terminal sequencing and identified as oxygen regulated protein 150 (Orp150). This *N*-terminally truncated cytoplasmic isoform, which expresses sialyl-TF is essential in NLS-nuclear protein import, reinforcing the evidence that ABL mediates its anti-proliferative effects as a consequence of the inhibition of NLS-nuclear protein import. The ABL lectin is another example of how a common edible lectin is able to interact with colonic epithelial cells.

1.9 Nuclear localisation sequence (NLS)-dependent nuclear protein import

A defining feature of eukaryotic organisms is the cell nucleus. The nuclear envelope is a barrier that nuclear proteins, which are synthesised in the cytoplasm, and RNA have to be transported across in regulated manner (Gorlich 1997). The import of proteins into the nucleus occurs through nuclear pore complexes (NPC), which allow the passive diffusion of macromolecules of less than ~40 kDa (Paine et al. 1975). The rate of transport across the nucleus is believed to be between ~100 and ~1,000 cargoes per minute per NPC (Ribbeck et al. 2001). Proteins greater in size up to around several million Dalton in weight are actively transported through the NPC if they contain the appropriate targeting signal using soluble transport factors and carrier molecules (Stewart 2007). An NPC is a huge molecular assembly of ~120 MDa made up of distinct sub complexes that spans the double membrane of the nuclear envelope [Figure 1.9]. The NPC is formed by an 8-fold symmetrical framework around a central pore, in the plane of the nuclear envelope its structure is best described as hour glass with an overall length of ~90 nm, it narrows towards the centre to ~40nm (Lim et al.

2006). Fibres also extend from the central body into the cytoplasm and nucleus; these fibres form a basket like structure in the nucleus but not the cytoplasm.

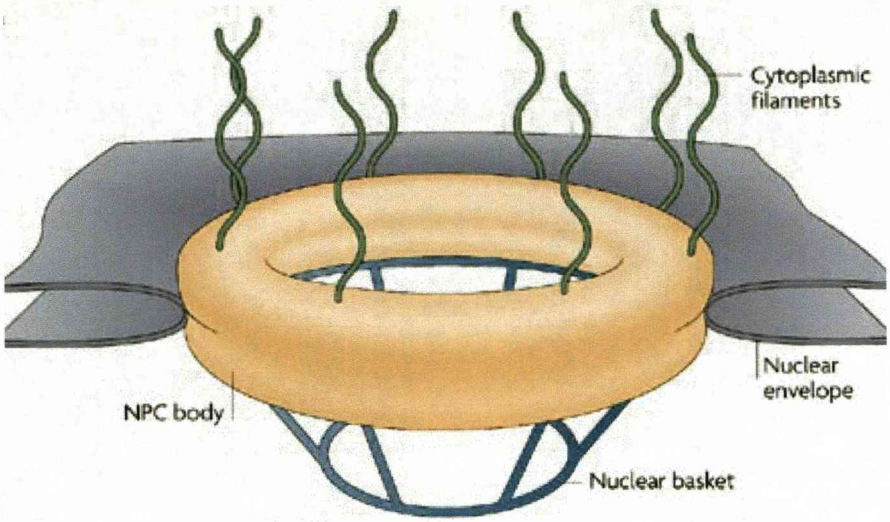


Figure 1.9: Nuclear pore complex (Stewart 2007)

The structure of the pore channel of ~30 nm through which the proteins are transported is not completely understood, but is thought to contain high concentrations of nuclear pore protein chains of little formal structure allowing the NPC to be plastic and deform on the transport of large molecules (Beck et al. 2004). The NPC is constructed of multiple copies of different proteins called nucleoporins, which are modularly assembled to form subcomplexes. The nucleoporins are located symmetrically around the NPC on both the cytoplasmic and nuclear side and are constructed of a number of common protein folds such as α-helical solenoids (Schwartz 2005).

There are a number of nuclear import pathways, each responsible for the import of specific macromolecules in to the nucleus (Pemberton et al. 2005). They share common characteristics and follow a sequence of protein recognition in the

cytoplasm, translocation through the NPC and release of the protein into the nucleus. The proteins are targeted by a short nuclear localisation sequence (NLS), which is different according to the pathway. In the classical nuclear protein import pathway, the NLS is bound by adaptor protein importin-α which forms an import complex with carrier protein importin-β in the cytoplasm, this import complex then is translocated through the NPC [Figure 1.10]. A NLS can be either monopartite with a single cluster of 4-5 basic residues, such as the SV40 large T-antigen, or bipartite with two clusters of basic residues, such as nucleoplasmin. The molecular recognition and attachment of the NLS is mediated by the tandem series of Armadillo repeats, which are responsible for the structure of importin-α, which has a concave inner surface allowing binding sites formed of an array of Trp, Asn and acidic residues (Fontes et al. 2000). A few cargo proteins can, however, bind directly to importin-β rather than through importin-α. The N-terminus of importin-α contains an importin-β binding domain (IBB), which contains a cluster of basic residues similar to that of the NLS, through which it binds importin-β. This IBB has an important auto-inhibitory role, due the similarity of its basic residues to the NLS it will compete for attachment to importin-α with NLS's when not attached to importin-β. This contributes to cargo release as the accumulation of NLS cargo in the nucleus is dictated by the affinity of NLS cargo for importin-α and the concentration of importin-α receptor (Kobe 1999).

Translocation of the trimeric complex of importin-α/β and NLS containing cargo is mediated by FG-nucleoporins in the NPC it is dissociated by RanGTP. When RanGTP binds to importin-β it induces a conformational change increasing the helicoidial pitch of importin-β causing the release of the IBB domain and freeing the importin-α cargo complex (Lee et al. 2005). The release of the cargo by importin-α is achieved, as the affinity to the cargo is reduced due to the IBB competing for the NLS

of the cargo. Dissociation is accelerated by FG-nucleoporins located at the nuclear face of the NPC. FG-nucleoporins such as Nup1, which has high affinity for the importinα/β import complex and can bind CAS the export carrier, increases the concentration of the complex at the nuclear face of the pore and increases its binding to RanGTP and CAS (Liu et al. 2005). Nup2 also concentrates these factors and also actively displace the NLSs from the importin-α (Matsuura et al. 2003).

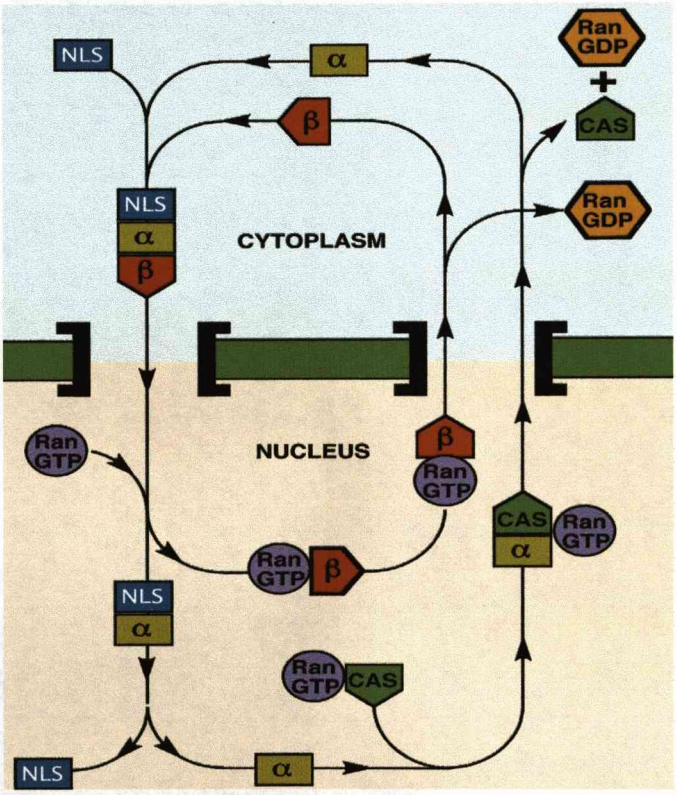


Figure 1.10: The NLS-nuclear protein import sequence (Stewart 2007)

The final step is the recycling of importin-α/β to the cytoplasm where Importin-β is complexed with Ran GTP and importin-α is actively exported with CAS. CAS coils around both RanGTP and importin-α, the IBB domain can only bind to CAS after the release of the cargo, thus preventing the cycling of the cargo back to the cytoplasm.

The formation of the CAS/importin-α/RanGTP complex has to contend with the Nup2 protein which aids the dissociation of the cargo. This is achieved through higher binding affinity of CAS than Nup2 to importin-α displacing the NLSs and revealing the IBB domain, these steps occur in a stepwise concerted fashion and Nup1 and Nup2 being utilised as scaffolds to aid the process (Matsuura et al. 2005). Once in the cytoplasm the export complexes of importin-β/RanGTP and CAS/importin-α/RanGTP have the RanGTP hydrolysed by RanGDP and accessory protein RanBP1 freeing the importin-α/β, and allowing them to repeat the cycle.

Glycobiology is key to understanding many biological processes particularly cell-cell and cell-protein interactions. There is a need for better understanding of mechanisms for altered glycosylation in disease and for the consequences of this altered glycosylation for cellular function. This thesis will focus on investigating the importance of glycosylation on cellular processes which are vital to the cell, with particular emphasis on the appearance of the oncofetal Thomsen-Friedenreich antigen on a cytoplasmic protein that is implicated in protein transport into the nucleus. This thesis will explore the possible role for this truncated antigen and investigate whether other cytoplasmic proteins also express the TF antigen. Finally this thesis will investigate the relationship between the structure of the Golgi apparatus, where the majority of glycosylation processes are thought to occur and altered glycosylation.

CHAPTER 2

GENERAL METHODS

This chapter contains details of all the major techniques and methods used in this thesis. Specific methods are described in each chapter.

2.1 Materials

All plastic including flasks (T25 cm^2, T75 cm^2 and T150 cm^2), 6 well, 12 well and 24 well plates all purchased from BD Bioscience (USA).

All chemicals were of analytical grade and purchased from Sigma-Aldrich (Dorset, UK) unless otherwise stated.

2.2 Cell lines

Human colon cancer cell-line HT29 was obtained from the European Collection of Animal Cell Culture at the Public Health Laboratory Service (Wiltshire, UK). The HT-29 cell line was first established from a colonic adenocarcinoma from 44 year old female Caucasian (Fogh 1971). When these cells are grown under the standard conditions, they form a multilayer of nonpolarized cells that display an undifferentiated phenotype (Cohen et al. 1999). If grown in a hexose-free medium they develop into a foetal small intestine-like state with the expression of brush border enzymes and development of micro villi; they are widely used for the study of epithelial malignancy in the colon.

The HT-29 MTX cells are a homogeneous, mainly gastric mucin (MUC5AC)-secreting population that has been obtained by stepwise adaptation of HT-29 cells to 10^{-6} or 10^{-5} M methotrexate (MTX) (Lesuffleur et al. 1990). They were a kind gift from Dr Lesuffleur (Unité de Recherches sur la Differenciation et la Neuroendocrinologie de Cellules Digestives, France). HT-29 MTX are a more differentiated cell line which

makes them suitable for experiments involving the effects of bacteria interaction on glycoconjugates.

2.2.1 Cell culture

Cells were grown as monolayers in Dulbecco's modified Eagle's medium (DMEM) (Sigma; Dorset, UK) supplemented with 10% FCS, 100 μg/ml penicillin, 100μg/ml streptomycin and 4 mM glutamine (all purchased from Sigma; Dorset, UK). Cells were maintained at 37°C in a humidified atmosphere of 5% CO_2, 95% air. The cells were released from the flask with 3ml trypsin (0.5mg/ml in versine) and routinely passaged at a 1:8 sub culture ratio when they had become 60% - 80% confluent. Cells at ~80% confluence were used for all experiments. The cells were used up for a maximum of 12 passages.

2.2.2 Cell freezing

In order to maintain cell stock levels 80-90% confluent cells were washed twice with PBS and released from the flasks with trypsin. The cell clumps were dispersed by gentle pipetting after the addition of DMEM routinely used for cell culture. The cell number was counted and the cell suspension was centrifuged at 1000g for 5 mins. Freezing medium [20% (v/v) FCS, 7% (v/v) dimethylsulphoxide (DMSO, Sigma) and 73% (v/v) DMEM] was prewarmed to 37 °C and was added to the pellet to make a cell density of 10^6 cells/ml. The cell suspension was immediately transferred to 1ml freezing vials (1ml/vial) and put into a box containing dry ice and kept at -80°C. 24 h later the freezing vials were transferred in to liquid nitrogen.

2.2.3 Cell thawing

Cells reaching 12 passages were discarded and new cells taking from stocks frozen in liquid nitrogen. The freezing vials were withdrawn from the liquid nitrogen

bank and placed in a 37°C water bath. Once the frozen cells had just melted, 20 ml of fresh 37°C culture medium was added and the mixture was centrifuged at 1000 g for 5 mins. The cell pellet was dispersed in 10 ml culture medium and seeded T75 cm^2 culture flasks with 2-5 x 10^5 cells per flask. After one day of culture the medium was replaced. The cells were used between 2-12 passages.

2.3 Protein measurement

All protein concentrations were determined using the Bicinchoninic Acid method (BCA) (Smith et al. 1985) with a commercially available kit (Sigma, UK). The BCA assay primarily relies on the reaction causing the peptide bonds in protein reduce Cu^{2+} ions from the cupric sulphate to Cu^{1+}, this is a temperature dependant reaction and therefore temperature was kept at 37°C. The amount of Cu^{2+} reduced is proportional to the amount of protein present in the solution. Next, two molecules of bicinchoninic acid chelate with each Cu^{1+} ion, forming a purple-coloured product that strongly absorbs light at a wavelength of 562 nm.

The kit consists of:

- BCA solution
- Copper (II) sulphate pentahydrate 4% solution.
- bovine serum albumin (BSA), for the standard curve

A standard curve was constructed and samples added to plate as below:

- 100 µl copper sulphate solution was added to 5ml of the BCA solution.
- 20 µl of a serially diluted BSA standard ranging from 0 – 4 mg/ml was plated in 96 well plate and the solution of unknown protein concentration, with 200 µl of the BCA/Copper sulphate solution
- The plate was then incubated at 37°C for 30 min

- The plate was then removed and allowed to cool before the absorbance read at 562 nm (Tecan, Sunrise)

2.4 Bacterial strains and growth conditions

HM427 *E. coli* was previously isolated from a patient with Crohn's disease, the bacteria was classified as being localised beneath the mucus layer (mucosa-associated) (Martin et al. 2004). K12 was also used, it is an *E.coli* which was first isolated in 1921 from the stool of a malaria patient and it has been maintained in laboratory stocks as a pure strain for the last 75 years. E. coli K12 was the bacterial strain of choice in biochemistry labs because it was easy to grow and amenable to metabolic studies. All isolates were grown on Columbia agar plates with overnight incubation in air, at 37°C.

2.5 SDS polyacrylamide gel electrophoresis (PAGE)

SDS-Page was performed to separate cellular proteins according to size following the Laemmli method (Laemmli 1970). 1mm and 1.5ml thick polyacrylamide gels were cast using a self assembly system (Biorad, Hemel Hempstead, UK). Solubilised proteins were separated by SDS-PAGE either on 6%, 10% or 12% gels with a 3% stacking gel.

The preparation and composition of the reagents was as follows:

Resolving buffer 4X: Tris-base 36.3 g, pH 8.8 then make up to 200 ml with H_2O

Stacking Buffer 4X: Tris-base 12.0 g, pH 6.8 then make up to 200 ml with H_2O

Running buffer: Tris-base 30.67 g, Glycine 64,04 g, 2.2 g SDS (Sigma, UK) and H_2O up to 4000 ml (pH8.3)

Ammonium persulphate: 10% Ammonium persulphate (w/v) (Sigma, UK)

SDS: 10% (w/v) (Sigma, UK)

Acrylamide: 30% (w/v) (Sigma, UK)

Sample buffer: 4X stacking buffer 2.5 ml, Glycerol 1.0 ml (Sigma, UK), Mercaptoethanol 0.5 ml (Sigma, UK), 20% SDS in H_2O 1.0 ml, 1% Bromothymol blue 25 µl (Sigma, UK).

Table 2.1: Composition of resolving gels

Reagent	6% resolving gel	10% resolving gel	12% resolving gel
Acrylamide (30%)	2.0 ml	3.33 ml	4.0 ml
Resolving Buffer	2.5 ml	2.5 ml	2.5 ml
Deionised H_2O	5.35 ml	4.2 ml	3.35 ml
10% SDS	100 µl	100 µl	100 µl
TEMED	5 µl	5 µl	5 µl
10% Ammonium persulphate	50 µl	50 µl	50 µl

Table 2.2: Composition of stacking gel

Reagent	Stacking gel
Acrylamide (30%)	1.33 ml
Stacking Buffer	2.5 ml
Deionised H_2O	6.1 ml
10% SDS	100 µl
TEMED	10 µl
10% Ammonium persulphate	50 µl

The sample under examination was mixed 1:1 with sample buffer and heated at 100°C fir 5 min in an eppendorff heater. Between 20 µl and 80 µl were loaded on to each lane on 6%, 10% or 12% resolving polyacrylamide gels which were run at 200V in running buffer (25 mM Tris, 192 mM glycine, 10% SDS) until the bromophenol blue reached the 0.5 cm from the bottom. After the electrophoresis the gels were stained with coomassie R250 (Sigma, UK) in destain solution (Ethanol 100ml, acetic acid 100ml (BDH Chemicals, UK, H_2O to 1000ml) or electrotransferred to a nitrocellulose membrane.

A prestained molecular weight standard marker (Sigma, UK) was used throughout the experiments. The markers were reconstituted with 0.5 mL of 8 M urea and 0.5 mL of 2 X sample buffer when first received.

Table 2.3: Composition of prestained marker

Prestained marker	kDa
α_2-Marcroglobulin from Human Blood Plasma	174
β-Galactosidase from *E. coli*	115
Lactoferrin from Human Milk:	90
Pyruvate kinase from Rabbit Muscle	58
Fumarase from Porcine Heart	48.5
Lactic dehydrogenase from Rabbit Muscle	36.5
Trisphosphate Isomerase from Rabbit Muscle	26.6

2.6 Western blotting and lectin blotting

To transfer the proteins resolved by electrophoresis to a nitrocellulose membrane, the gel was first soaked in transfer buffer (Tris 6.06 g, Glycine 28.83 g, methanol 400 ml and H_2O to 2000ml). The gel was then sandwiched in a transfer cassette between a nitrocellulose membrane and 3 mm filter paper either side. On each outermost side a transfer sponge was placed and the cassette placed into the transfer apparatus (Bio-Rad, Hemel Hempstead, UK) with an ice pack for 1 h at 100V, in transfer buffer.

After transfer the membranes were blocked with PBS-buffered saline (PBS) containing 1% (w/v) BSA, 0.1% (v/v) Tween-20 (PBS-T) for 1h in sealed container placed on a rolling platform. Membranes were then probed with appropriate primary antibodies [Table 2.4], followed by 3 washes of 10 minutes each in a sealed container placed on a rolling platform in PBS-T. The membranes were then incubated with peroxidase-conjugated secondary anti Ig antibodies (Dako; Glostrup, Denmark), or lectins [Table 2.5] which are peroxidise-conjugated or biotin-conjugated in which case Extravidin peroxidise (Sigma; Dorset, UK) was used as a secondary reagent followed

by 6 washes in PBS-T of 10 minutes each in sealed container placed on a rolling platform and a final wash in PBS for 10 minutes. Signal detection was achieved using a chemiluminescence Super-signal immunoblotting detection kit (Pierce; Rockford IL, USA) which was detected with a Fluor-S multi-imager (Bio-Rad; Hemel Hempstead, UK). Equal loading was confirmed using an anti-tubulin antibody. Densitometric analysis of immunoreactive protein bands was performed using Quantity One software (Bio-Rad; Hemel Hempstead, UK).

Table 2.4: Description of antibodies

Antibody (Source)	Raised in/purified from	Monoclonal/ polyclonal	Stock concentration	Dilution			Buffer
				WB	IP	IH	
Orp150 (IBL Hamburg: 10301)	Mouse	Monoclonal	1mg/ml	1:5000	1:200	n/a	PBS-T
Hsp70 (Bioquote: SPA-810)	Mouse	Monoclonal	1mg/ml	1:2000	n/a	1:200	PBS-T
Hsp70 (Sigma: H5147)	Mouse	Monoclonal	1mg/ml	1:5000	n/a	1:200	PBS-T
Hsp90 (Abcam AB58950)	Mouse	Monoclonal	1mg/ml	1:5000	1:500	n/a	PBS-T
Tubulin (Sigma)	Mouse	Monoclonal	1mg/ml	1:1000	n/a	n/a	PBS-T
Erp72 (BD Bioscience)	Mouse	Monoclonal	1mg/ml	1:1000	n/a	n/a	PBS-T
Giantin (Sigma)	Rabbit	Monoclonal	1mg/ml	1:5000	1:500	1:500	PBS-T
Ran (BD bioscience: 610340)	Mouse	Monoclonal	1mg/ml	1:5000	1:500	1:500	PBS-T

Table 2.4: Description of antibodies used in experiments. WB= western blot, IP= immunoprecipitation, IH= immunohistochemistry

Table 2.5: Description of lectins

Lectin (Cat number)	Purified from	ligand	Stock concentration	Dilution		Buffer
				WB	Purification	
ABL (EYlabs) Biotinylated (BA-50001-01)	*Agaricus bisporus* (common mushroom)	TF/sialyl TF	1mg/ml	1:2000	1ml/1ml (v/v)	PBS
JAC Biotinylated (B-1155)	*Artocarpus integrifolia* (Jack fruit)	TF/sialyl TF	1mg/ml	1:2000	1ml/1ml (v/v)	PBS
MAL II Biotinylated (B-1265)	*Maackia amurensis*	Sialic acid (α2,6 linked)	1mg/ml	1:2000	n/a	PBS
PNA-Biotinylated (B-1075)	*Arachis hypogaea* (Peanut)	TF	1mg/ml	1:2000	n/a	PBS
VVA-Biotinylated (B-1235)	*Vicia villosa* (Hairy vetch seeds)	Tn	1mg/ml	1:2000	n/a	PBS

Table 3: Description of lectins used in experiments JAC, MAL II, PNA and VVA sourced from Vector (Peterborough, UK), ABL sourced from EY labs (CA,USA) except WB= western blot

2.7 Slot blotting

Nitrocellulose membrane was soaked in PBS for 10 minutes before use. Cells were directly lysed in slot blot buffer (4 x stacking buffer 2.5 ml, Glycerol 1.0 ml (Sigma, UK), Mercaptoethanol 0.5 ml (Sigma, UK), 20% SDS in H_2O 1.0 ml) and were loaded on to the respective well after dilution with 100µl of Tris buffer to ensure even distribution of proteins. The vacuum pump was then allowed to run for 10 mins, before removal of membrane washing 3 times in PBS and blocking with 5% BSA in PBS-T in sealed container placed on a rolling platform. The nitrocellulose membrane could then be used for western/ lectin blotting.

2.8 Statistical analysis

Sample groups were analysed using one-way analysis of variance (ANOVA) followed by selected pair-wise comparisons of treatment means using Bonferroni's modified t-test (StatsDirect v2.3.1; StatsDirect Ltd; Sale, UK). Differences were considered significant when $P<0.05$.

CHAPTER 3

HYPOTHESES AND AIMS

3.1 HYPOTHESES

1. Cytoplasmic Orp150 is modified by sialyl-TF and is involved in NLS-dependent nuclear protein import.

2. Unsubstituted or sialic acid-substituted TF carbohydrate structures may also occur on cytosolic proteins other than Orp150.

3. Alterations in cellular *O*-glycosylation will cause an alteration in the NLS-dependent nuclear protein import of Hsp70.

4. Bacteria may interact with the colon epithelial cells to disrupt the Golgi apparatus and affect glycosylation.

3.2 AIMS

- To purify and characterise the different isoforms of Orp150.

- To characterise the role of Orp150 in NLS-nuclear protein import.

- To purify sialyl-TF expressing cytoplasmic glycoproteins.

- To characterise purified proteins utilising mass spectrometry and lectin binding.

- To analyse the effects of bafilomycin, TNFα, 5-CDP and benzyl GalNAc on TF and sialic acid expression on HT-29 MTX cells.
- To evaluate the effect of inhibitors of glycosylation on NLS-dependent nuclear protein import by studying the nuclear localisation of Hsp70 upon heat shock.

- To utilise siRNA to downregulate the Galtransferase responsible for the generation of Core1 glycans by analysing TF expression on HT-29-MTX cells.
- To investigate the effect of the siRNA Galtransferase on NLS-dependent nuclear protein import.
- To investigate the effect of bacterial supernatant HM427 on the structure of the Golgi and TF expression in colon epithelial cells.
- Release of the pro-inflammatory cytokine, IL-8, to be measured as a positive control to monitor the response of epithelial cells to bacterial interactions.

CHAPTER 4

PURIFICATION OF CONSTITUTIVE CYTOPLASMIC ORP150 AND CHARACTERISATION OF ITS GLYCOSYLATION

4.1 HYPOTHESIS

Cytoplasmic Orp150 is modified by sialyl-TF and is involved in NLS-dependent nuclear protein import.

AIMS

- To purify and characterise the different isoforms of Orp150
- To characterise the role of Orp150 in NLS-nuclear protein import

4.2 INTRODUCTION

It was traditionally thought that glycosylation was limited to cell membrane and secreted molecules, as a result of the believed restricted localisation of glycotransferases to the ER/Golgi. It has now been shown that glycosylation exists on certain cytoplasmic and nuclear proteins, this was best exemplified by the discovery of *O*-GlcNAc (Hart et al. 1989). It was recently shown by our group that a truncated cytoplasmic constitutively expressed isoform of Orp150 is glycosylated by the addition of sialyl-TF antigen (Yu et al. 2002) and this is so far one of only two reports that demonstrate this carbohydrate antigen on a cytoplasmically localised protein. The other is alpha-synuclein, a protein that is ubiquitinated by parkin in a process that is altered in Parkinson's disease (Shimura et al. 2001). Further study of Orp150 and its intriguing glycosylation may lead to a better understanding of *O*-glycosylation and possibly to uncovering of a novel glycosylation mechanism.

4.2.1 Oxygen-Regulated protein 150 KDa (Orp150)

Oxygen-Regulated protein 150 KDa (Orp150) has been demonstrated to have a number of roles and functions, the best described of which is as an endoplasmic reticulum localised stress protein (Tamatani et al. 2001). Orp150 was first purified as a novel stress protein in cultured rat astrocytes which had been subjected to hypoxia (Kuwabara et al. 1996). Astrocytes are the most abundant cell in the central nervous system, which are involved in the nurture of neurons and their protection from cellular stress. It was revealed that Orp150 protein levels that were induced by 24 h of hypoxia, rose slightly on reoxgenation and returned to normal after 24 h (Kuwabara et al. 1996). The Orp150 expression was the result of *de novo* protein synthesis, as it could be inhibited by cycloheximide, and other forms of stress such as 2-deoxyglucose, hydrogen peroxide, cobalt chloride or tunicamycin failed to induce expression of Orp150 indicating that the Orp150 stress protein is induced selectively under oxygen deprivation (Kuwabara et al. 1996).

Orp150 was later found to be expressed in cultured human aortic smooth muscle cells and mononuclear phagocytes that were exposed to oxygen deprivation, and thought to play a part in a protective mechanism in human atherosclerotic plaques allowing mononuclear phagocytes to withstand cellular stress on exposure to hypoxia and modified low density lipoprotein (Tsukamoto et al. 1996). Ikeda and colleagues were the first to clone the cDNA encoding the human stress form of Orp150 from hypoxic astrocytes (Ikeda et al. 1997). A deduced amino acid sequence of 999 residues indicates the presence of a signal peptide at the *N*-terminus and an ER retention sequence (KDEL) at the *C*-terminus. This form of Orp150 also shows close sequence similarity with Chinese hamster 170 kDa glucose-regulated protein (Grp170) with the *N*-terminal portion of the sequence also showing similarity with the well conserved

ATP binding domains of the Hsp70 family of proteins. Ikeda and colleagues also demonstrated that the induction of Orp150 in U373 astrocytoma cells was not limited to hypoxia, but also induced by 2-deoxyglucose and tunicamycin treatment (Ikeda et al. 1997). When the entire human Orp150 gene was sequenced, analysis of the transcription initiation sites and transcriptional regulatory sequences uncovered at least three distinct mRNA species from alternative promoters (Kaneda et al. 2000). In a detailed study Kaneda and colleagues demonstrate that two of the mRNA species begin from alternative exon 1 (1A or 1B), and the third one starts from exon 2, which is located 6 nucleotides upstream of the initial AUG initiation codon. The transcript beginning with exon 1B was preferentially induced by hypoxia and a cis–acting segment which has a role in the stress-dependent induction was discovered at the 5'-end of exon 1A. This could account for the selective induction of transcription from exon 1B. The induction of Orp150 is distinct from the unfolded protein response (UPR), or ER stress response pathway (Yoshida et al. 1998), and the Orp150 gene showed no enhancers similar to the hypoxia-inducible factor 1 (HIF-1) (Semenza et al. 1991). *In vitro* analyses of translation of the third mRNA, indicates the constitutive expression of an *N*-terminally truncated form, lacking the ER signal peptide as a result of altered translation and suggesting localisation in the cytosol. Little is known about the function of Orp150, however the stress-dependent induction from exon 1B and ER localisation would suggest a role in protection against ER stress induced deletions (Kaneda et al. 2000).

Other studies of Orp150 show a role in the regulation of tumour angiogenesis via processing of vascular endothelial growth factor (VEGF) (Ozawa et al. 2001, Ozawa et al. 2001). Thus, reduction of Orp150 by adenoviral mediated antisense ORP150 cDNA transfer, caused a reduction in tumorigenicity and angiogenicity in

human prostate cancer cells (Miyagi et al. 2002). Orp150 protein levels have been shown to be elevated in breast cancer (Stojadinovic et al. 2007) and anti-Orp150 autoantibody levels in Type 1 diabetic patients are higher compared with non-diabetic subjects, suggesting that ER stress is increased in Type 1 diabetes (Nakatani et al. 2006). Orp150 has also been shown to protect dopaminergic neurons against MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine), a drug used to induce experimental Parkinsons disease (Kitao et al. 2007).

4.2.2 Cytoplasmic Orp150 as a ligand for edible mushroom lectin (*Agaricus bisporous)*

Experiments investigating the effects of TF-binding lectins on cellular proliferation demonstrated that PNA lectin stimulates proliferation of HT-29 human colon cancer cells (Ryder et al. 1992). This effect was also seen *in vivo,* stimulating the proliferation of the rectal epithelium in patients who had low-level expression of TF in their colonic mucosa and who ate a pack of peanuts per day for 5 days (Ryder et al. 1998). ABL, (*Agaricus bisporus*, the edible mushroom lectin), and jacalin lectins both display the opposite effect on HT-29 colonic epithelium cells by reversibly inhibiting proliferation with no apparent toxicity to the cells (Yu et al. 1993, Yu et al. 2001). It was further shown that ABL needed to be internalised to have its antiproliferative effect as ABL immobilised on agarose beads showed no effect on proliferation. Electron microscopy of gold-conjugated ABL revealed that ABL was internalised to the endocytotic vesicles and multivescular bodies. This was also confirmed by confocal microscopy of isothiocyanate-conjugated ABL which also inhibited proliferation and showed perinuclear accumulation, which signified a possible interference with nuclear protein import. This was confirmed when ABL prevented heat shock protein 70 translocation from the cytoplasm to the nucleus upon heat stress.

Further to this, in digitonin-permeabilised HT-29 cells, nuclear uptake of a NLS-peptide conjugated to BSA was inhibited by pre-incubation with ABL, indicating that ABL specifically blocks NLS-dependent nuclear protein import (Yu et al. 1999). Pulse labelling experiments following the location of the ABL have highlighted the nuclear localisation and reversibility of the ABL binding and hence reversibility of its anti-proliferative effect (Yu et al. 2000).

The determination of a cytoplasmic ligand for ABL was achieved through ABL lectin affinity chromatography of digitonin-released cytosolic proteins. The major glycoprotein purified expressed sialyl-TF and was identified by *N*-terminal sequencing as the *N*-terminally truncated form of Orp150.

4.2.3 Orp150 and NLS-dependent nuclear protein import

Thus, ABL inhibits the proliferation of HT-29 cells, and its intracellular ligand was shown to be Orp150, extractable from a digitonin-released cytosolic extract. The NLS-dependent nuclear import of proteins is also inhibited by ABL. NLS-dependent nuclear protein import mediates nuclear import of large proteins (>40-60 kDa). The classical NLS presented on a protein to be translocated in to the nucleus contains short stretches of basic amino acids which are recognized by an adapter protein, importin-α. Importin α then binds to importinβ, and the cargo-importin complex then translocates into the nucleus through nuclear pore complexes (Nakielny et al. 1999). RanGTP is responsible for the dissociation of the cargo-importin complex (Gorlich et al. 1996). The subsequent return of the importin-α is achieved by forming a trimeric complex with cellular apoptosis susceptibility gene protein (CAS) and RanGTP which is then translocated to the cytoplasm. Importin-β is bound to RanGTP before its return to the cytoplasm. Conversion of RanGTP to Ran GDP by GTPase-activating protein (RanGAP1) and Ran-binding protein (RanBP1) causes the dissociation of the Ran-

importin complexes freeing the importin proteins for reuse. RanGDP then returns to the nucleus with NTF2 (nuclear transport factor 2) and is converted back to RanGTP (Gorlich et al. 1996) .The localisation of Ran is therefore predominately in the nucleus with rapid oscillating movements to the cytoplasm. Orp150 is the intracellular ligand for ABL and therefore is implicated in the NLS-dependent nuclear protein import, the localisation of Ran upon depletion of Orp150 protein is examined in this study.

Further experiments have now been performed to characterise the glycosylation of Orp150 and assess its role in NLS-dependent nuclear protein import.

4.3 METHODS

4.3.1 Materials:

The anti-Orp150 antibody was obtained from IBL (Hamburg). Biotinylated Mushroom lectin (ABL), Hrp-ABL lectin, Biotinylated Jacalin (Jac), Hrp-Jac were all obtained from Vector (Peterborough, UK). Peroxidase-conjugated secondary anti Ig antibodies were obtained from Dako (Glostrup, Denmark). Anti Hsp70 antibody was obtained from Sigma (Poole; UK). ER resident proteins ERp72 and protein disulfide isomerase PDI obtained from BD Biosciences (Oxford; UK). Jacalin-bound agarose beads obtained from Vector (Peterborough, UK). siRNAOrp150 transfection reagents obtained from Dharmcon (Perbio science, UK)

4.3.2 Glucose starvation of HT-29 cells

A T75cm^2 flask of HT-29 human colon cancer cells, was cultured as a monolayer to 60% confluence in DMEM at 37°C in a humidified atmosphere of 5% CO_2, 95% air, then washed 2 times with PBS, and then incubated for a further 24h in glucose-free DMEM. The monolayers were then washed 3 times with PBS before being directly lysed in Lammelli's sample buffer and run on a 6% SDS PAGE electrophoresis gel, before immunostaining with anti-Orp150 antibody.

4.3.3 Lectin purification

Ten T75 flasks of HT-29 cells were grown to 60% confluence, before starvation of glucose for 24 h. The cells were released with 3 ml trypsin and washed 3 times with PBS each time centrifuge at 70 *g* for 1 min (1000 rpm; MSE microcentaur, Sanyo Gallenkamp PLC, UK). The cells were collected then suspended in 15ml sterile PBS and sonicated (*5 x 10s* pulses at 28% amplitude; GEX 400 Ultrasonic Processor; Sigma) on salt ice followed by ultra centrifugation for 1 h at 4°C (Beckman's). 10 ml

of jacalin-bound agarose beads (Vector, UK) were added and left to incubate for 12 h at 4°C. The beads were loaded 20 cm x 1cm column plugged with glass fibre enabling the beads to be washed with 60 ml sterile PBS, removing the unbound proteins. The proteins bound to the jacalin lectin were eluted with 20 ml 0.8 M galactose. The resulting eluate was loaded in to viscous tubing and subjected to dialysis in 2 litres of water which was changed at regular intervals and left overnight at 4°C. The volume was then reduced by freeze-drying in 6 equal aliquots at -40°C.

4.3.4 Digitonin permeabilisation of cells

HT-29 cells were grown as a monolayer to 60% confluence in a T150cm^2 flask, washed 2 times with PBS before incubation in DMEM without glucose for 24 h. Cells were then washed 2 times in ice cold PBS and the flask put on ice. Five ml ice-cold digitonin (50µl/ml) was added for 5 min to the flask to puncture the outer membrane of the cells. The digitonin-released fraction was then extracted and freeze dried at -40°C overnight. The cells remaining in the plate were lysed directly with sample buffer. Samples were then run on SDS electrophoresis gel and transferred to nitrocellulose membrane and western blot analysis with anti Orp150 (IBL, Hamburg) antibody performed.

4.3.5 siRNA Orp150 treatment

Cell plating:

HT-29 cells were diluted in antibiotic-free DMEM with 5% FCS to a plating density of 5.0 x 10^4 cells/ml. 100 µl of cells were placed into each well of a 96 well plate and incubated for 24 h.

Transfection with siRNA:

To perform the experiment in triplicate the following protocol was followed: a 2 μM siRNA solution in 1X siRNA buffer (Dharmacon) was prepared, from this 17.5 μl was added to 17.5 μl serum-free/antibiotic-free medium. In a separate tube 1.4 μl of DharmaFECT4 (Dharmacon) was added to 33.6μl of FCS-free/antibiotic-free DMEM. These tubes were pipetted up and down and left for 5 minutes before mixing them together and leaving to stand for a further 20 minutes. To this solution 280 μl antibiotic-free DMEM was added and to each well 100 μl was added. The plate was incubated at 37°C in a humidified atmosphere of 5% CO2, 95% air for 48 h. In each experiment non-specific siRNA (Dharmcon) was used as a negative control.

4.3.6 Hsp70 and Ran localisation

HT-29 cells were seeded at $1x10^4$ on glass cover slips, 24 h prior to siOrp150 treatment. After 2 days of siRNA treatment the cells were heat-treated for 1 hour at 42°C, and then fixed for10mins in freshly prepared paraformaldehyde followed by 3 washes with PBS. The fixed cells were then blocked with 300 μl of 5% goat serum (Dako; Glostrup, Denmark) for 1 hour at room temperature, followed by the addition of anti Hsp70 antibody (Bioquote, York) 1:200 (v/v) concentration or anti-Ran antibody (Bioscience, Cambridge) 1:500 concentration, for 2 h. The cells were then washed 2 times with PBS and then the FITC conjugated secondary antibody (Dako; Glostrup, Denmark) was added, at 1:500 (v/v), for 1 h in the dark. The cells were then washed 3 times in PBS and the cover slips mounted on glass slides with a PI nuclear counter-stain mounting solution. The localisation of Hsp70 or Ran was then visualised using an Olympus camera and the percentage nuclear Hsp70 or Ran calculated used AQM software.

Hsp70: Experiments were performed 5 times, each time 10 individual cells for each category were chosen at random from the slide for AQM analysis. Inter assay coefficient of variance (COV) 8%, intra assay COV 8.71% for all experiments.

4.3.7 2-D electrophoresis

HT-29 cells were grown to 60% confluence in a 24 well plate, prior to direct lysis of each well with 150 µl 2-D lysis buffer (8 M urea, 2 M thio urea, 4% Chaps, 1% DTT, 5 nM sodium vanadate, 40m M Tris) on ice. The sample was then left on a shaker for 1 h before centrifugation at 8000 g for 5 min. For the initial isoelectric focusing, 125 µl of sample was placed in a strip holder and a 4-7non-linear IPG (immobilised pH gradient) strip gel side down was applied and covered in mineral oil.

Focusing conditions for 7 cm IPG strips

Ramp	Linear
Temp	20°C
Rehydration	active@50v for 12 hours
Pause after hydration	No
Step 1	250V for 15min (linear ramp)
Step 2	4000V for 2 h
Step 3	4000V to 20000V/hours (rapid ramp)
Step 4	500V hold

The IPG strips were then rehydrated for 15 mins gel side up in 12 ml equilibration buffer (30% glycerol (v/v), 6 M urea, 2% SDS (w/v) and 50ml 1.25 M Tris-HCL to pH 6.8 in distilled H_2O) containing 240 mg DTT, followed by 15 mins in 12 ml equilibration buffer containing IAA (iodoacetamide).

The 6% 1-D SDS-PAGE gel was prepared previously and after removal of the butan-2-ol from the surface the iso-electrical focusing strip was laid on top ensuring a good contact between the two. A prestained marker with Coomassie blue was added at this point in a well on the end. Melted agarose was added to seal the strip in place. The gel was then run at 200V in running buffer (25 mM Tris, 192 mM glycine, 10% SDS) and electrotransferred to nitrocellulose membrane for 1 h, at 100V, in transfer buffer (25 mM Tris, 192 mM glycine and 20% (v/v) methanol). Identical gels were Coomassie stained and samples cut out from the gel.

In gel digest for 2D PAGE:

Bands were extracted using a scalpel and placed in an eppendorff tube followed by destaining with 50 mM ACN (Acetonitrile), 50mM ammonium bicarbonate at 37°C for 15 min. This was repeated until stain removed, and supernatant discarded. Gel plugs were then dehydrated in 100% ACN until opaque (10 µl/plug) and incubated at 37°C for approx 30-45 min. Gel plugs were then rehydrated in 50 mM ammonium bicarbonate and trypsin (9 µl ammonium bicarbonate and 1 µl trypsin stock, 100 ng/µl) over-night at 37°C, and stopped with 2 µl formic acid.

Supernatants were removed and analysed using an UltiMate nano-liquid chromatograph (LC Packings) connected to a Waters (Manchester, UK) Q-TOF Micro electrospray tandem mass spectrometer, operated in positive ion mode. Chromatography was using a µ-Precolumn C18 cartridge (LC Packings) connected to a PepMap C18 column (3µm 100Å packing; 15cmx 75µm i.d.), using a linear gradient of 5%(v/v) solvent B [0.1% v/v formic acid in 80% (v/v) acetonitrile in water] in solvent A [0.1% (v/v) formic acid in 2% (v/v) acetonitrile in water] to 100% solvent B over 60min at a flow rate of 200nl/min. The spectrometer was operated in Data

Directed Analysis (DDA) mode, where a survey scan was acquired from m/ζ 400-1500, with switching to MS/MS on multiply charged ions. MS/MS mass spectrum acquired over mass range 80-2000Da.

For the MS/MS data, MS/MS ion searches of the NCBI database were undertaken using the MASCOT search engine (http://www.matrixscience.com) to yield protein identifications. Searches were performed without restriction of protein Mr or pI and one trypsin miscleavage was allowed. Peptide mass tolerance and fragment mass tolerance were set to 2.0kDa and ±0.8kDa, respectively. Furthermore, partial peptide sequences were determined by manual interpretation of MS/MS data using the PepSeq software within the MassLynx package (Waters). The resulting sequences were then used to search the NCBI database using the BLAST algorithm and the option to search for short nearly exact matches (www.ncbi.nbm.nih.gov/BLAST/). Thus, high confidence identifications were made when statistically significant MASCOT search scores (<0.05) were consistent with the protein experimental pI and mW and when the manually-derived partial peptide sequences matched the database protein sequence.

4.4 RESULTS

4.4.1 Demonstration that Orp150 has two isoforms; a constitutive and a stress-inducible form

HT-29 colon cancer epithelial cells, when grown under normal conditions, have a single band after SDS-PAGE and Orp150 immunoblotting corresponding to constitutively expressed Orp150. Upon glucose starvation another band appears of slightly lower molecular weight representing the stress inducible form [Figure 43.1]

Figure 4.1: Immunoblot of two isoforms of Orp150

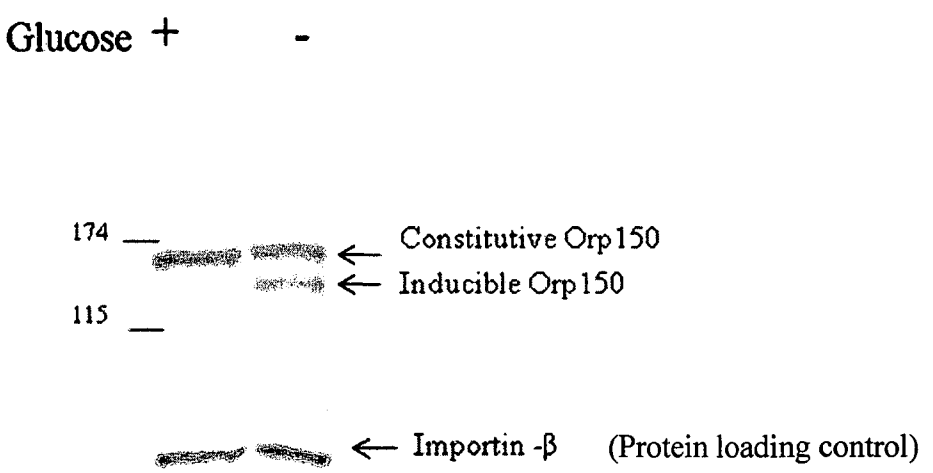


Figure 4.1. Anti-Orp150 immunoblot. A T75cm^2 flask of HT-29 human colon cancer cells was cultured to 60% confluence and then washed twice with PBS, and then incubated for a further 24 h in glucose free-medium. The cells were released with trypsin then lysed in sample buffer and the cell extracts electrophoresed on an SDS PAGE (6% w/v) electrophoresis gel. Two distinct bands are observed, a constitutively expressed isoform and a slightly lower molecular weight stress-inducible form.

4.4.2 The constitutive but not the inducible form of Orp150 can be isolated by Jacalin or AB- lectin affinity purification

HT-29 cells were grown to 60% confluence under normal conditions, then released by with trypsin and cells recovered by centrifugation. The cells were sonicated then centrifuged, and the resulting whole cell lysate was subjected to lectin affinity chromatography purification with agarose-linked jacalin or ABL which both bind TF in its native or sialylated form. Both jacalin and ABL were able to isolate Orp150, however jacalin is also able to isolate 2 other proteins of molecular weight ~110kDa and ~90kDa [Figure 4.2]. Jacalin binding glycoproteins of a similar molecular weight have been found in human astrocytes and shown to have *N*-terminal sequence similarity with Hsp70 protein (Ikeda, J., et al. 1997).

Figure 4.2: Immunoblot of glycoproteins purified by Jacalin and ABL lectin affinity purification

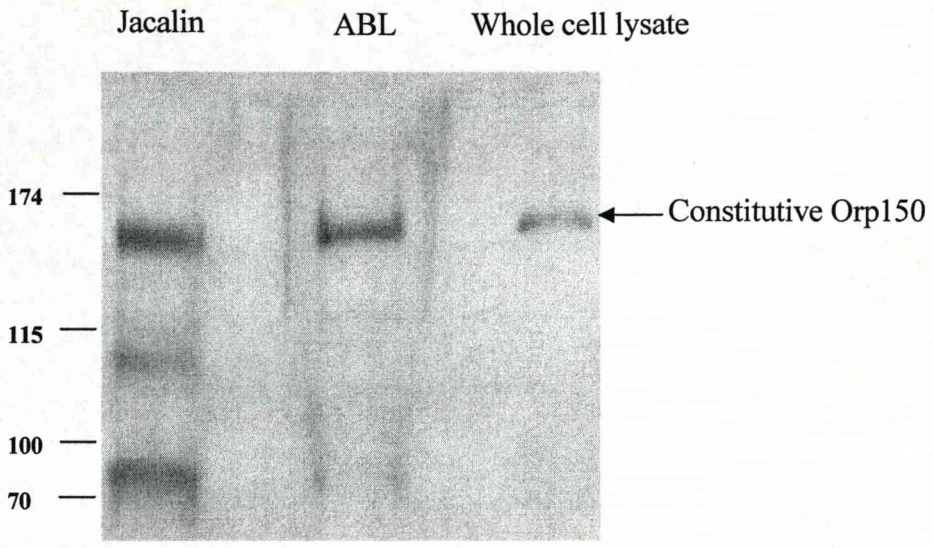


Figure 4.2: Anti Orp150 immunoblot. Jacalin and mushroom lectins conjugated to agarose beads are both able to isolate constitutive Orp150 protein from a whole cell lysate. The jacalin lectin is shown to bind at least two other proteins.

Following this, HT-29 glucose-starved cells were subjected to lectin affinity chromatography, and it is shown that only the constitutive form and not the stress inducible form is isolated by the jacalin lectin [Figure 4.3].

Figure 4.3: Jacalin affinity purification of constitutive Orp150

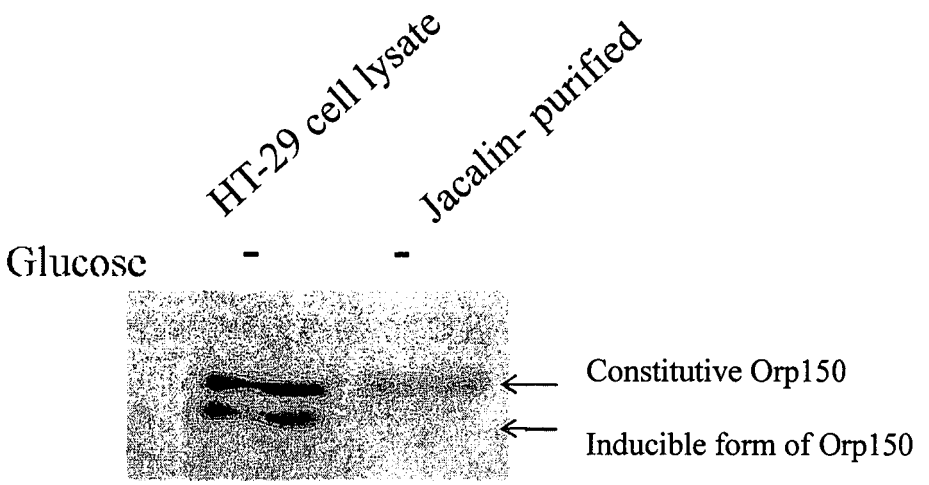


Figure 4.3: Anti-Orp150 immunoblot. Jacalin affinity purification: 1 flask of HT-29 cells was grown to 60% confluence and then starved of glucose for 24 h. The cells were extracted with trypsin and placed in PBS and washed. The solution was then sonicated and ultra-centrifuged for 1 h. To the resulting supernatant, jacalin-bound agarose beads were added and left to incubate for 12 h. The beads were washed and the bound proteins eluted with 0.8 M Galactose. A dialysis against water was then performed before freeze drying and running samples on SDS PAGE 6% at 200V. The jacalin lectin is able to bind constitutive Orp150, and release the protein again after washing with 0.8 M galactose. The lectin recognises the sialyl-TF or TF structure which therefore seems to be expressed only on the constitutive form.

4.4.3 but.... mass spectrometry of Orp150 fails to reveal sialyl TF

Lectin affinity purification with jacalin was used to purify constitutive Orp150 [Figure 4.3] to be analysed by nano LC-ESI-MS/MS on a Q-STAR mass spectrometer (Imperial College London). The returned data indicated the presence of 4 high mannose N-glycopeptides the most abundant of which were $Man_{5-8}GlcNAc_2$. One further glycopeptide was observed at m/z 747^{3+} indicating the presence of a single HexNAc. No evidence for the presence of sialyl-TF or TF was returned. Possible explanations for the lack of identification of sialyl-TF might include: variation in glycosylation under different conditions and loss of or damage to *O*-glycans during processing for mass spectrometry, this is discussed later.

Figure 3.3: Purification of constitutive Orp150

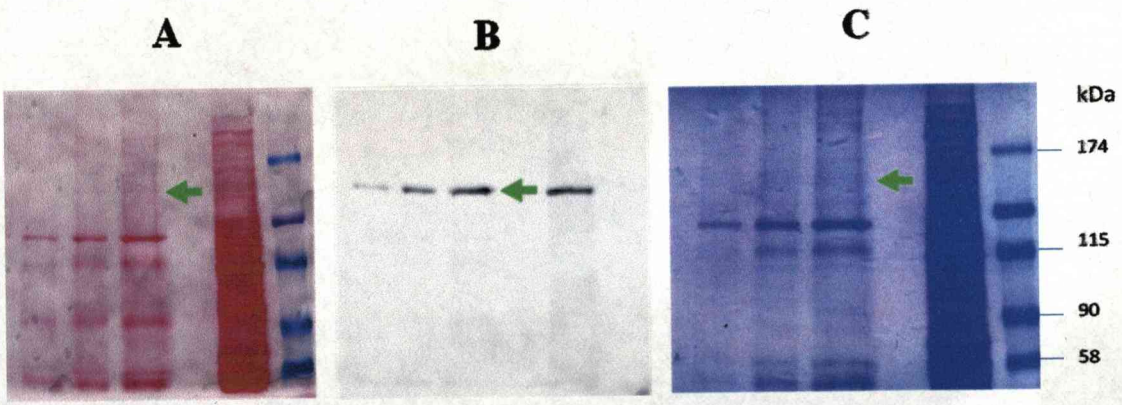


Figure 4.3: Five 150 cm^2 flasks of 60% confluent HT-29 cells were harvested by scraping into 15ml ice cold PBS. The cells were sonicated and ultra centrifuged for 1 h. Jacalin bound agarose beads were added to the supernatant and left to incubate for 12 h. The beads were washed and the bound proteins eluted with 0.8 M galactose. A dialysis against water was then performed before freeze drying and running samples on SDS electrophoresis gel stained by coomassie (C). A ponceau S red stain (A) then an anti- Orp150 western blot (B) was performed on an SDS-PAGE gel run at the same time. The green arrow indicates Orp150.

4.4.4 The constitutive Orp150 isoform is localised in the cytoplasm

The localisation of the 2 isoforms of Orp150 was determined through digitonin permeabilisation, allowing the cytoplasmic contents to be isolated. Immunoblotting demonstrates that the constitutive isoform is present in the digitonin-released cytoplasmic fraction; the stress inducible form is not [Figure 4.4].

Figure 4.4: Representative immunoblot showing localisation of constitutive Orp150

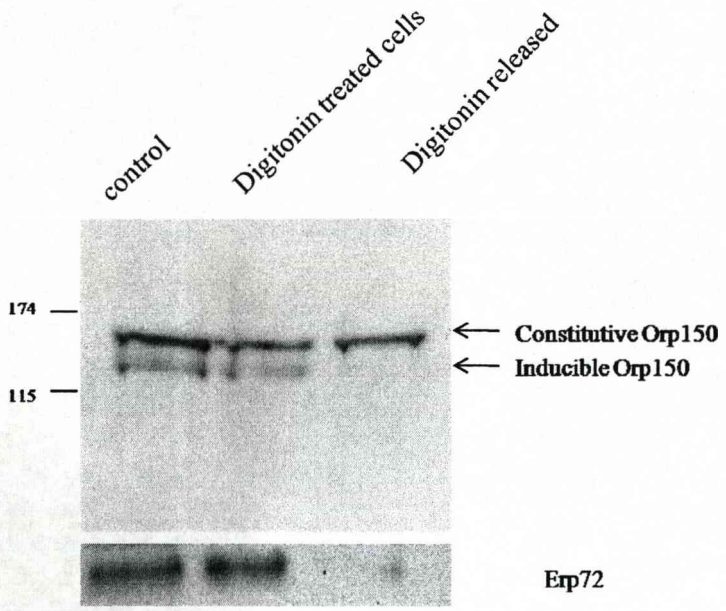


Figure 4.4: Anti-Orp150 immunoblot. HT-29 cells were grown to 60% confluence in a T150cm^2 flask then starved for 24h of glucose. Cells were washed 2 times in ice cold PBS. 5ml digitonin (50 µl/ml) was added for 5 min to puncture the outer membrane of the cells. The digitonin extract was then freeze-dried. The cells remaining on the plate were lysed directly with sample buffer. Digitonin selectively permeabilises the outer cell membrane whilst keeping the internal membranes and compartments intact, yielding a cytoplasmic fraction (Holaska et al. 2001). This is confirmed by the lack of Erp72, which is an ER localised protein, in the digitonin-released sample. The

localisation of the constitutive form thus appears to be in the cytoplasm. HT-29 cells were starved of glucose to induce the stress form of Orp150 as shown in the control lane. The two isoforms of Orp150 are clearly seen in the control and digitonin-treated fractions, however, the inducible isoform is not seen in the digitonin-released fraction.

4.4.5 Silencing RNA used to down regulate Orp150 protein expression in HT-29 cells to allow assessment of its role in nuclear protein import

The mushroom lectin, ABL, has previously been shown to reversibly inhibit cellular proliferation with no apparent toxicity to the cell (Yu et al. 1993). Further investigation has shown that ABL most likely causes this inhibition of proliferation by preventing NLS-dependent nuclear protein import. The internal ligand for ABL was revealed by lectin affinity purification utilising ABL, which results in the isolation of Orp150 (Yu et al. 2002). The use of siRNA to downregulate the Orp150 protein expression should be an effective tool to assess the role of Orp150 in NLS-dependent nuclear protein import.

Silencing RNA directed towards Orp150 caused a decrease in Orp150 protein expression, as measured by densitometry after immunoblotting [Figure 4.5]. This decrease was first significant after 2 days with an average (±SD) 71 ± % reduction from control ($p<0.005$) [ANOVA] (n=6) inter assay coefficient of variance 7.1% and also observed on Day 3 65 ± 2% (n=3) ($p<0.005$). After 4 days Orp150 protein expression increased again with only a 12 ± 1% (n=3) reduction observed [Figure 4.6]. These data were obtained from 3 time course experiments over 4 days, and a further 3 experiments for siRNA transfection over 2 days. Any further experiments with siRNA Orp150 utilised a 2 day transfection, as it resulted in the optimum reduction of Orp150 protein expression.

Figure 4.5: Representative immunoblot of Orp150 protein expression after siRNA treatment.

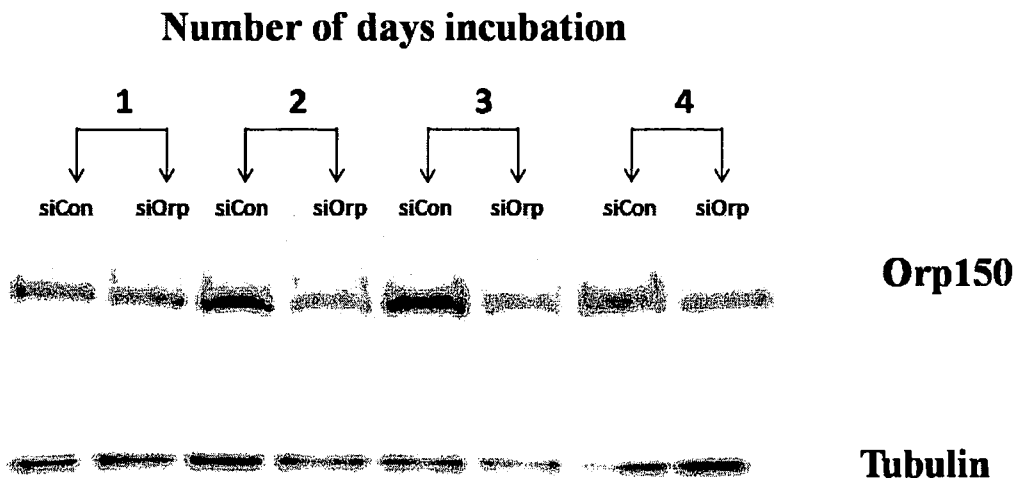


Figure 4.5: Anti-Orp150 western immunoblot. HT-29 cells were treated with siRNA Orp150 for 1-4 days prior to lysis and SDS-PAGE. The representative immunoblot shows each day's control on the left and treated with siRNA on the right. Statistical analysis of densities is displayed in Figure 4.6.

Figure 4.6: Orp150 protein reduction after siRNA treatment

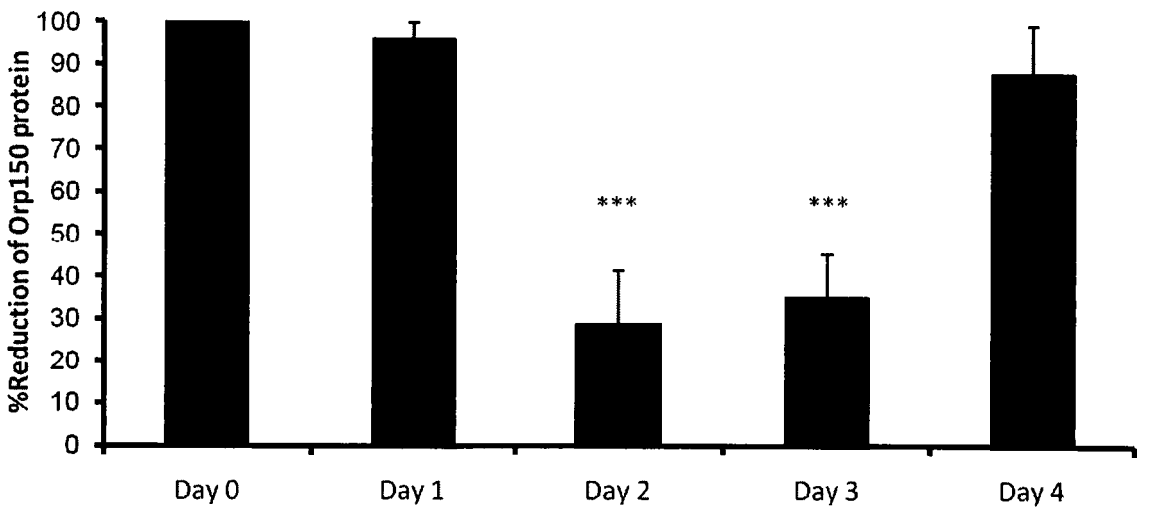


Figure 4.6: A 4 day time course incubation with siRNA to silence Orp150 results in knock down of Orp150 protein expression, which by days 2 and 3 had reached 71 ± 2% and 65 ± 2% respectively (n=3). This was (*** $p<0.001$) compared to day 0.

Following this time course experiment the effect of siRNA Orp150 was studied at 2 Days incubation [Figure 4.7]. It was shown that siRNA Orp150 reduced Orp150 protein expression after a 2 day incubation by an average 63 ± 6%.

Figure 4.7: Representative immunoblot of 2 day siRNA Orp150 treatment of HT-29 cells

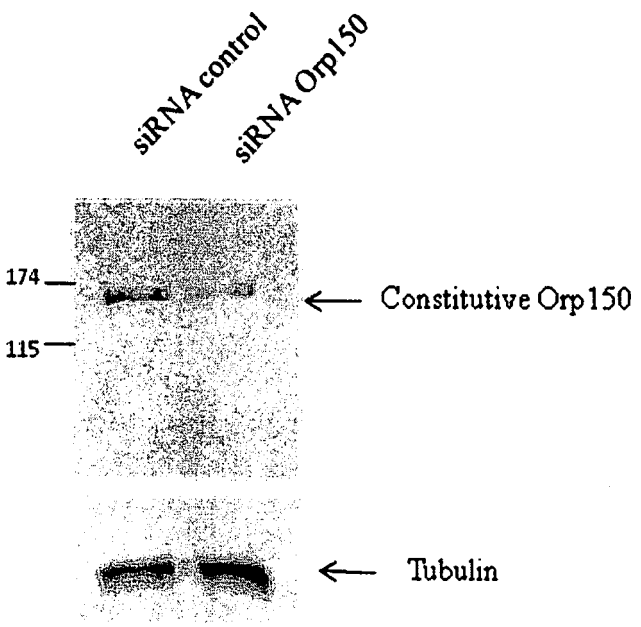


Figure 4.7: Anti-Orp150 western immunoblot. $1x10^5$ HT-29 cells were treated with siRNA Orp150 or siRNA control for 2 days prior to direct lysis and SDS-PAGE.

Table 4.1: Percentage reduction of Orp150 protein with 2 day siRNA Orp150 treatment

	Expt 1	Expt 2	Expt 3	Expt 4	Expt 5	Expt 6	Average
Density of siRNA control band	75	67	69	70	75	66	70 ±4
Density of siRNA Orp150 band	24	20	30	29	27	25	26±4
% Reduction	68	70	56	58	64	62	63±6

Figure 4.8: Percentage Orp150 reduction after 2 day siRNA orp150 treatment

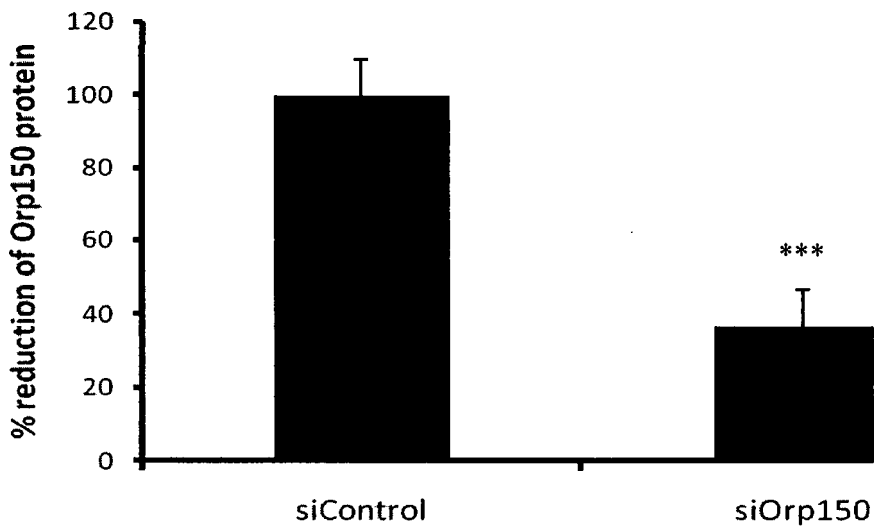


Figure 4.8: HT-29 cells with 2 day treatment with siRNA Orp150 show an average 63 ± 6% reduction of Orp150 protein expression ($p<0.001$, n=6)

4.4.6 A reduction of Orp150 protein expression using siRNA leads to a subsequent reduction of Hsp70 nuclear localisation upon heat shock

The role of Orp150 in NLS-dependent nuclear protein import was investigated by reducing the Orp150 protein expression with siRNAs. HT-29 cells were transfected with siRNA Orp150 or siRNA Control for 2 days prior to investigation into localisation of Hsp70. Hsp70 can clearly been seen to translocate from the cytoplasm and concentrate in the nucleus after heat treatment (1 h at 42°C) [Figure 4.9]. This movement was quantified with AQM software and the % nuclear fluorescence calculated, each experiment was repeated 5 times, each time 10 cells were randomly selected from slide, with the inter assay coefficient of variance <8%, intra assay coefficient of variance <8% for all experiments.

For the control cells nuclear fluorescence (±SD) was 46±4% (n=50), compared with heat treated control 58±3% (n=50), ($p<0.001$). HT-29 cells transfected with siRNA control under normal conditions displayed a nuclear fluorescence of 46±4% (n=50) which is comparable to the control cells. SiRNA control transfected cells after heat treatment displayed a nuclear fluorescence of 59±2% (n=50) which is also comparable to heat treated control cells. SiRNA Orp150 transfected cells after heat treatment had a nuclear fluorescence of 51±3% (n=50, which when compared to siRNA control heat treated cells is a 61% reduction in Hsp70 translocation into the nucleus ($p<0.001$) [Figure 4.10, 4.11].

Figure 4.9: Hsp70 localisation in HT-29 cells

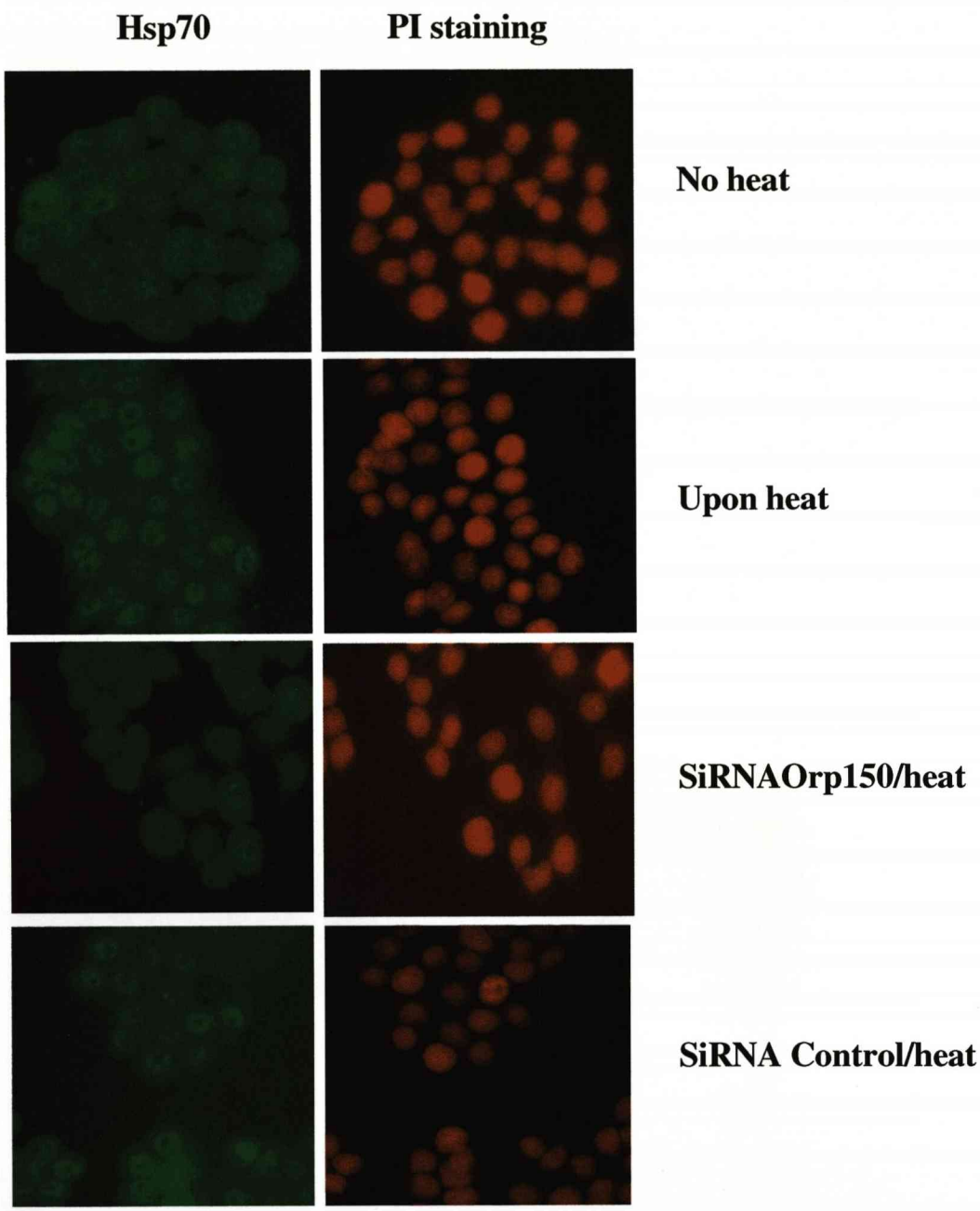


Figure 4.9: Representative immunohistochemistry (x40 objective) of the localisation of Hsp70 as visualised in Green. The Red is a nuclear PI stain. The Hsp70 is seen to be localised more strongly in the nucleus under heat shock in control cells. This effect is reduced in the siRNA Orp150-treated cells, but is unaffected in the siRNA control cells. The overall average reduction (n=5), in nuclear fluorescence after transfection with siRNA Orp150 is 60% compared to the siRNA control (COV 4%).

Figure 4.10: Hsp nuclear fluorescence with heat treatment after 2 day SiRNA Orp150 treatment

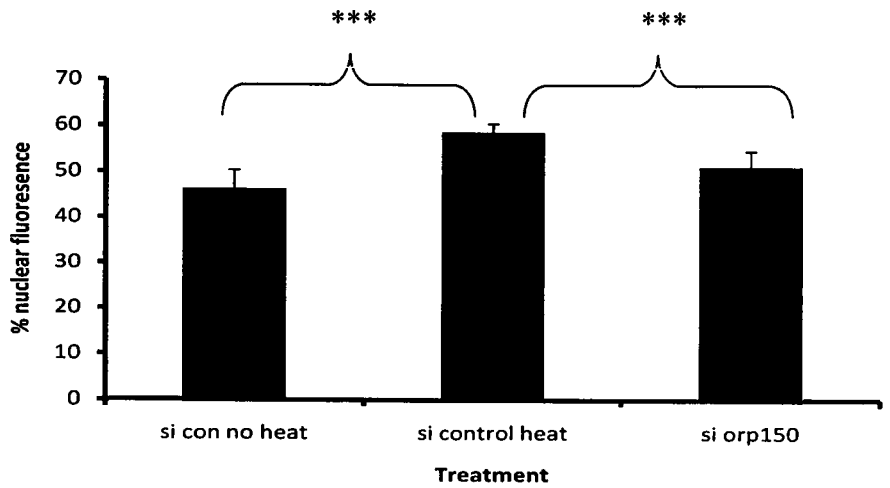


Figure 4.11: Percentage inhibition of Hsp70 localisation following heat treatment after 2 day SiRNA Orp150 treatment

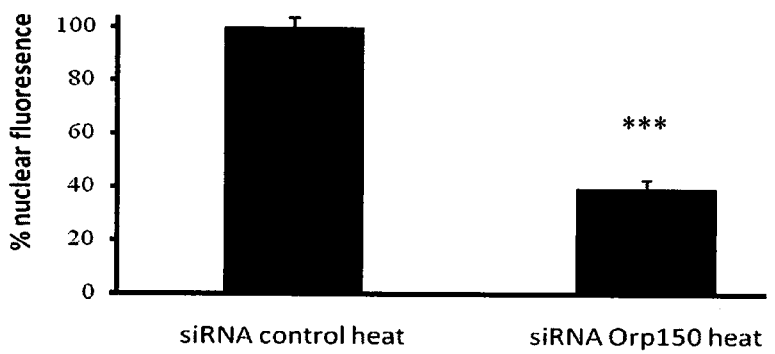


Fig 4.10 and 4.11: 2 day Treatment of HT-29 cells with siRNA Orp150 results in a subsequent reduction in Hsp70 nuclear fluorescence after heat treatment when compared to siRNA control heat treated cells. Hsp70 nuclear fluorescence was 59±2% for siRNA control cells (n=50) and siRNA Orp150 cells 51±3% (n=50), siRNA control with no heat treatment mean was 46±4% (n=50) [Figure 4.10]. Thus there was an overall mean reduction of Hsp70 nuclear localization after 2 day treatment with siRNA Orp150 of 61% when compared to siRNA control cells (p=<0.001 ***) [Figure 4.11]

4.4.7 Hsp70 protein level was not affected by siRNA treatment

The protein expression level of Hsp70 was measured by measuring the density of bands on immunoblots from 3 repeated experiments [Figure 4.12]. The overall protein levels were seen as expected to increase upon heat shock, control density mean (±SD) was 43±7 arbitrary densiometric units (ADU) and after heat shock 65±6 ADU ($p<0.001$). Transfection with siRNA control caused no significant change, no heat 40±10 ADU, and heat 63±12 ADU. Transfection with siRNA Orp150 also caused no significant change, no heat was 41±11 ADU and heat was 65±6A U [Figure 3.13]

Figure 4.12: Representative immunoblot showing Hsp70 protein expression is not affected by siOrp150 treatment

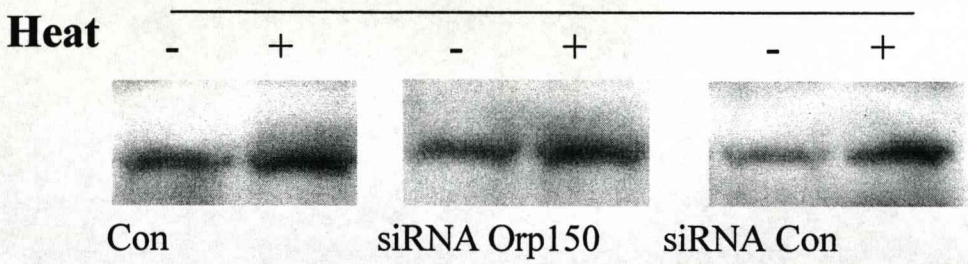


Figure 4.12: Anti-Hsp70 immunoblot. The Hsp70 protein expression of HT-29 cells with or without 2 day siRNA control or Orp150 treatment and with or without heat stress was calculated via western blotting and subsequent densitometry of bands. Overall no change in Hsp70 protein expression was observed in response to siRNAOrp150 although heat stress as expected caused an increase in expression.

Figure 4.13: Hsp70 protein expression with siRNA Orp150 treatment

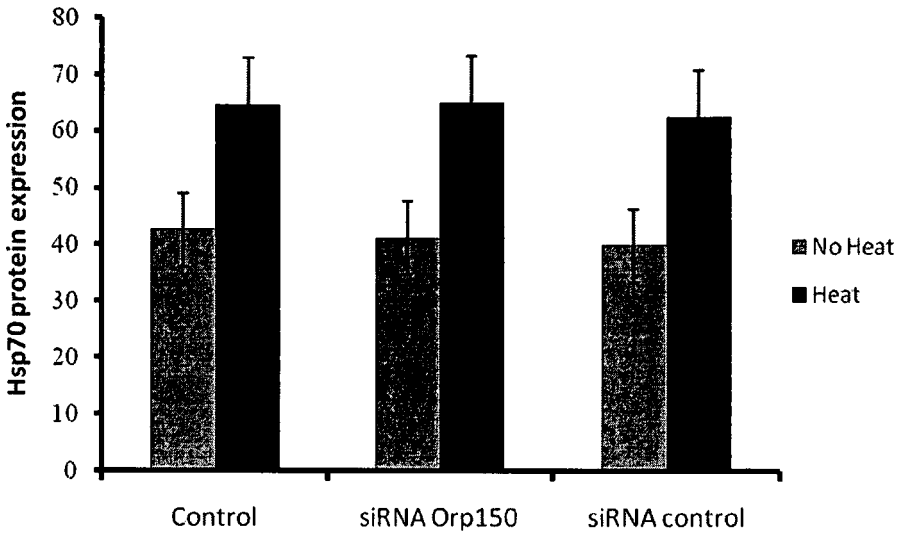


Figure 4.13: HT-29 cells with or without siRNA treatments and with or without heat stress. Overall Hsp70 protein expression is seen to increase with heat stress but protein expression was not altered by siRNA treatments. Transfection with siRNA control caused no significant change, no heat mean (±SD) 40±10 ADU, and heat 63±12 ADU. Transfection with siRNA Orp150 also caused no significant change, no heat 41±10 ADU and heat was 65±14 ADU.

4.4.8 Localisation of Ran is unaffected by reduced Orp150 protein levels

Previous investigations have shown that Ran is co-immunoprecipitated with Orp150 and Ran is also isolated by ABL affinity purification of cell extracts as shown by immunoblotting (Yu et al. 2002), therefore, it is possible that Orp150 may form a functional complex with Ran inside the cell. An interaction of Orp150 and Ran would imply that Orp150 could be involved in the cycling of Ran-GTP and Ran GDP between the nucleus and the cytoplasm, a process that is vital in the control of nucleocytoplasmic transport. Ran resides predominantly in the nucleus, shuttling rapidly between the nucleus and cytoplasm. This investigation, therefore, assessed whether the nuclear localisation of Ran is disrupted by reducing Orp150 protein levels in HT-29 cells by siRNA and also observed with the effects of heat stress.

Ran localisation was unaffected by siRNA Orp150 with and without heat stress. Ran localisation has been shown to be spread from the nucleus into the cytoplasm under heat stress (Miyamoto et al. 2004), this was not observed in the present study, however, there was an overall background level of Ran observed in the cytoplasm. The experiment was repeated 3 times and measured in 20 cells chosen at random. Ran was FITC labeled and the nuclear fluorescence calculated as a measure of Ran localisation. For all experiments the inter assay coefficient of variance was less than 3.4% and the intra assay coefficient of variance was less than 8.5%. The control cells showed a nuclear fluorescence (±SD) of 58980±4921 fu (fluorescent units) (n=60), which was comparable to siRNA control cells 59368±2761 fu (n=60) (ns). The heat-treated control cells showed no significant increase in Ran nuclear localisation with a nuclear fluorescence of 59883±5114 fu (n=60), neither did siRNA control transfected cells show significant change in Ran localisation after heat treatment with a nuclear fluorescence of 60523±3585 fu (n=60). The siRNA Orp150 transfected cells

showed similar nuclear Ran fluorescence to controls 60144±4507 fu (n=60) and after heat treatment 61356±4198 fu (n=60) [Figure 4.14 and 4.15].

Thus siRNA Orp150 had no significant effect on the localisation of Ran.

Figure 4.14: Localisation of Ran after siRNA treatments, with or without heat stress

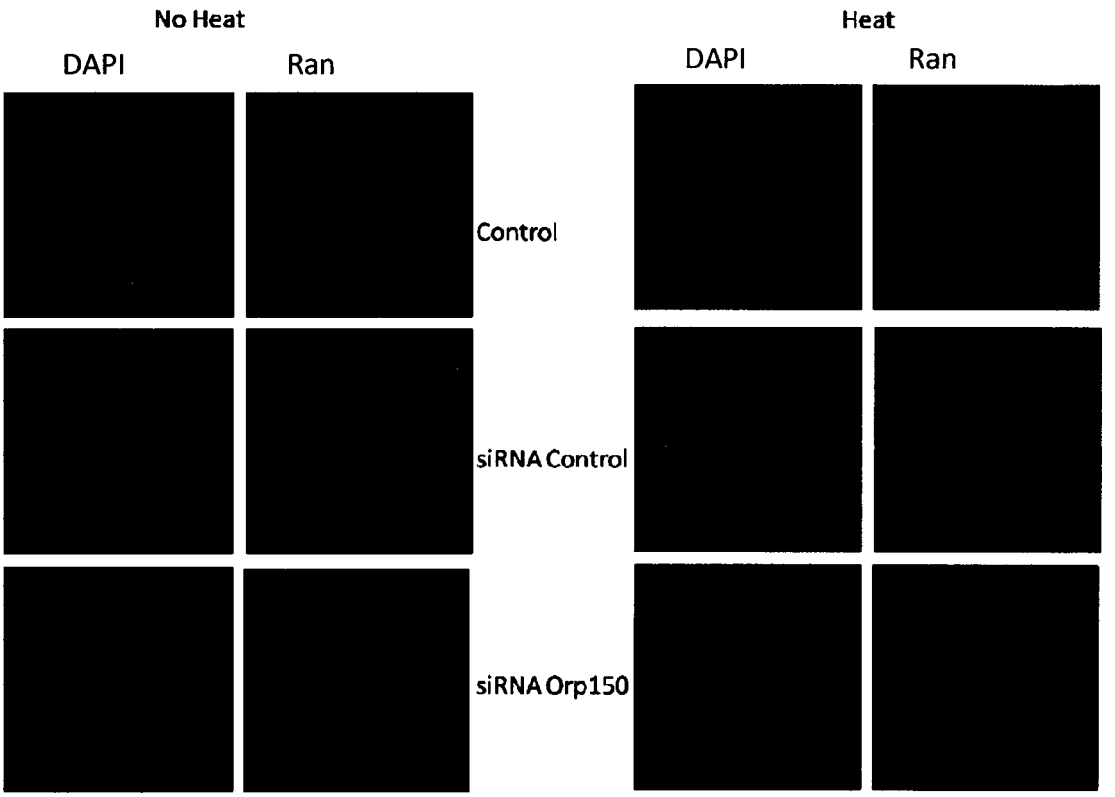


Figure 4.14: Representative immunohistochemistry of Ran localisation (green), nuclear DAPI counter stain (Blue) (images captured with a x40 objective). HT-29 cells with or without siRNA control or Orp150 treatment and with or without heat stress (42°C for 1h), were fixed on coverslips. The localisation of Ran is predominantly nuclear and appears unaffected by siRNA Orp150 treatment or heat treatment.

Figure 4.15: Ran localisation measured by nuclear fluorescence

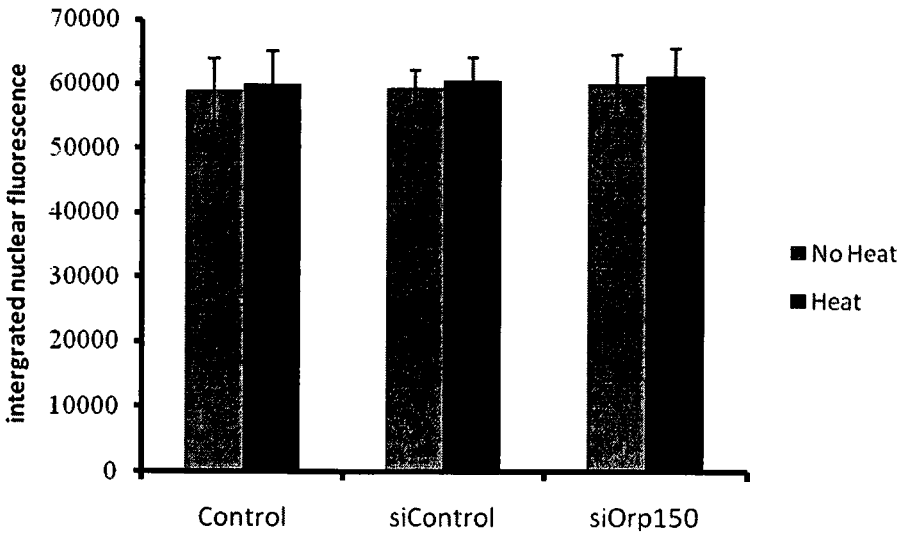


Figure 4.15: HT-29 cells with or without siRNA treatment and with or without heat stress were fixed on coverslips. Calculation of nuclear fluorescence as a measure of Ran localisation using AQM software reveals no significant difference with or without heat, or after siRNA treatment.

4.4.9 Constitutive Orp150 can be visualised as 2 isoforms using 2-D analysis

At least 3 splice variant mRNAs have been identified for Orp150, as described in the introduction to this chapter (Kaneda et al. 2000). In this thesis a constitutively expressed cytoplasmic isoform and a stress inducible isoform have already been investigated utilising 1-D SDS PAGE electrophoresis and these were seen as two distinct bands [Figure 4.1] The current experiment uses 2-D SDS PAGE electrophoresis to investigate the possibility of a further isoform of Orp150 which could not be visualised by 1-D electrophoresis. A whole cell lysate of HT-29 cells cultured under normal conditions was subjected to 2-D electrophoresis after which Orp150 western blotting revealed 2 individual spots, whereas 1-D electrophoresis showed only one band. Subsequent ABL lectin blotting reveals that only one of these spots is bound by the lectin [Figures 4.16 and 4.17]

Figure 4.16: Orp150 immunoblot of 4-7 linear 2-D 6% electrophoresis gel

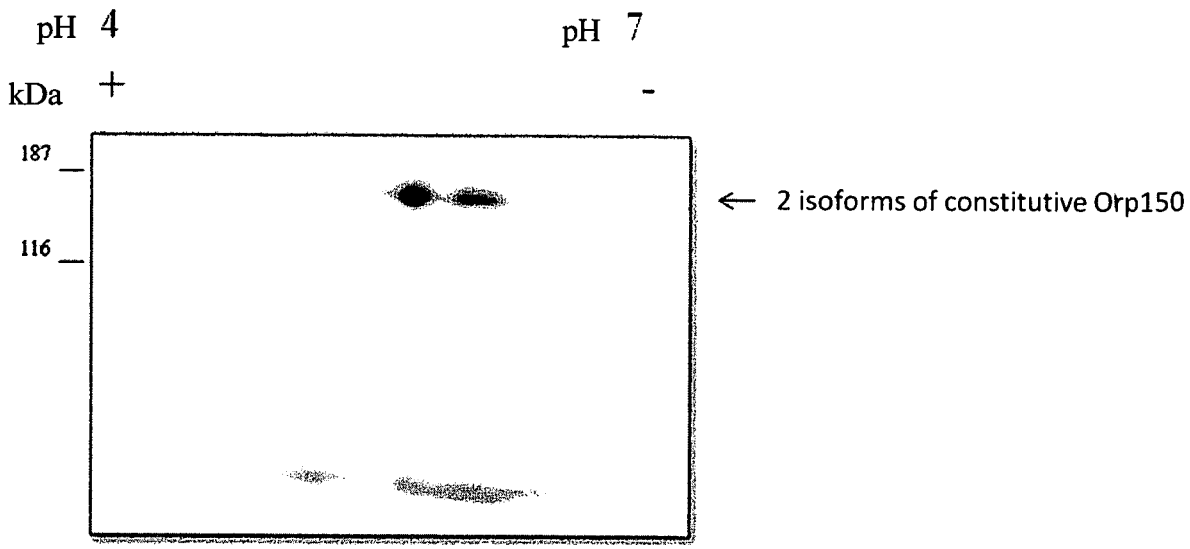


Figure 4.16: Anti Orp150 western immunoblot. HT-29 cells were lysed directly in 2-D lysis buffer and run on 4-7 nonlinear strips before 1-D electrophoresis on a 6% poly acrylamide gel. The resulting anti-Orp150 western blot reveals 2 distinct protein spots of the same molecular weight. Experiment repeated in duplicate

Figure 4.17: ABL biotinylated lectin blot of 4-7 linear 2-D 6 % electrophoresis gel

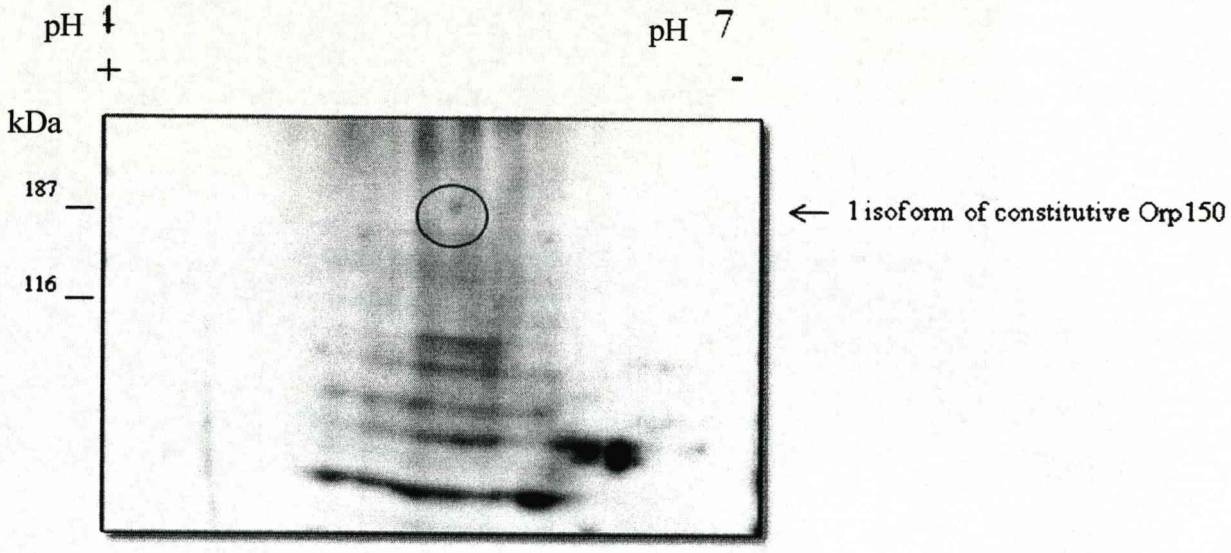


Figure 4.17: ABL lectin western immunoblot. 2-D electrophoresis analysis [Figure 4.16] reveals 2 possible isoforms of the constitutively expressed isoforms of Orp150 as shown by the 2 distinct spots after Orp150 antibody western blotting. Of the 2 forms of the constitutively expressed isoforms, only one appears to be bound by ABL lectin, as indicated by the circle. The ABL blot is performed on the same membrane as figure 4.16 after treatment with stripping buffer.

3.5 DISCUSSION

The initial experiments in this chapter clearly demonstrate 2 forms of Orp150. For the first time a constitutively expressed cytoplasmic isoform of Orp150 released by digitonin permeabilisation is shown to be distinct from the stress inducible ER localised form, with an apparent lower molecular weight on a 1-D SDS-PAGE gel. This constitutive cytoplasmic isoform is recognised by both ABL and jacalin lectins suggesting that it is modified by the oncofetal TF antigen in its sialylated form. It was also shown by using a whole cell lysate that had been subjected to 24 h glucose starvation that only the constitutive isoform can be isolated using jacalin affinity chromatography, indicating that the inducible form is not modified by sialyl-TF. Initial work to immunoprecipitate Orp150 in sufficient quantities for analysis failed. A thorough examination of the protocol and experimentation with the parameters still failed to isolate Orp150, suggesting that the antibody was not suitable. Therefore a lectin purification method was utilised to isolate Orp150 during these experiments.

2-D electrophoresis demonstrated that the constitutive Orp150 band seen on 1D gels contained two distinct proteins of the same molecular weight, of which only one was recognized by the sialyl TF binding ABL lectin. A detailed study by Kaneda and colleagues demonstrated that there are 3 mRNA's for the Orp150 protein produced by alternative promoters, one of which contains an inducible element (Kaneda et al. 2000). This would fit with the discovery that the constitutive band can be separated into two proteins.

Lectin affinity purification with Jacalin was used to purify constitutive Orp150 to be analysed by nano LC-ESI-MS/MS on a Q-STAR mass spectrometer [Figure 4.3]. The returned data indicated the presence of 4 high mannose *N*-glycopeptides the most abundant of which were $Man_{5-8}GlcNAc_2$. One further

glycopeptide was observed at m/z 747^{3+} indicating the presence of a single HexNAc but the analysis did not return any evidence for the presence of sialyl-TF. This evidence is contrary to my experimental data through a lectin binding approach which gives strong evidence for the presence of sialyl-TF. However, it is possible that the lack of sialyl-TF under mass spectrometry analysis could have occurred due to the low abundance of constitutive Orp150 provided for the mass spectrometry analysis. The 1-D SDS-PAGE gel when stained with coomassie showed only the faintest band that had to be aligned with identical anti-Orp150 western blots. Even with the high sensitivity of the mass spectrometry it is possible that there was a degradation of the sample and this coupled with a low abundance of protein meant that no sialyl-TF was revealed. The evidence from ABL and jacalin lectin blotting and purification is still compelling especially with the known specificity of the lectin binding. An alternative explanation might be that ABL and jacalin have some affinity for high mannose *N*-glycans. It has been shown that jacalin has some affinity for galactose terminated *N*-glycans but this has not been shown for ABL.

Orp150 has previously been described as the ligand for ABL lectin which is able to reversibly inhibit cellular proliferation of HT-29 cells (Yu et al. 2002). It was not understood how an ER-associated protein would be involved in NLS-dependent nuclear protein import, but this can now be explained in part by the presence of this cytoplasmic isoform. This is similar in some respects to calreticulin, also a known ER-associated protein, that has a cytoplasmic isoform that is involved in nuclear export signal (NES) -related nuclear protein import by acting as a receptor and forming an assembly with NES-protein and RanGTP (Holaska et al. 2001). In this study it is shown that a reduction in Orp150 by siRNA caused a subsequent reduction in Hsp70

translocation into the nucleus upon heat stress. This confirms earlier evidence of a role for Orp150 in NLS-dependent nuclear protein import.

Although previous work had shown that Orp150 co-associated with RanGTPase (Yu et al. 2002) siRNA Orp150 was shown in the present study to have no effect on the cellular localisation of Ran. Further studies are needed to elucidate the mechanism by which Orp150 is involved in NLS-dependent nuclear protein import.

CHAPTER 5

IDENTIFICATION OF CYTOPLASMIC PROTEINS EXPRESSING *O*-LINKED TF

5.1 HYPOTHESIS

Unsubstituted or sialic acid-substituted TF carbohydrate structures may also occur on cytosolic proteins other than Orp150.

AIMS

- To purify sialyl-TF expressing cytoplasmic glycoproteins
- To characterise purified proteins utilising mass spectrometry and lectin binding

5.2 INTRODUCTION

The previous chapter has presented evidence for an *N*-terminally truncated cytoplasmic form of Orp150 which expresses sialyl-TF. This is one of only two reports to date of a cytoplasmic glycoprotein expressing sialyl-TF; the other is the protein alpha-synuclein (Shimura et al. 2001). The appearance of sialyl-TF on cytoplasmic proteins is not readily explained by conventional knowledge of the *O*-glycosylation process and, therefore, warrants close investigation. This study sets out to investigate the presence of other cytoplasmic proteins that express this novel form of cytoplasmic glycosylation.

5.2.1 Cytoplasmic glycosylation

In the past it was thought that glycosylation was limited to secreted and cell membrane proteins due to the restricted localisation of required glycosyltransferases to the Golgi and ER. However there is now clear evidence for the existence of cytoplasmic glycosylation, exemplified by the discovery of *O*-GlcNAc (Hart et al. 1989, Hart et al. 1989) which is β-N-acetylglucosamine *O*-linked to the hydroxyl groups of Ser/Thr. The enzymes responsible for the addition of *O*-GlcNAc, *O*-GlcNAc transferase (OGT), and removal, β-N-acetylglucosaminidase (*O*-GlcNAcase), have been characterised (Iyer et al. 2003) and shown to be localised in the cytoplasm (Hart et al. 1989). The biological role of *O*-GlcNAc is still being uncovered, but so far it has been shown to be involved in a regulatory mechanism, which shares similarities with phosphorylation such as the dynamic cycling of the proteins (Comer et al. 2000). There are reports of cytoplasmic structures expressing various other sugars such as galactose, glucose and mannose, *N*-acetylgalactosamine, and sialic acid (Hart, G.W. 1999), mostly demonstrated by lectin binding, but these intra-cellular glycoconjugates have generally not been identified. The functional roles of these other forms of *O*-glycosylation have not been elucidated, although it has been shown that concealment of *O*-GlcNAc on nucleoporin p62 by a further attachment of galactose has no effect on protein import (Miller et al. 1994). Earlier work from our group has demonstrated that ABL specifically blocks NLS-dependent nuclear protein import (Yu et al. 1999), and that the internal ligand for the lectin is an *N*-terminally truncated isoform of Orp150, implying the expression of sialyl-TF on this protein. The role for sialyl TF in this process is unclear, but it has been suggested that some sugars such as glucose-, fucose-, mannose- or *N*-acetylchitobiosides could serve as nuclear localisation signals in a mechanism separate from the classical nuclear NLS dependent pathway (Duverger et

al. 1995, Duverger et al. 1996). This pathway also utilises energy and can be inhibited by wheat germ lectin, but does not require cytosolic transport factors (Duverger et al. 1995). From the strong evidence for cytoplasmic glycosylation it is plausible that there are other cytoplasmic pathways in existence. The expression of sialyl-TF on proteins in the cytoplasm could either be the result of an as yet unknown glycosylation pathway or the entry of traditionally glycosylated proteins into the cytoplasm. In this chapter experiments are described that investigate the possible existence of other cytoplasmic glycoconjugates that express TF or sialyl-TF.

5.2.2 Mucin type *O*-glycosylation and the sialyl-TF antigen

Mucin type *O*-glycosylation is a post-translational modification in which the monosaccharide GalNAc is added from a nucleotide donor, UDP-GalNAc, to the serine or threonine residues of the fully folded and assembled protein, catalysed by a polypeptide GalNAc transferase (GalNAcT). This feature sets it apart from the other forms of *O*-glycosylation such as *O*-GlcNAc (Hart 1997), *O*-Mannose and *O*-Fucose (Harris et al. 1993). A family of GalNAc transferases is responsible for the addition of GalNAc to the serine and threonine residues of which up to 20 members have been identified (Ten Hagen et al. 2003). This process occurs in the Golgi apparatus (Colley 1997) and specific regions of the endoplasmic reticulum or an inter ER-Golgi compartment (ERGIC) (Bieberich et al. 2000, de Graffenried et al. 2004, Young 2004). The *O*-GlcNAc transferase is present in the cytoplasm and nucleus (Hart et al. 1989) but there is no evidence to date that any of the GalNAc transferases are active in the cytoplasm.

Core structures are built up after the action of the GalNAcTs; many *O*-glycans contain the Core 1 structure formed by the addition of galactose in a β1-3 linkage to

GalNAc (Galβ1-3GalNAcα-Ser/Thr), which is catalysed by the glycosyltransferase: Core 1 β1-3 glycosyltransferase (Core 1 GalT). This Core 1 disaccharide is also called the Thomsen Friedenreich antigen which in its unsubstituted form behaves as an onco-fetal antigen as it is only seen in the fetus and in cancer and other disease states but not in normal epithelia. It is the substrate for many glycosyl and sialyl transferases and in normal healthy tissue is usually concealed by further glycosylation.

5.3 METHODS

5.3.1 Materials:

Biotinylated edible mushroom lectin, *Agaricus bisporus,* (ABL), Hrp-ABL lectin, Biotinylated Jacalin (Jac), Hrp-Jac were all obtained from Vector (Peterborough, UK). Peroxidase-conjugated secondary anti-Ig antibodies were from Dako (Glostrup, Denmark). Digitonin was obtained from Sigma (Poole; UK) and the anti Hsp90 mouse monoclonal antibody from Abcam (Cambridge; UK). Control mouse immunoglobulins were obtained from Santa Cruz biotechnology (Heidelberg, Germany).

5.3.2 Digitonin permeabilisation of cells

HT-29 cells were grown as a monolayer to 60% confluence in a T150cm^2 flask, washed twice in PBS before incubation in DMEM without glucose for 24 h. Cells were washed twice in ice cold PBS. Five ml ice-cold digitonin (50 µl/ml) was added for 3mins to the flask to puncture the outer membrane of the cells. The digitonin-released fraction was then extracted and freeze dried at -40°C overnight. The cells remaining in the plate were lysed directly with sample buffer. Samples were then run on SDS electrophoresis gel, either 6% or 12% as described in methods chapter and transferred to nitrocellulose membrane and lectin blot analysis performed.

5.3.3 Lectin affinity purification

Jacalin lectin affinity purification

Ten T75cm^2 flasks of HT-29 cells were grown to 60% confluence. The cells were released with 3 ml trypsin (1 mg/ml) and washed 3 times in PBS each time centrifuged at 70*g* for 1min (1000rpm; MSE microcentaur, Sanyo Gallenkamp PLC, UK) to collect cells. The cells were then suspended in 15 ml sterile PBS and sonicated

(*5 x 10s* pulses at 28% amplitude; GEX 400 Ultrasonic Processor; Sigma) on salt ice followed by ultra centrifugation at 500,000 *g* for 1 h at 4°C (100,000rpm; Beckman TL 100 Ultracentrifuge, Minnesota, USA). Ten ml of jacalin bound agarose beads (4mg lectin/ml gel) (Vector, UK) were added and left to incubate for 12 h on an orbital shaker at 4°C. The beads were loaded into a 20cm x 1cm column plugged with glass fibre enabling the beads to be washed with 60 ml sterile PBS, removing unbound proteins. The proteins bound to the jacalin lectin were eluted with 20ml 0.8M galactose. The resulting eluate was subjected to dialysis in 2 litres of deionised water which was changed at regular intervals and left overnight at 4°C. The volume was then reduced by freeze drying in 6 equal aliquots at -40°C.

ABL lectin affinity

A T75cm^2 flask of HT-29 cells was grown to 60% confluence. The cells were then washed 3 times in PBS before removal with 3 ml of trypsin (1 mg/ml) and washed a further 3 times in PBS and each time centrifuged at *7 0g* for 1 min (1000 rpm; MSE microcentaur, Sanyo Gallenkamp PLC, UK) to collect cells. The cells were then suspended in 2 ml sterile PBS and sonicated (*5 x 10s* pulses at 28% amplitude; GEX 400 Ultrasonic Processor; Sigma) on salt ice followed by ultra centrifugation at 500,000 *g* for 1 h at 4°C (100,000 rpm; Beckman TL 100 Ultracentrifuge, Minnesota, USA). One hundred µl of ABL bound agarose beads (4mg lectin/ml gel) (TCS group, Buckingham, UK) were added and left to incubate on an orbital shaker for 12h at 4°C. The beads were collected by pulse centrifugation, 170*g* for 15s (1500rpm; MSE microcentaur, Sanyo Gallenkamp PLC, UK) and washed 5 times in PBS. Sixty µl of sample buffer was then added to release the proteins and beads were then boiled for 5 minutes to dissociate the lectin-ligand from the beads. The beads were collected by centrifugation and SDS-PAGE performed as described in methods chapter.

5.3.4 Immunoprecipitation

Immunoprecipitation of Hsp90 was performed in order to isolate the protein to be utilised for lectin analysis to verify its glycosylation. HT-29 cells were grown to 60% confluence in a T150cm^2 flask and washed twice in the flask with ice-cold PBS. One ml ice-cold modified RIPA buffer containing protease and phosphatase inhibitors (Perbio science, Northumberland, UK) was added and adherent cells scraped off with a plastic cell scraper that had been cooled in ice-cold distilled water. The cell suspension was then transferred into a centrifuge tube and the suspension placed on an orbital shaker at 4°C for 15 minutes to lyse cells. The lysate was centrifuged at 15,000 *g* in a precooled centrifuge at 4°C for 15 minutes and the supernatant was immediately transferred into a fresh centrifuge tube and the pellet discarded.

The cell lysate was pre-cleared to reduce non-specific binding of proteins to the agarose by adding 50 µl of Protein A/ G agarose beads slurry per 1 ml of cell lysate and incubating at 4°C for 10 minutes on a rocker or orbital shaker. The Protein A/G beads were removed by centrifugation at 170 *g* at 4°C for 10 minutes (1500rpm; MSE microcentaur, Sanyo Gallenkamp PLC, UK) and the supernatant transferred in to a fresh centrifuge tube. The protein concentration of the cell lysate was calculated using a bicinchoninic acid assay (Sigma, Poole; UK) and the cell lysate diluted to 5µg/µl total cell protein with PBS. Five µl of the Hsp90 antibody (1mg/ml) (Abcam, Cambridge; UK) was added to 1ml of cell lysate in an Eppendorff. To another 1ml of cell lysate, 5µl of control mouse immunoglobins (1 mg/ml) (Santa Cruz biotechnology, Heidelberg, Germany), was added.

The cell lysate/antibody mixture was gently rocked on an orbital shaker for 2 hours at 4 °C. The immunocomplex was captured by adding 50 µl Protein A/G agarose bead slurry and gently rocking on an orbital shaker for 1 h at 4 °C. The agarose beads

were collected by pulse centrifugation for 15 seconds in the microcentrifuge at 170*g* (1500 rpm; MSE microcentaur, Sanyo Gallenkamp PLC, UK). The supernatant was discarded and the beads washed 5 times with 1 ml ice-cold PBS.

The agarose beads were resuspended in 60 µl 2x sample buffer and mixed gently. The agarose/sepharose beads were then boiled for 5 minutes to dissociate the immunocomplexes from the beads. The supernatant was collected following centrifugation at 170 *g* for 10 minutes at 4°C (1500 rpm; MSE microcentaur, Sanyo Gallenkamp PLC, UK) and SDS-PAGE performed as described in the methods chapter. For some samples the supernatant was transferred to a fresh microcentrifuge tube and stored at –20°C for later use.

5.4 RESULTS

5.4.1 ABL lectin binds proteins in a cytoplasmic fraction of HT-29 cells

A cytoplasmic fraction of HT-29 cells was first prepared by digitonin-selective membrane permeabilisation. A T150 cm^2 flask was washed 2 times in PBS before the addition of 5ml ice cold digitonin (50 μg/ml) for 3 min to puncture the outer membrane of the cells. The digitonin-released fraction was then extracted and freeze dried at -40°C overnight, the remaining cells were lysed directly with sample buffer. After separation on a 12% SDS-PAGE gel an ABL mushroom lectin blot was performed [Figure 5.1]. This demonstrated the appearance of bands in the cytoplasmic fraction that are not apparent or less apparent in the remaining digitonin-treated cells. This suggests the existence of sialyl-TF-expressing proteins in the cytoplasm. To ensure that the digitonin treatment resulted in a pure cytoplasmic fraction the presence of two endoplasmic reticulum (ER) localised proteins, disulfide isomerase (PDI) and Erp72, were tested for by western blotting. They were both absent from the cytoplasmic fraction.

Figure 5.1: ABL blot of a digitonin-released fraction, resolved on a 12% SDS-PAGE gel

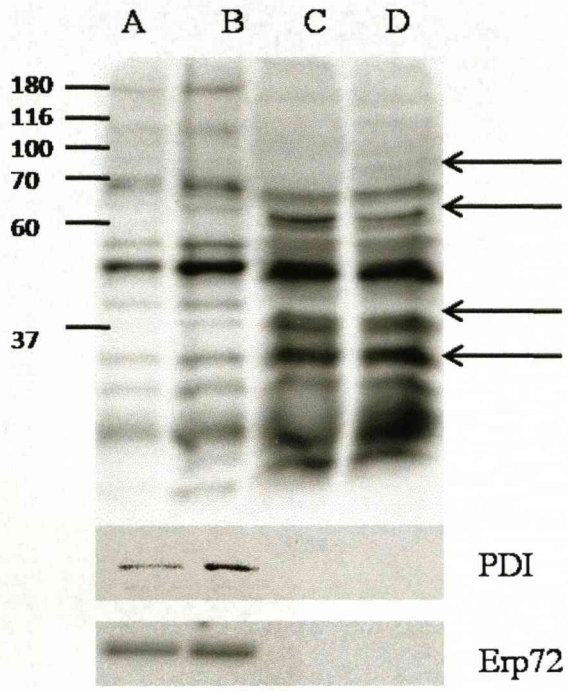


Figure 5.1: 12% electrophoresis gel. ABL-biotinylated blot. Lane **A**: Whole cell lysate, Lane **B:** Remaining digitonin-treated cells, Lane **C**: Digitonin-released fraction 10 μg, Lane **D**: Digitonin- released fraction 20 μg.

HT-29 cells were grown to 60% confluence in a T150cm^2 flask. Cells washed twice with ice cold PBS. Five ml digitonin (50μl/ml) was added for 5mins to puncture the outer membrane of the cells. The digitonin-released fraction was then extracted and freeze-dried. The cells remaining in the plate were lysed directly with sample buffer. Erp72 and PDI are ER-localised proteins, so demonstrating that the ER had not been punctured during the digitonin treatment. Four ABL-binding glycoproteins are shown by arrows that are relatively over expressed in the cytoplasmic fraction.

5.4.2 Mass spectrometry analysis of an ABL purified sample with bands excised from a 12% SDS electrophoresis gel reveals no clear protein matches due to insufficient purity

To isolate the glycoproteins that express sialyl-TF in the cytoplasm, lectin affinity purification with ABL of the cytoplasmic fraction was utilised. A cytoplasmic fraction was prepared and the agarose-bound ABL beads added to the solution. The beads were washed and the bound proteins eluted by directly adding sample buffer. The proteins were run on a 12% SDS gel, an identical copy was removed for staining using coomassie R250 and the other transferred on to a nitrocellulose membrane and used for an ABL lectin blot [Figure 5.2].

Bands that occurred in the ABL purified fraction and 1 band from the cytoplasmic fraction that was recognised by ABL were cut from the gel and analysed by mass spectrometry. None of the bands gave a clear identification however, due to several proteins existing in one band.

Figure 5.2: Coomassie stained gel and ABL blot of an ABL purified cytoplasmic fraction resolved on a 12% SDS-PAGE gel

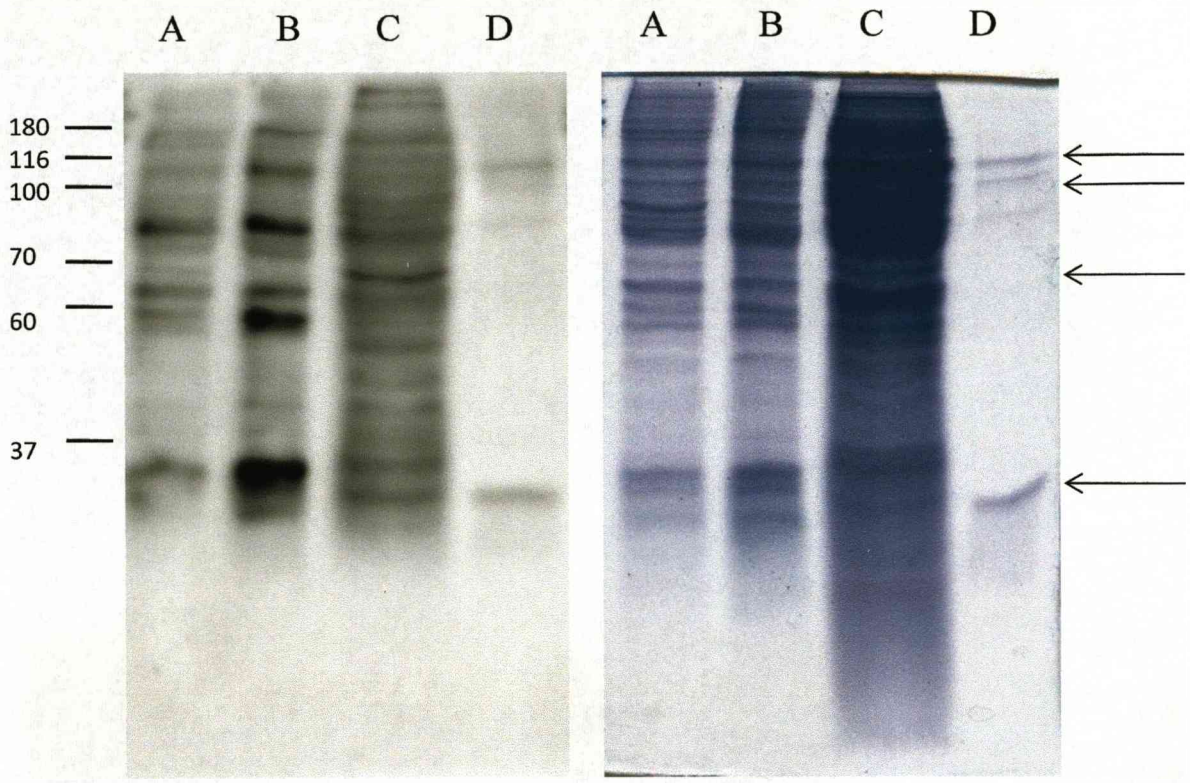


Figure 5.2: 12% SDS-PAGE, ABL-biotin blot and coomassie stained gel. Lane **A**: Whole cell lysate, Lane **B**: Remaining digitonin-treated cells, Lane **C**: Digitonin-released fraction 10 µg, Lane **D**: ABL lectin affinity purified. An ABL lectin affinity purification of the cytoplasmic fraction was performed to isolate cytoplasmic proteins expression sialyl-TF. Arrows mark bands excised from gel which were further analysed by mass spectrometry. None of the bands analysed contained a sufficiently pure protein for a clear identification.

5.4.3 Mass spectrometry analysis of an ABL purified sample with bands excised from 6% SDS electrophoresis gel reveals a few possible candidate proteins

In order to separate proteins further with an aim of identifying glycoconjugates localised in the cytoplasm which express sialyl-TF, a 6% SDS PAGE gel was used to resolve the proteins purified with the ABL lectin. This was to overcome the problems encountered with the 12% SDS PAGE gel, which did not result in sufficient separation. Proteins were purified with ABL lectin as before and after resolving on the 6% SDS PAGE gel, either stained with coomassie [Figure 5.3] or an identical copy transferred to nitrocellulose and utilised for ABL lectin blotting [Figure 5.3]. Two protein bands were selected for extraction and mass spectrometry analysis. The data received from the mass spectrometry analysis [Tables 5.1-5.8] revealed that Hsp90 β is the major candidate for the identity of Protein 1 with 6 peptides identified and 10.6% coverage of the sequence. The other peptides identified suggest the presence of a number of proteins including spermatogenesis associated 1, tubulin tyrosine ligase-like family, member 11 (Homo sapiens), pyruvate kinase 3 isoform 2 (Homo sapiens) and programmed cell death 6 interacting protein (Homo sapiens), however only one 1 peptide of each was identified. In the protein 2 band, two major candidates arose, eukaryotic translation elongation factor 1 alpha 1 which had 3 peptides identified with 10.2% coverage of the sequence and tubulin, beta, 2 (Homo sapiens), which also had 3 peptides identified and 8.3% coverage of the sequence. Another protein with 1 peptide identified was nuclear transport factor like export factor 1 (Homo sapiens).

The most promising identification was deemed to be Hsp90β, which is of a comparable molecular weight compared to the band excised and also with the strongest identification, with most peptides identified.

Figure 5.3: Coomassie stained gel and ABL blot of an ABL purified cytoplasmic fraction resolved on a 6% SDS-PAGE gel

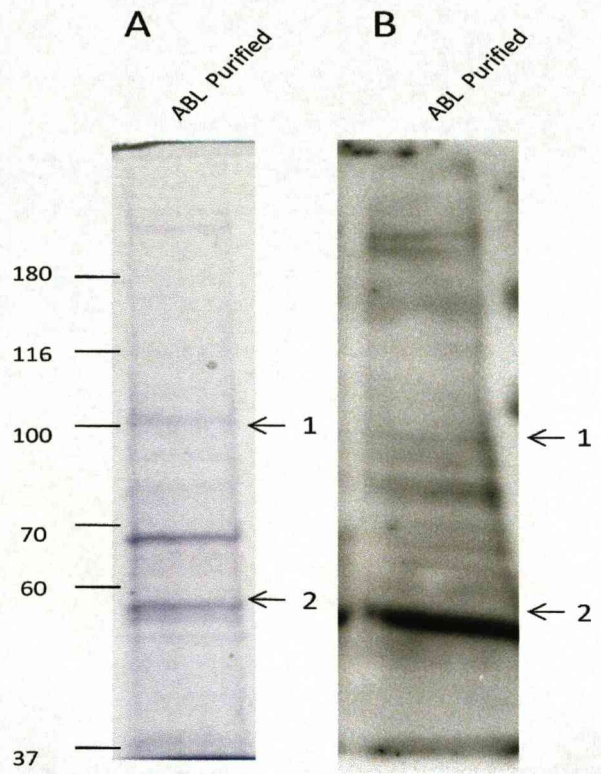


Figure 5.3: Coomassie stained gel (A) and an ABL-biotin blot (B). A cytoplasmic fraction was obtained by treatment with digitonin. This fraction was utilised in an ABL lectin purification and the proteins isolated run on a 6% SDS-PAGE gel. Two bands were excised and analysed by mass spectrometry. Protein 1 was identified as having 6 peptides homologous to human Hsp90β and protein 2 as having 3 peptides homologous to eukaryotic translation elongation factor 1 alpha 1.

The peptide sequences identified by mass spectrometry for protein 1 are detailed below. The significance of the peptides identified were assessed using Mascot. Mascot incorporates a probability based implementation of the Mowse algorithm. The Mowse algorithm is an excellent starting point because it accurately models the behaviour of a proteolytic enzyme. Matches using mass values (either peptide masses or MS/MS fragment ion masses) are always handled on a probabilistic basis. The total score is the absolute probability that the observed match is a random event.

Given an absolute probability that a match is random, and knowing the size of the sequence database being searched, it becomes possible to provide an objective measure of the significance of a result. A commonly accepted threshold is that an event is significant if it would be expected to occur at random with a frequency of less than 5%. This is the value which is reported in the tables.

Table 5.1: Heat shock 90kDa protein 1, beta [Homo sapiens]

6 peptides, 65 spectra, 10.6% coverage

Residues	Peptide sequence	p-value
42-54	R.ELISNASDALDK.I	9.9999
73-83	K.IDIIPNPQER.T	9.9999
83-96	R.TLTLVDTGIGM+16TK.A	9.9999
205-220	K.HSQFIGYPITLYLEK.E	0.00034
539-551	K.EGLELPEDEEEK.K	0.0037
625-640	K.HLEINPDHPIVETLR.Q	0.00045

Table 5.2: spermatogenesis associated 1

1 peptides, 1 spectra, 3.2% coverage

Residues	Peptide sequence	p-value
10-19	K.IPIDNYPIQ.T	0.00058

Table 5.3: tubulin tyrosine ligase-like family, member 11 [Homo sapiens]

1 peptides, 1 spectra, 2.0% coverage

Residues	Peptide sequence	p-value
186-197	P.VQGLCPHGKPR.D	0.008033

Table 5.4: pyruvate kinase 3 isoform 2 [Homo sapiens]

1 peptides, 1 spectra, 2.1% coverage

Residues	Peptide sequence	p-value
33-44	R.LDIDSPPITAR.N	0.0118

Table 5.5: programmed cell death 6 interacting protein [Homo sapiens]

1 peptides, 2 spectra, 1.8% coverage

Residues	Peptide sequence	p-value
358-374	K.M+16VPVSVQQSLAAYNQR.K	2.0116

The peptide sequences identified by mass spectrometry for protein 2 are detailed below.

Table 5.6: eukaryotic translation elongation factor 1 alpha 1

3 peptides, 4 spectra, 10.2% coverage

Residues	Peptide sequence	p-value
6-21	K.THINIVVIGHVDSGK.S	0.0011109
248-256	R.LPLQDVYK.I	0.0026363
267-291	R.VETGVLKPGM+16VVTFAPVNVTTEVK.S	0.0469230

Table 5.7: tubulin, beta, 2 (Homo sapiens)

3 peptides, 3 spectra, 8.3% coverage

Residues	Peptide sequence	p-value
63-78	R.AVLVDLEPGTM+16DSVR.S	9.999
163-175	R.IM+16NTFSVVPSPK.V	0.00102
242-252	R.FPGQLNADLR.K	0.03151

Table 5.8: NTF2-like export factor 1 (Homo sapiens)

1 peptides, 1 spectra, 7.9% coverage

Residues	Peptide sequence	p-value
38-49	R.LYMGTATLVWN.G	0.0192

5.4.4 Investigation into the glycosylation of Hsp90 reveals possible expression of sialyl-TF

To confirm the presence of Hsp90β in the sample of cytoplasmic ABL purified proteins and to assess Hsp90β glycosylation, the sample was again resolved on another 6% SDS PAGE gel. The subsequent anti-Hsp90 (Sigma, Poole, UK) western blot with an antibody specific for both Hsp90 α and β reveals the presence of Hsp90 in the ABL purified sample. An ABL lectin blot reveals numerous protein bands, which would be expected, careful alignment with the Hsp90 western blot shows that there may be a corresponding band, further suggesting the presence of TF or sialyl-TF on Hsp90 [Figure 5.4]

Figure 5.4: ABL lectin blot and Hsp90 blot of cytoplasmic ABL purified proteins resolved on a 6% SDS-PAGE gel

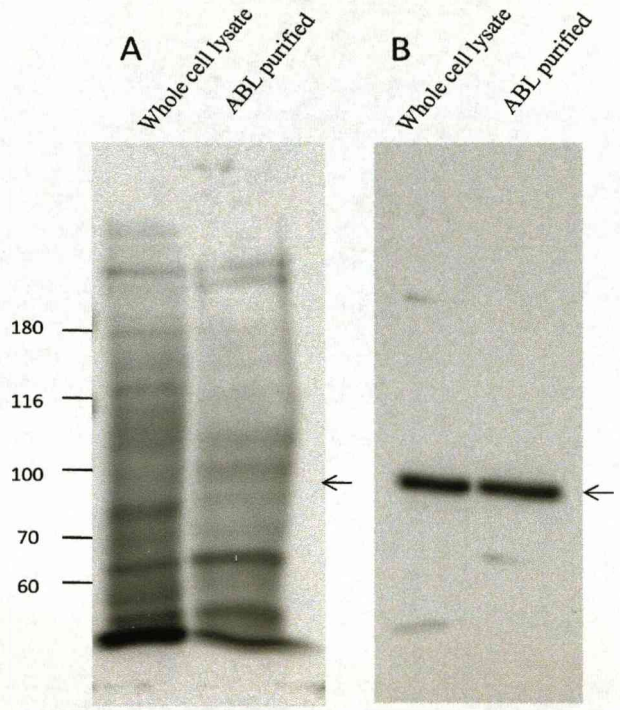


Figure 5.4: ABL lectin blot (A) and anti- Hsp90 western blot (B). The same sample of cytoplasmic proteins purified with ABL lectin that was analysed by mass spectrometry was resolved on a 6% SDS PAGE gel. The anti-Hsp90 western blot shows a clear band in the ABL purified proteins lane, indicating that ABL lectin is capable of identifying and isolating Hsp90.

5.4.5 Hsp90 immunoprecipitation and subsequent ABL lectin blot reveals possible TF or sialyl-TF expression

To further confirm the presence of sialyl-TF on Hsp90β, immunoprecipitation was performed to isolate the protein. HT-29 cells were seeded at $1x10^5$ and grown until 60% confluent. The cells were extracted in ice cold RIPA buffer and sonicated on salt ice. The cells were then centrifuged at 15,000 *g* at 4°C for 30 min. The supernatant was removed and 5 µl of the anti-Hsp90 antibody (1µg/ml, mouse monoclonal) was added, and left for 2 h at 4°C before the addition of 50 µl Protein A/G agarose (Perbio, Northumberland, UK) and then left for a further 2 hours. The beads were washed 5 times with PBS and sample buffer added, before resolving on a 6% SDS-PAGE gel. An Hsp90 immunoblot reveals that Hsp90 is precipitated by the antibody and that it is not precipitated by the control mouse immunoglobins. An ABL lectin blot was performed on an identical blot showing the presence of a band corresponding to Hsp90 band, thus implying that ABL can recognise Hsp90 and that therefore Hsp90 expresses sialyl-TF [Figure 5.5].

Figure 5.5: Immunoprecipitation of Hsp90 and ABL lectin blotting

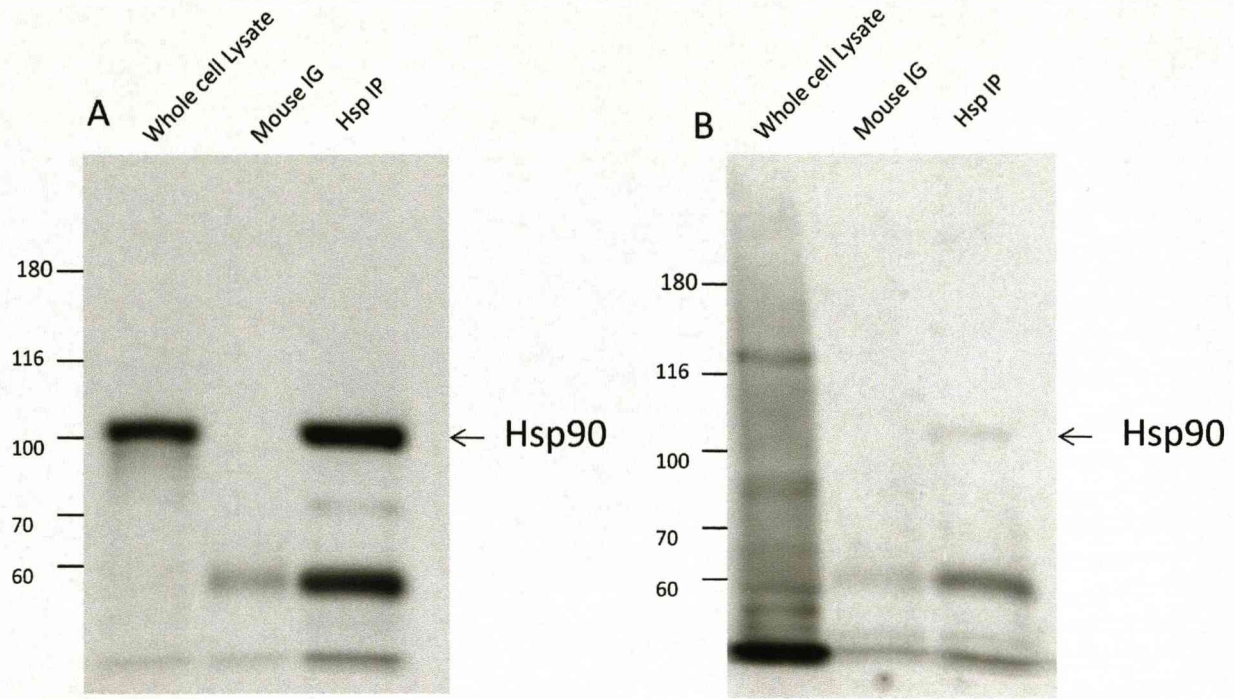


Figure 5.5: Anti-Hsp90 western blot (A) and ABL lectin blot (B): HT-29 cells were seeded at $1x10^5$ and grown until 60% confluent. The cells were extracted in ice cold RIPA buffer and sonicated on salt ice. The cells were then centrifuged at 14,000 rpm at 4°C for 30 mins. The supernatant was removed and the anti-Hsp90 antibody added, and left for 2 hours at 4°C before the addition of Protein A/G agarose and then left for a further 2 hours. The beads were washed 5 times with PBS and sample buffer added, before resolving on a 6% SDS-PAGE gel. An Hsp90 western blot reveals that Hsp90 protein has indeed been precipitated and that none was isolated by the control mouse immunoglobins [Figure A]. An ABL lectin blot was performed on an identical blot showing the presence of a band corresponding to Hsp90. Alignment of the blots supported the binding of ABL to Hsp90, implying the presence of sialyl-TF.

5.4.6 Jacalin lectin purification also isolates Hsp90

The carbohydrate binding specificities of ABL and Jacalin are very similar and both are able to recognise sialyl-TF. This further experiment utilises jacalin lectin to purify a whole cell lysate as previously described in an earlier chapter. An Hsp90 western blot reveals that Hsp90 is isolated by jacalin affinity purification, implying the presence of sialyl-TF [Figure 5.6]

Figure 5.6: Hsp90 immunoblot of jacalin lectin purified sample

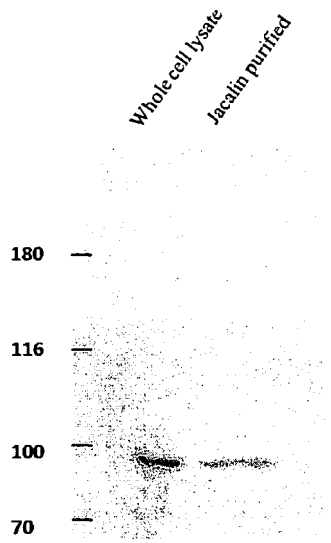


Figure 5.6: 6% SDS-PAGE, Anti-Hsp90 immuno-blot. HT-29 cells were grown to 60% confluence and then starved of glucose for 24 h. The cells were extracted with trypsin and placed in PBS and washed. The solution was then sonicated and ultra centrifuged for 1 h. Jacalin-bound agarose beads were added and left to incubate for 12 h. The beads were washed and the bound proteins eluted with 0.8 M Galactose. Dialysis against deionised water was then performed before freeze drying and running samples on a 6% SDS-PAGE electrophoresis gel. An Hsp90 western blot reveals that Hsp90 is isolated by the jacalin lectin purification, implying the expression of sialyl-TF.

5.5 DISCUSSION

The previous chapter demonstrated the presence of a truncated cytoplasmic isoform of Orp150 that was bound by ABL. ABL is known to bind the onco-fetal carbohydrate TF antigen and its sialylated form, thus implying the expression of TF or sialyl-TF on Orp150. Orp150 was not bound by PNA which recognises TF only in its unsubstituted form, further treatment of Orp150 with sialidase then allows PNA to bind implying that it is sialyl-TF and not TF expressed on Orp150 (Yu et al. 2002). This discovery led to an investigation to uncover other possible glycoconjugates in the cytoplasm which also express sialyl-TF or unsubstituted TF. To date various sugars have been shown to be expressed by proteins in the cytoplasm but apart from glycoproteins expressing *O*-GlcNAc, few of the proteins have been identified. In the experiments detailed here ABL lectin was shown to bind to at least 4 protein bands which are apparent in the cytoplasmic fraction but to a lesser extent in the remaining cells after digitonin treatment [Figure 5.1]. This initial discovery was followed by ABL lectin purification of cytoplasmic proteins which after resolving on a 6% SDS-PAGE gel led to 2 bands being further analysed by mass spectrometry. This led to the identification of Hsp90β with 6 peptides identified and 10.6% coverage of the sequence [Figure 5.3]. Hsp90β was selected for further analysis due to the strong identification of the peptides and was also easily identified amongst a few other proteins in the band analysed. The probable expression of sialyl-TF on Hsp90β was shown by ABL lectin blotting after immunoprecipitation of Hsp90.

The digitonin-released cytoplasmic fraction was free from 2 known ER localised proteins, Erp72 and PDI, giving confidence that a cytoplasmic fraction free from contaminates was achieved. The binding specificities of the lectins have been studied extensively; ABL lectin is very specific in its binding for the Galβ1-3GalNAc

as well as to sialic acid modified Galβ1-3GalNAc (Irazoqui et al. 1999). Jacalin lectin is also highly specific for the TF and sialyl-TF antigen but it has also been demonstrated that glycopeptides containing multi-antennary *N*-linked oligosaccharides with terminal α-galactose residues can bind jacalin (Do et al. 1998). The known specificities of the lectin bindings should allow for the isolation of glycoconjugates that express sialyl-TF and TF.

The evidence from this investigation suggests that Hsp90β expresses sialyl-TF or TF and that there may possibly be other proteins with similar glycosylation in the cytoplasm. The presence of this glycosylation is intriguing and the mechanisms by which Hsp90β acquires this modification or how it appears in the cytoplasm need further investigation. Hsp90 is an abundant molecular chaperone which is found in the cytosol of eukaryotic cells. It is involved in the folding of cell regulatory proteins and in the re-folding of stress denatured polypeptides (Bagatell et al. 2004). It also plays a role in cellular signalling as it is essential in maintaining the activity of key signalling factors which include protein kinases and steroid hormone receptors (Picard et al. 1990, Xu et al. 1993). In vertebrates there exist two distinct genes that encode a constitutive form, Hsp90β, and an inducible form Hspα. The functional differences between the two forms of Hsp90 are poorly understood and further homologues of Hsp90 have also been found in the endoplasmic reticulum: Glucose regulated protein 94 (Grp94) and mitochondrion TRAP1 (Bagatell et al. 2004). All of these proteins have a similar structural plan and are therefore expected to have similar mechanisms of action. Their structure contains a constitutive heterodimer with its main intersubunit contacts within the COOH-terminal 190 residues (Nemoto et al. 1995). There is also a highly conserved NH_2-terminal domain of about 25kDa which is the binding site for ATP. Polypeptide binding by Hsp90 has been shown to be ATP-dependent (Panaretou

et al. 1998) and cytosolic Hsp90 interacts with a range of co-chaperones in multichaperone complexes. Most of the co-chaperones bind via helix-turn-helix tetratricopeptide repeat (TPR) motifs, which are found fused to a series of different functional domains such as protein phosphatase (PP5) (Chen et al. 1996). Much work at present is focused on the complex interaction of chaperones in the development of cancer. Chaperones are upregulated under stress conditions to maintain the normal protein folding environment and they have been shown to be upregulated in a variety of tumours. It has been shown that chaperones can serve as biochemical buffers at the phenotypic level for the genetic instability that is characteristic of many human cancers, thus supporting the concept that their altered utilisation during oncogenesis is critical to the development of human cancers, allowing tumour cells to tolerate the mutation of multiple critical signalling molecules that would otherwise be lethal (Bagatell et al. 2004). The discovery that Hsp90 complexes can be inhibited by antibiotics such as geldanamycin (GA), which bind to the NH_2-terminal domain (Whitesell et al. 1994) has also furthered understanding of the role of heat shock proteins in tumorigenesis. The glycosylation of Hsp90 has not been extensively investigated, but there are many *N*-glycosylation sites on Hsp90, as deduced from the amino acid sequence and work by Hart and colleagues identified Hsp90 as an *O*-GlcNac modified protein (Wells et al. 2002).

The present investigation demonstrates that Hsp90β is modified with sialyl-TF or TF but the possible role for this modification on this chaperone is uncertain. Furthermore the mechanism by which Hsp90 could be modified by sialyl-TF or TF and exist in the cytoplasm needs further investigation. To further confirm the presence of sialyl-TF or TF on Hsp90β, a carbohydrate mass spectrometry analysis is required. Hsp90β was successfully immunoprecipitated in this study and, therefore, it should be

uncomplicated to isolate the protein in sufficient quantity to be analysed. This study showed the possible existence of a number of proteins that express sialyl-TF or TF; these also warrant further investigation as they could lead to a better understanding of cellular glycosylation. Further experiments to identify other cytosolic proteins that express sialyl-TF could utilise 2-D electrophoresis to yield further protein separation, combined with lectin blotting. Subcellular fractionation such as differential centrifugation or sedimentation through a sucrose gradient to back up evidence on the localisation of the proteins could also be performed.

CHAPTER 6

EFFECT OF ALTERED GLYCOSYLATION ON NLS-DEPENDENT NUCLEAR PROTEIN IMPORT

6.1 HYPOTHESIS

Alterations in cellular *O*-glycosylation may cause an alteration in the NLS-dependent nuclear protein import of Hsp70

AIMS

- To analyse the effects of bafilomycin, TNFα, 5-CDP and benzyl GalNAc on TF and sialic acid expression on HT-29 MTX cells
- To evaluate the effect of inhibitors of glycosylation on NLS-dependent nuclear protein import by studying the nuclear localisation of Hsp70 upon heat shock
- To utilise siRNA to downregulate the Galtransferase responsible for the generation of Core1 glycans by analysing TF expression on HT-29-MTX cells
- To Investigate the effect of the siRNA Galtransferase on NLS-dependent nuclear protein import

6.2 INTRODUCTION

The functional role of glycosylation is becoming revealed and it is now clear that complex carbohydrates are involved in many biological processes such as protein folding, structural stability and oligomerization and also in the immune response and host pathogen interactions (Merry et al. 2005). A possible role in NLS-dependent nuclear protein import for the constitutively expressed cytoplasmic isoform of Orp150 has been shown in a previous chapter and it has also been shown that this isoform

expresses the sialyl-TF antigen. The functional importance of glycosylation and the effects of known *O*-glycosylation inhibiting drugs in the NLS-dependent nuclear protein import are not yet known and are investigated in this chapter. siRNA was used in this study to down-regulate the Gal-transferase responsible for the addition of galactose to *N*-acetylgalactosamine is utilised to further explore the role of the TF antigen in NLS-dependent nuclear protein import.

6.2.1 Heat shock proteins

The Hsp70 family encompasses at least 11 proteins which are all closely related. Of these proteins some act as molecular chaperones ensuring the correct early folding of nascent protein chains, others are stress inducible and implicated in the protection and repair of stress induced protein damage (Tavaria et al. 1996). Heat stress induces the expression of Hsp72/73 in the cytoplasm, which is then transported into the nucleus via the NLS-dependent nuclear protein import mechanism, this movement is examined in this chapter and visualised with FITC conjugated antibodies.

6.2.2 *O*-glycosylation inhibiting drugs

Four differently acting *O*-glycosylation inhibitors have been selected for use in these experiments:

6.2.2.1 5-cytosine diphosphate (5-CDP)

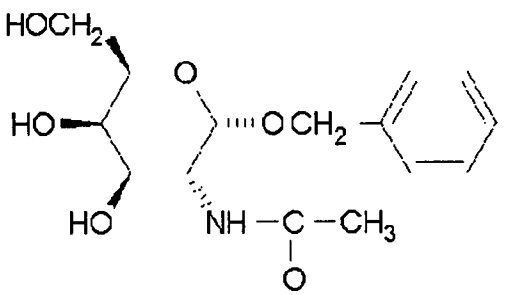
Alterations of carbohydrate structure and composition of cell surface glycoproteins and glycolipids have been shown to have an influence on the metastatic potential of tumour cells (Kobata et al. 2005). There has been shown to be a direct correlation between tumour metastasis and number of sialic acids (Zhao et al. 2007),

with hypersialylation being the result of increased sialyltransferase activity (Collard et al. 1986). The inhibition of sialyltransferases can be achieved with a number of compounds. Some of the most effective of these are the family of cytosine phosphates with increasing inhibitory potency from 5-cytosine monophosphate (5-CMP), 5-cytosine diphosphate (5-CDP) to 5-triphosphate (5-CTP) for inhibition of the α-2, 6-sialyltransferase of rat liver. The best inhibitor of α2-3-sialyltransferase from the porcine submandibular gland was 5-CDP which inhibited both sialyltransferases equally at 85% and 89% (Kleineidam et al. 1997). In the present experiments 5-CDP was selected for its overall inhibition of sialyltransferases.

6.2.2.2 GalNAc-α-*O*-benzyl

GalNAc-α-*O*-benzyl (benzyl-GalNAc) is an inhibitor of mucin type *O* glycosylation (Huet et al. 1998). Short term exposure to benzyl-GalNAc has been demonstrated to reduce mucus secretion, to decrease sialylation of newly synthesised mucins and to increase TF antigen expression in post-confluent HT-29 colonic cancer cells (Huet et al. 1995). These effects have also been shown in LS174T cells (Kuan et al. 1989) and KATO II cells (Byrd et al. 1995). Permanent exposure to benzyl-GalNAc leads to dramatic changes in HT-29 cells such as reduced mucus secretion, swelling of the cells with intracellular accumulation of numerous vesicles (Hennebicq-Reig et al. 1998) and causes apical membrane proteins such as MUC1, DPP-IV, and CEA to accumulate in the cytoplasm and, therefore, no longer be expressed at the apical membrane, without modification of polarised cells. Exposure to benzyl-GalNAc causes a decrease in the NeuAcα2-3 sialylation of these proteins, thus suggesting a possible role for sialylation for the targeting of apical membrane proteins (Huet et al. 1998). These effects are reported to be the result of the metabolism of GalNAc-α-*O*-

benzyl into Gal β1-3 GalNAc-α-*O*-benzyl, which is responsible for the competitive inhibition of Gal β1-3 GalNAc α2-3sialyltransferase (Huet et al. 1995). Further to this the massive accumulation of benzylated oligosaccharides in the HT-29 cells is also thought to alter cellular processes (Zanetta et al. 2000). These results cannot always be readily extrapolated to other cell lines, this is clearly demonstrated by the lack of effect of benzyl-GalNAc on Caco-2 cells. This is explainable as the main sialyltransferase expressed in these cells is ST6Gal I, which is not affected by benzyl-GalNAc. However, HT-29 MTX cells treated with benzyl-GalNAc have profound changes in mucin oligosaccharide chains. Exposure of HT-29-MTX cells to benzyl-GalNAc caused a decrease in intracellular mucins, sialic acid and galactose content and an increase in the TF antigen and Tn antigen expression. The expression of MUC5Ac was also inhibited and no overlying mucus layer detected (Delannoy et al. 1996, Hennebicq-Reig et al. 1998).

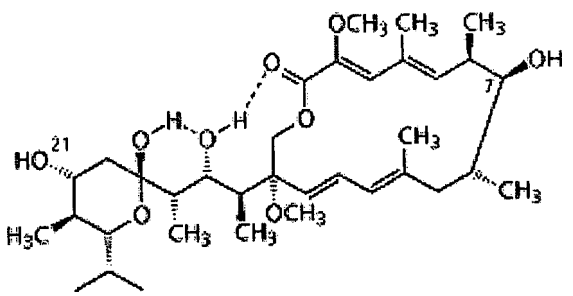


Benzyl-GalNAc

6.2.2.3 Bafilomycins

The family of bafilomycins A_1, B_1, C_1 and D are macrolide antibiotics with a 16-membered lactone ring, first isolated from the fermentation of *Streptomyces* spp. (Werner et al. 1984). They all inhibit growth of Gram positive bacteria and fungi in a disc diffusion assay and bafilomycin C_1 has been reported to inhibit the enzymatic

activity of the Na^+,K^+, ATPase inhibitors (Huang et al. 1984). Bafilomycin A_1 has been shown to be a useful tool in distinguishing among the three main types of ATPases, as it has no effect on the F_1F_0 ATPases of bacteria and mitochondria, the E_1E_2 ATPases such as the K^+ ATPase from ox brain and the Ca^{2+}ATPase from the sarcoplasmic reticulum are moderately sensitive. The membrane ATPases from neurospora vacuoles, chromaffin granules are extremely sensitive, making it a relatively specific potent inhibitor of vacuolar H^+ATPases (V-ATPases) in both prokaryotic and eukaryotic organisms (Bowman et al. 1988). Structurally the vinylic methoxy group adjacent to a carbonyl function, the dienic system and the hydroxyl at position 7 are important for the V-ATPase inhibitory activity (Gagliardi et al. 1999). Bafilomycin can therefore, be used experimentally to inhibit Golgi acidification.



Bafilomycin A1

6.2.3 Effect of Golgi pH on glycosylation

Altered glycosylation may result from altered expression or localisation of glycotransferases (Brockhausen et al. 2001, Sewell et al. 2006, Taylor-Papadimitriou et al. 1999). However, the molecular basis for increased TF expression cannot be so readily explained, due to a poor correlation between TF expression and the observed enzymatic changes in cancer cells. Altered MUC1 glycosylation in breast cancer cells is an example of this, with predominant changes involving a change from Core 2, to Core 1 type structures, as a result of a lack of the Core 2 *N*-

acetylglucosaminyltransferase 1 (C2GnT1) or competition from the α2-3-sialyltransferase (α2-3ST1), which is seen to be increased in breast cancer cells (Dalziel et al. 2001). However, C2GnT 1 is not consistently altered and breast cancer cells produce large amounts of unsialylated MUC1-associated oligosaccharide chains, even though there is an increase in terminal sialyltransferase activity (Brockhausen et al. 1995).

The pH of the Golgi apparatus is crucial for normal cellular glycosylation to occur. The Golgi apparatus under normal conditions has a pH, which progressively becomes more acidic, from pH6.7 at the *cis*-Golgi to pH6.3 at the *trans*-Golgi (Paroutis et al. 2004). This acidic pH is crucial for controlling the localisation of glycosyltransferases and ensuring their optimum activity (Varki 1998). Bafilomycin A_1 is able to specifically inhibit the vacuolar H^+ATPases of the Golgi (Moriyama et al. 1989) and causes an alkalinisation of the Golgi apparatus (Wu et al. 2000). This alkalinisation of the Golgi by bafilomycin A_1 causes structural changes in the Golgi apparatus (Kellokumpu et al. 2002) [Figure 6.1] that correlate with an increase in cancer-associated carbohydrate antigens such as TF in breast and colorectal cancer cells (Rivinoja et al. 2006).

The addition of bafilomycin A_1 or the weak base ammonium chloride (NH_4Cl) and subsequent increase in Golgi pH of HeLa and LS174T cells causes re-localisation of *N*-acetylgalactosaminyltransferase 2; β1-2 *N*-acetylglucosaminyltransferase I, and β1-4 galactosyltransferase 1, normally localised to the Golgi stack, the medial/trans-Golgi and the trans-Golgi/TGN, respectively (Axelsson et al. 2001). Increase of the Golgi pH in cultured LS174T goblet-differentiated cells with bafilomycin A_1 has been shown to mimic the decreased mucin sulphation and increased TF antigen expression seen in colon cancer (Campbell et al. 2001).

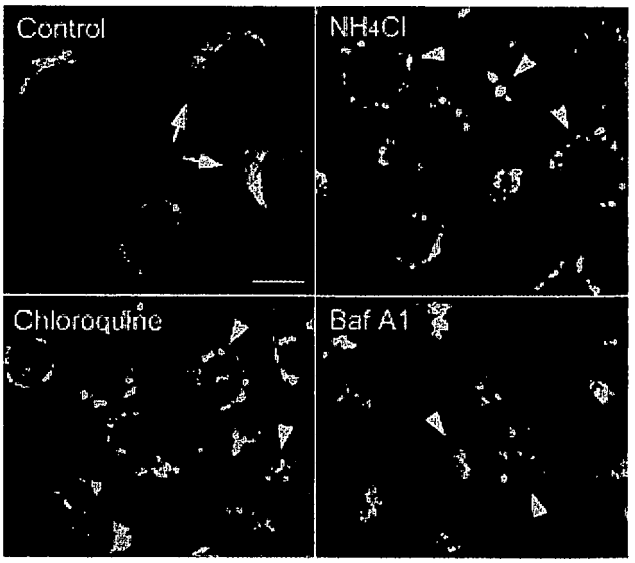


Figure 6.1: Disorganisation of the Golgi apparatus in cultured NRK cells treated with drugs that block Golgi acidification (Kellokumpu et al. 2002).

6.2.4 RNA interference and RNA silencing

The impact of specific glycosyltransferases can be assessed in a cell line by using RNA interference or silencing. The RNA interference (RNAi) pathway was first observed in petunias, in a phenomenon then called cosuppression, where the introduction of a pigment producing gene under the control of a powerful promoter caused the suppression of both the introduced gene and the homologous endogenous gene (Napoli et al. 1990). Cosuppression was found to exist in other species of plants and fungi where it was called "quelling" (Cogoni et al. 1996). A further discovery was made from the injection of dsRNA into the gonads of *Caenorhabditis elegans,* which resulted in potent gene silencing and established dsRNA as the inducer of RNAi (Fire et al. 1998). Short interfering RNAs (siRNAs) were first implicated in plants as part of the post translational gene silencing in plants (PTGS) (Hamilton et al. 1999) which led to the work on the *Drosophila* system, which demonstrated that dsRNA was processed

into siRNAs of 21-25bp in length which could then cause mRNA cleavage corresponding to the introduced dsRNA (Elbashir et al. 2001), forming the basis of the RNAi pathway. Higher eukaryotes have many antimicrobial defence mechanisms, which are based on the recognition of conserved molecular patterns which includes double stranded RNA (dsRNA). The RNA interference (RNAi) pathway is an antiviral immune response to dsRNA, which is a key component in the genomic replication of viral DNA and not normally present in eukaryotic cells (Cullen 2002). This ancient and highly conserved innate cellular response can be induced artificially by the addition of exogenous dsRNA or short interfering RNA (siRNA) stimulating the RNAi pathway. Early efforts to utilise the RNAi phenomenon for gene suppression were disrupted, as it was found that dsRNA larger than 30bp caused an interferon-mediated response causing apoptosis of the cells. The RNAi pathway is by nature extremely sequence specific, responding to dsRNAs by selectively degrading mRNAs that are homologous in sequence to the dsRNA inducer. RNAi is, therefore, capable of blocking the expression of not only viral, but host cell genes, upon the introduction of homologous dsRNA. This enables the specific knockdown of proteins by the introduction of sequence specific dsRNAs [Figure 6.2].

Figure 6.2: General pathway of RNAi in vitro

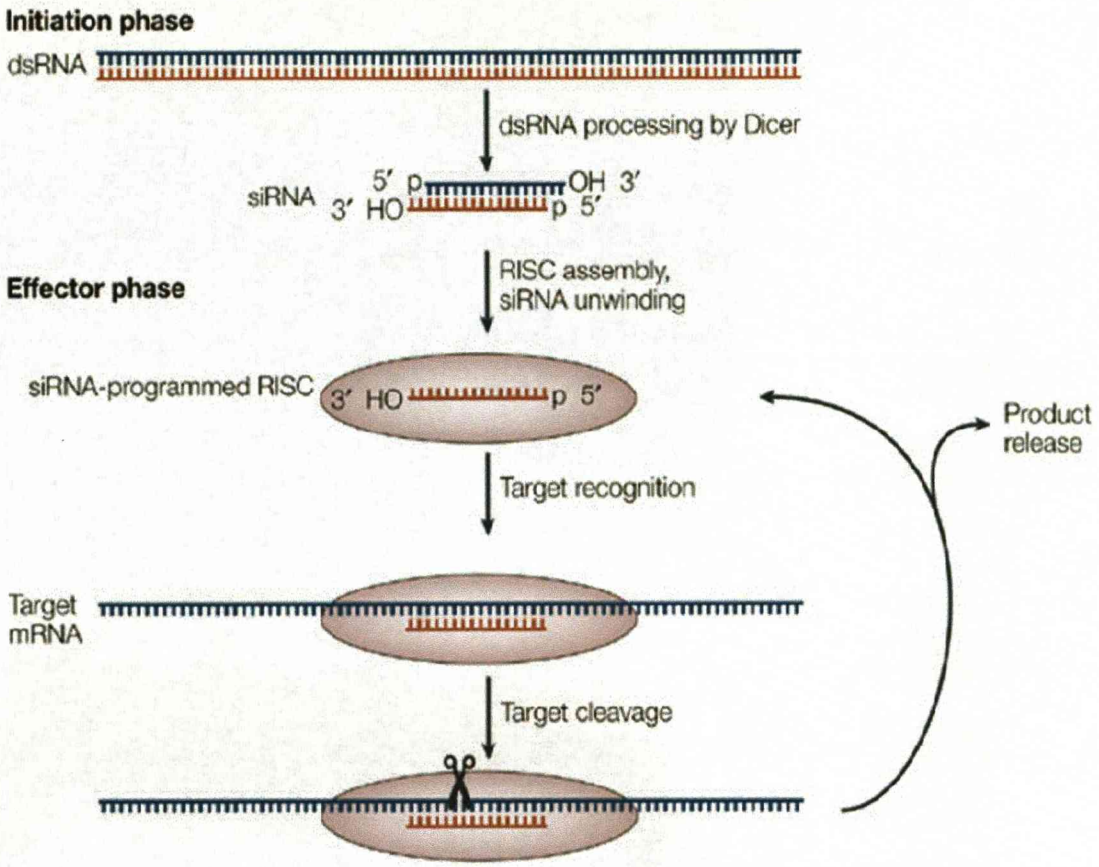


Figure 6.2: The general pathway of RNAi *in vitro* (Sontheimer 2005).

The mechanism for RNA silencing by inducing the RNAi pathway in mammalian cells *in vitro* commences with the introduction of dsRNA which is cleaved in to 21-23bp siRNA duplexes by the ribonuclease-III enzyme Dicer (Doi et al. 2003) [Figure 5.2]. siRNAs bear 5′-phosphate groups and 2-bp 3′ overhangs, both of which are important for subsequent siRNA-induced silencing complex (siRISC) assembly (Sontheimer 2005). Alternatively the siRNAs can be chemically synthesised and be introduced directly into the cytoplasm with use of transfection reagents. During the effector phase of the RNAi pathway the siRNA is unwound (Martinez et al. 2002) by

an unwindase enzyme leading to the assembly of RISC. It is this activated effector complex that recognises the target by siRNA to mRNA base pairing, and cleavage of the mRNA strand is achieved through its endoribonuclease activity (Liu et al. 2004). This process is accelerated by the presence of ATP, which increases enzyme turnover by promoting siRNA-product unwinding and product release (Haley et al. 2004). The remaining 'guide' strand associates with the PAZ domain of an Argonaute (Ago2) protein (Lingel et al. 2004) at which point the RISC complex is ready to mediate its effects and cleave the specific mRNA.

The formation of the RISC complex is still being elucidated, but the main features of this process are now understood with the majority of the work being achieved in the *Drosophila melanogaster* system [Figure 6.3]. After dsRNA processing the siRNA will either stay attached to the Dicer (Dcr2)-R2D2 heterodimer it was generated by or be taken up by another Dcr2-R2D2 complex in a formation previously known as R1. The R2D2 will bind the more thermodynamically stable end of the siRNA and the Dcr2 the less stable end, this asymmetric binding facilitates the determination of the guide strand of siRNA from the strand which will be loaded into the RISC complex later on. The siRNA then enters the RISC-loading complex (RLC) which was known before as R2, and associated with other as yet unknown RISC factors and one of the siRNA strands (the 'passenger' strand) is discarded [Figure 6.3]

Figure 6.3: The assembly of RISC in the *Drosophila melanogaster*

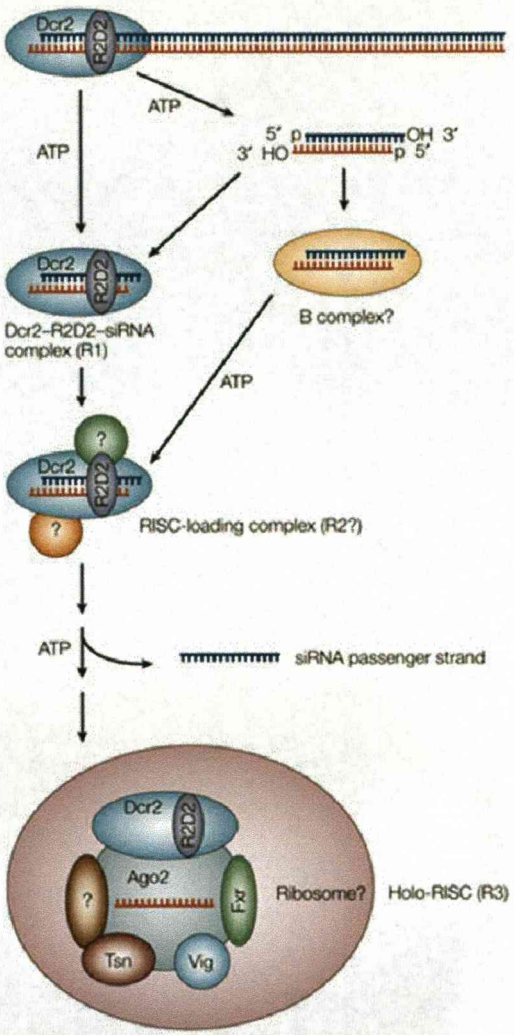


Figure 6.3: The assembly of RISC (Sontheimer 2005)

6.2.5 SiRNA Galtransferase

After the addition of GalNAc to the serine or threonine residues of the glycoprotein, the formation of Core 1, by the addition of galactose is controlled by a core β1-3 Galtransferase. To date there is only one known Galtransferase specific for transfering galactose onto GalNAc on *O*-linked chains (Ju et al. 2002). In the present study RNA silencing is employed to suppress this transferase and, therefore, prevent the formation of Core 1 and also preventing any further elongation. The importance

of the TF antigen in NLS-dependent nuclear protein import can thus be assessed by using siRNA technology to suppress the formation of the TF antigen with analysis of Hsp70 translocation in to the nucleus upon heat shock.

6.2.6 Tumour Necrosis factor (TNFα)

Although TNFα is not a classical glycosylation inhibitor; the alterations in glycosylation observed in colon cancer and inflammatory bowel disease have been induced in cultured goblet cell differentiated cell lines by TNFα. Alterations such as aberrant mucin synthesis and expression, increased TF antigen expression and reduction in mucosal sulphation have been shown (Campbell et al. 2000, Campbell et al. 2002). TNFα also increased cell surface expression of the cancer related carbohydrate antigen sialyl Lewis^x (Kuninaka et al. 2000) and Lewis^y in response to interferon gamma and interferon alpha (Flieger et al. 2001).

6.3 METHODS

6.3.1 Hsp70 localisation

HT-29 cells were seeded at $1x10^4$ on glass cover slips, 24 h prior to siOrp150 treatment. After 2 days siRNA treatment, the cells were heat-treated for 1h at 42°C, and then fixed for 10 min in freshly prepared paraformaldehyde followed by 3 washes with PBS. The fixed cells were then blocked with 300 µl of 5% (v/v) goat serum (Dako; Glostrup, Denmark) for 1 h at room temperature, followed by the addition of anti-Hsp70 antibody (Bioquote, York) 1:200 concentration, for 2 h. The cells were then washed 2 times with PBS and then the FITC conjugated secondary antibody (Dako; Glostrup, Denmark) was added, concentration 1:500, for 1 h in the dark. The cells were then washed 3 times with PBS and the cover slips mounted on glass slides with a PI nuclear counter-stain mounting solution. The localisation of Hsp70 was then visualised using an Olympus camera and the % nuclear Hsp70 calculated used AQM software.

Hsp70: Experiments were performed 5 times and the slides were blind labeled, each time 10 individual cells for each category were chosen at random from the slide for AQM analysis. Inter assay coefficient of variance 8.3%, intra assay coefficient of variance 8.7% for all experiments.

Hsp70 control experiment =Inter assay coefficient of variance (COV): 8.3%,

Intra assay COV 4.1%

Hsp70 heat control experiment = Inter assay coefficient of variance (COV): 8.0%,

Intra assay COV 3.0%

6.3.2 *O*-Glycosylation inhibitors

HT-29-MTX cells were grown as monolayers in 24 well plates to 60% confluence; they were then treated with the inhibitors at the following final concentrations chosen from previous work in the laboratory and literature: 5-CDP: 1 mM (Kleineidam et al. 1997), TNFα: 2 ng/ml (Campbell et al. 2000, Campbell et al. 2002) , benzyl GalNAc 2 nM (Huet et al. 1998), bafilomycin 0.01 μM (Campbell et al. 2001)

6.3.3 FACS analysis

HT-29-MTX cells were grown as monolayers in 6 well plates to 60% confluence and treated with the *O*-glycosylation inhibitors, as described previously. The cells were washed 3 times with PBS in the well before addition of a non enzymatic dissociation solution, 1 ml/well (Sigma; Dorset, UK), the cells were incubated at 37°C until dissociated. The cells were washed twice with 1ml ice cold PBS and collected each time by centrifugation at 70 *g* for 1 min (1000 rpm; MSE microcentaur, Sanyo Gallenkamp PLC, UK). The cells were then fixed in 1% paraformaldehyde for 10 minutes on ice and then washed 3 times with PBS. Following this the cells were incubated with 200 μl of either biotinylated PNA (Vector, UK) or *Vicia villosa* (VVA) (Vector, UK) at a concentration of 1mg/ml, in a PBS solution containing 1% BSA. Again the cells were washed 3 times with PBS, before incubation with a 1:200 dilution of Avidin-FITC in a PBS-BSA 1% solution, in the dark. The cells were washed 3 times with PBS and resuspended in 200μl PBS ready for the FACS analysis. The experiment was repeated 3 times. Each time 10,000 gated events were recorded.

6.3.4 siRNA Galtransferase

Cell plating:

HT-29 cells were diluted in antibiotic-free DMEM with 5% FCS to a plating density of 5.0 x 10^4 cells/ml. 100µl of cells were placed in to each well of a 96 well plate and incubated for 24 h.

Transfection:

To perform the experiment in triplicate the following protocol was followed: A 2 µM siRNA solution in siRNA buffer (Dharmacon) was prepared, from this 17.5 µl was added to 17.5 µl serum free/antibiotic free medium. In a separate tube 1.4 µl of DharmaFECT4 (Dharmacon) was added to 33.6 µl of FCS-free/antibiotic-free DMEM. These tubes were pipetted up and down and left for 5 min before mixing them together and leaving to stand for a further 20 min. To this solution 280 µl antibiotic free DMEM was added and to each well 100µl was added. The plate was incubated at 37°C in a humidified atmosphere of 5% CO2, 95% air for required time.

6.4 RESULTS

6.4.1 O-glycosylation inhibitors increase expression of TF in HT-29 MTX cells

Initial experiments to analyse the effects of the *O*-glycosylation inhibitors on HT-29 MTX cells were performed by investigating the expression of TF and sialic acid. HT-29 MTX cells are a homogeneous, mainly MUC5AC (normal gastric mucin) secreting population of well-differentiated goblet cells that has been obtained by stepwise adaptation of HT-29 cells to 10^{-6} or 10^{-5} M methotrexate (MTX) (Lesuffleur et al. 1990). HT-29-MTX cells were cultured for 2 days prior to the addition of the *O*-glycosylation inhibitors, bafilomycin 100 nM, TNFα 2 ng/ml, 5-CDP 1 mM, and benzyl GalNAc 2 nM. Subsequent lectin blotting with PNA lectin was utilised to assess the expression of the TF antigen. An initial time course experiment ranging from 0-3 days was first completed. It revealed that 48 h incubation with the *O*-glycosylation inhibitors caused the greatest increase in TF expression as assessed by PNA lectin blotting and densitometric analysis of the bands [Figure 6.4]. The time course was repeated 2 times. By Day 1 only benzyl GalNAc showed a significant increase in TF ($p<0.05$) [ANOVA]. By Day 2 bafilomycin, 5-CDP and TNFα ($p<0.05$) and benzyl GalNAc ($p<0.001$) all showed significant increase in TF, this was also observed on day 3 [Figure 6.5]. Consequently, 48 h incubation was used during subsequent experiments.

Figure 6.4: Representative slot blots of PNA lectin binding. Time course of the effect of *O*-glycosylation inhibiting drugs on PNA lectin binding.

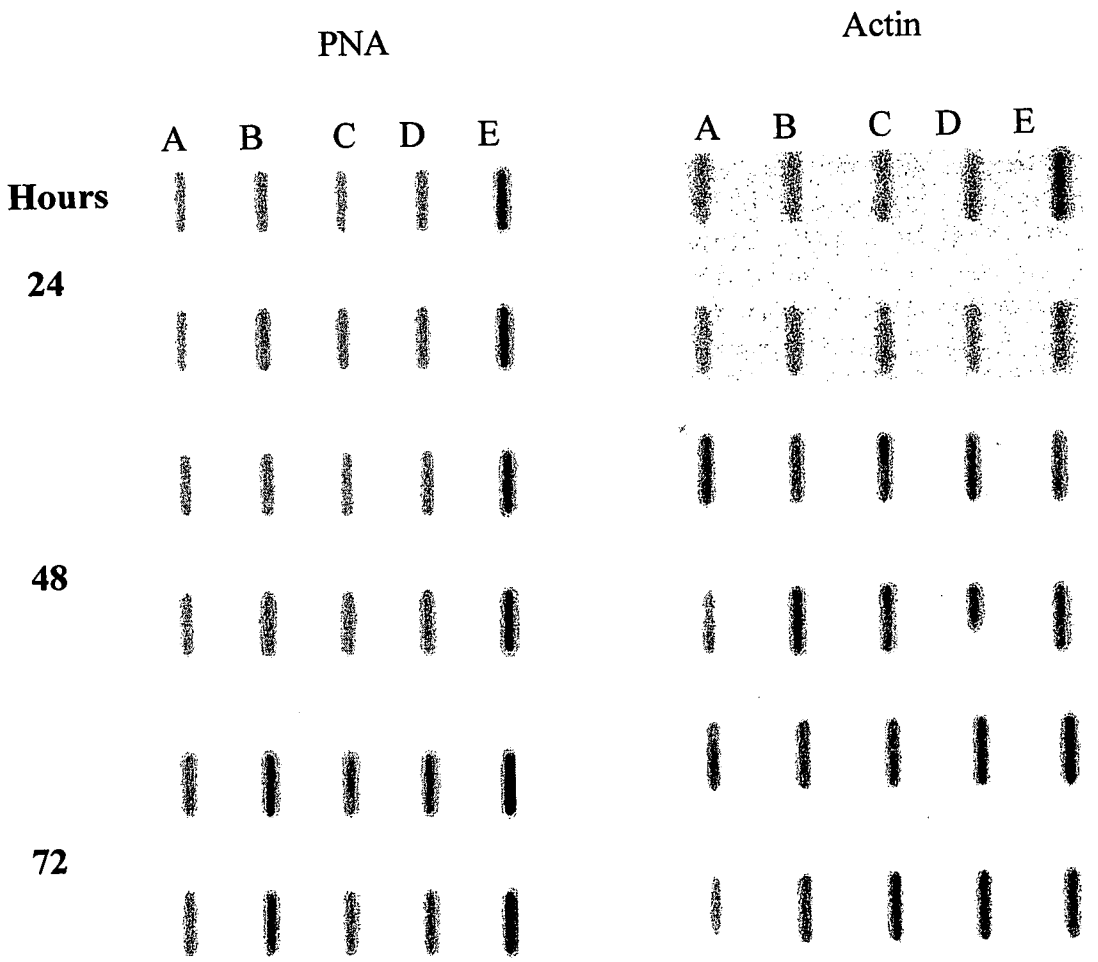


Figure 6.4: PNA lectin blot. Lane A: control, Lane B: bafilomycin, Lane C: TNFα, Lane D: 5-CDP, Lane E: benzyl GalNAc.

HT-29 MTX cells were grown to 60% confluence in 24 well plates before the addition of the *O*-glycosylation inhibitors at appropriate concentrations for 0-3 days. At the end of each time point the cells were washed 3 times with PBS and lysed directly with sample buffer. The samples were then subjected to slot blot analysis and lectin blotting with PNA lectin.

Figure 6.5: Mean density of PNA binding after inhibitor treatment

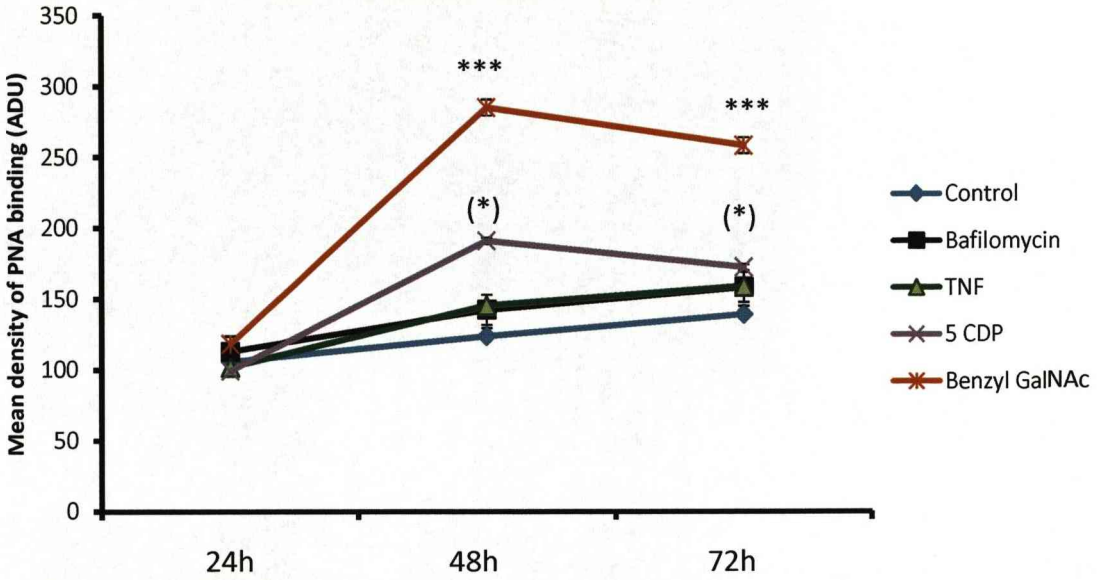


Figure 6.5: Time course (n=2) of the 4 *O*-glycosylation inhibiting drug treatments. HT-29 MTX cells were grown to 60% confluence in 24 well plates before the addition of the *O*-glycosylation inhibitors at appropriate concentrations for 0-3 days. At the end of each time point the cells were washed 3 times with PBS and lysed directly with sample buffer. The samples were then subjected to slot blot analysis and lectin blotting with PNA lectin, with analysis of the density of bands using Quantity One software (Bio-Rad; Hemel Hempstead, UK). By day 1 only benzyl GalNAc showed significant increase of PNA binding ($p<0.05$) [ANOVA], but by day 2 and 3 all inhibitors show significant increase in PNA binding (benzyl GalNAc ***= $p<0.005$, all others (*) =$p<0.01$) [ANOVA], the greatest overall increase was observed on day 2.

Further investigation of the effect of the *O*-glycosylation inhibitors at the 48h time point also demonstrated a significant increase in PNA binding. The experiment was repeated 4 times and the PNA binding assessed after slot blotting [Figure 6.6]. Benzyl GalNAc caused the most significant increase in PNA binding ($p<0.001$), bafilomycin, TNFα and 5-CDP ($p<0.05$). [ANOVA] [Figure 6.7]

Figure 6.6: Representative slot-blot of the effect of *O*-glycosylation-inhibiting drugs on PNA lectin binding after 48h

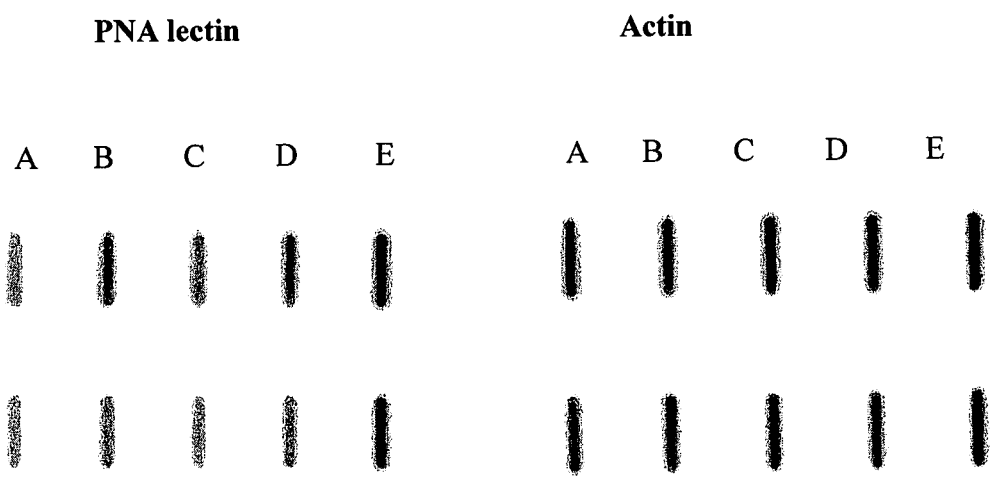


Figure 6.6: PNA lectin blot. Lane A: control, Lane B: Bafilomycin, Lane C: TNF alpha, Lane D: 5-CDP, Lane E: Benzyl GalNAc.

HT-29 MTX cells were grown to 60% confluence in 24 well plates before the addition of the *O*-glycosylation inhibitors at appropriate concentrations for 2 days. The cells were then washed 3 times with PBS and lysed directly with sample buffer. The samples were then subjected to slot blot analysis and lectin blotting with PNA lectin, with densitometric analysis of the bands using Quantity One software (Bio-Rad; Hemel Hempstead, UK).

Figure 6.7: TF expression determined by PNA lectin binding in HT-29 MTX cells treated for 48 h with inhibitors

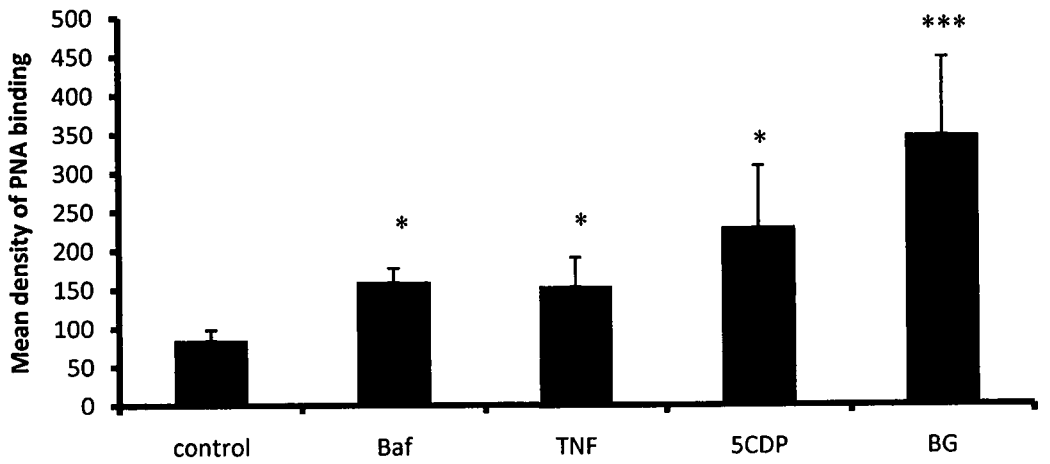


Figure 5.6: The density of PNA binding after treatment with *O*-glycosylation inhibitors for 48 h as determined by analysis of the density of bands using Quantity One software (Bio-Rad; Hemel Hempstead, UK). Each *O*-glycosylation inhibitor showed significance (n=4) (* = $p<0.01$, *** = $p<0.001$), with benzyl GalNAc causing the greatest increase in TF expression ($p<0.001$) [ANOVA].

6.4.2 Benzyl GalNAc increases cell surface expression of TF on HT-29 MTX cells

Flow cytometry was utilised to further analyse the effects of the *O*-glycosylation inhibitors on the expression of the TF antigen, using a PNA-FITC conjugate. The experiment was repeated 3 times and each analysis was of 10,000 gated events [Figure 6.9]. The control gated mean (±SD) was 9±1 and only benzyl GalNAc appears to increase surface binding of PNA lectin as compared to control cells, with a gated mean of 37±1 (n=3, $p<0.005$). HT-29-MTX cells treated with bafilomycin had a gated mean of 12±1.3, TNFα a gated mean of 10±1 and 5-CDP a gated mean of 13±3 (All n=3, and $p>0.05$). A graph of the gated mean shows clearly the increase in PNA binding [Figure 6.8].

Figure 6.8: Gated mean value of FACS analysis of PNA binding

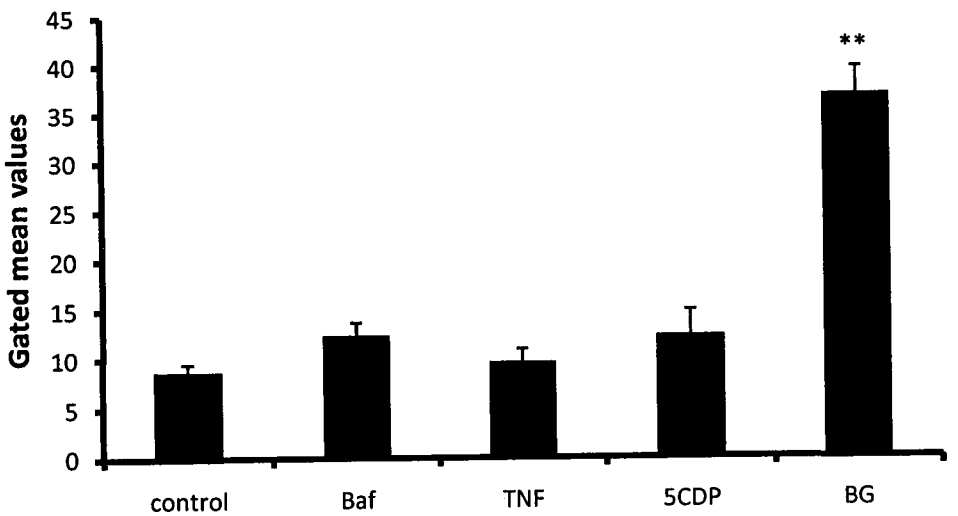


Figure 6.8: Gated mean values of HT-29-MTX cells which have been incubated with PNA-FITC prior to FACS analysis. Benzyl GalNAc, $p<0.005$ (**). All others not significant.

Figure 6.9: FACs analysis of TF expression after inhibitor treatment

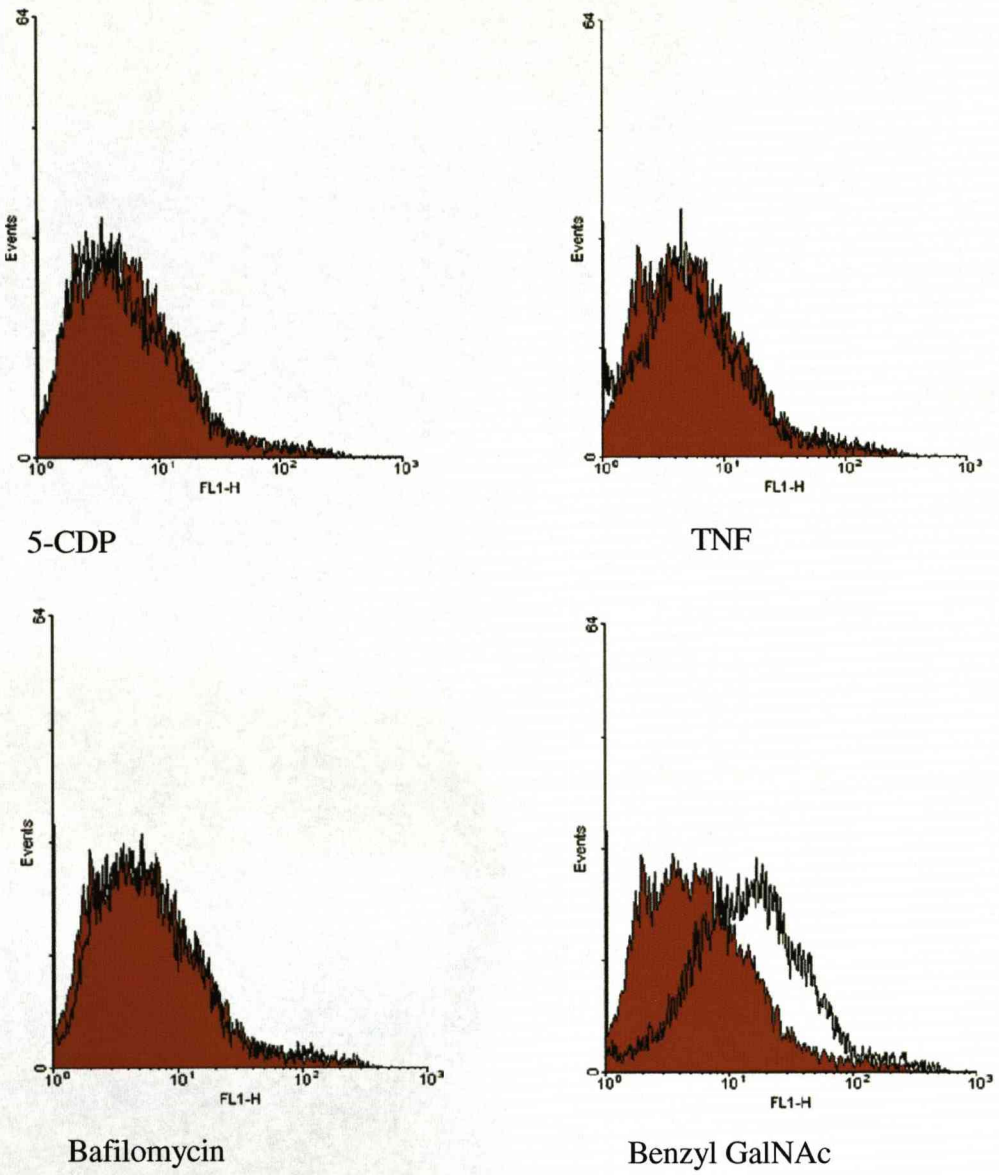


Figure 6.9: FACS analysis of HT-29 MTX cells that were treated with the *O*-glycosylation inhibitors for 48 h prior to incubation with PNA-FITC conjugate. Only Benzyl GalNAc causes an increase of surface TF expression compared to control cells with a mean gated value of 37 (n=3, $p<0.005$). All other treatments had no significant effect.

6.4.3 O-glycosylation inhibitors decrease sialic acid content of HT-29 MTX cells

The effect of *O*-glycosylation inhibitors on the expression of sialic acid was assessed. HT-29 MTX cells were cultured for 2 days prior to the addition of the *O*-glycosylation inhibitors, bafilomycin 100 nM, TNFα 2 ng/ml, 5-CDP 1 mM, benzyl GalNAc 2 nM and a further incubation of 48 h. Subsequent lectin blotting with MAL II lectin was utilised in order to assess the expression of sialic acid. The experiment was repeated 3 times, control mean density of MAL II binding was 122 ± 13 ADU. The only statistically significant change in sialic acid expression resulted from benzyl-GalNAc with a mean density 93 ± 5 ADU ($p<0.05$) and 5-CDP with a mean density of 94 ± 8 ADU ($p<0.05$) [Figure 6.10 and 6.11].

Figure 6.10: Effect of *O*-glycosylation inhibiting drugs on MAL II lectin binding

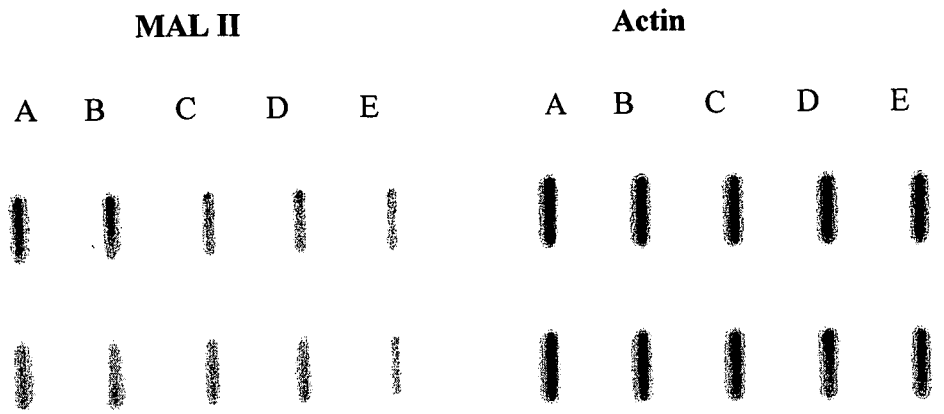


Figure 6.10: Representative MAL II lectin blot. Lane A: control, Lane B: Bafilomycin 0.01μM, Lane C: TNF alpha 2ng/ml, Lane D: 5-CDP 1mM, Lane E: Benzyl GalNAc 2nM.

HT-29-MTX cells were grown to 60% confluence in 24 well plates before the addition of the inhibitors under test at appropriate concentrations for 48 h. The cells were then washed 3 times with PBS and lysed directly with sample buffer. The samples were then subjected to slot blot analysis with MAL II lectin, with analysis of the density of bands using Quantity One software (Bio-Rad; Hemel Hempstead, UK).

Figure 6.11: Sialic acid expression determined by Mal II lectin binding to treated HT-29 MTX cells

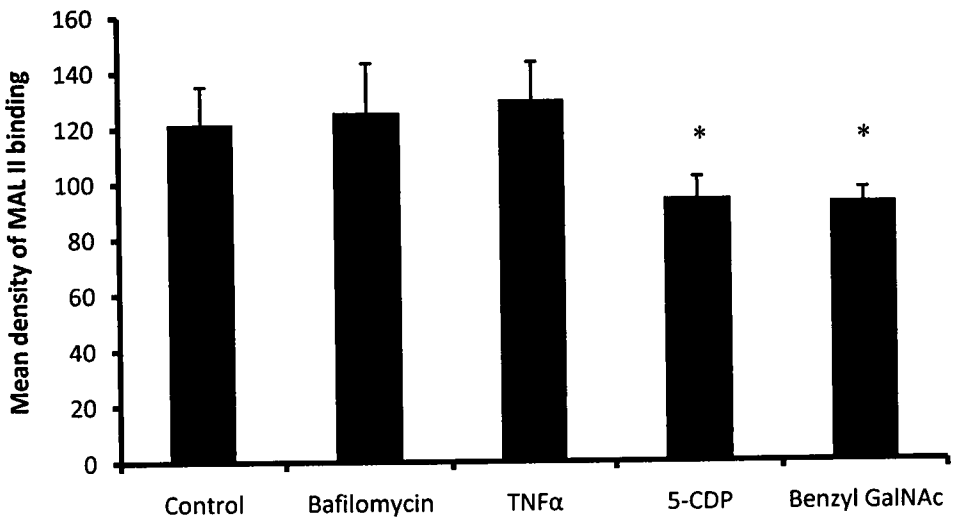


Figure 6.11: The density of MAL II binding after treatment with *O*-glycosylation inhibitors for 48 h as determined by analysis of the density of bands using Quantity One software (Bio-Rad; Hemel Hempstead, UK). Benzyl GalNAc and 5-CDP caused the greatest decrease in sialic acid with a mean MALII binding density of 93±5 ($p<0.05$).

6.4.4 Benzyl GalNAc causes a decrease in cell surface sialic acid expression

The surface expression of sialic acid was assessed using FACS analysis, using *Maackia amurensis* lectin II (MAL II), a MAL-FITC conjugate was used in these experiments. MAL II appears to bind only particular carbohydrate structures that contain sialic acid and unlike *Sambucus nigra* (SNA) which seems to prefer structures with α2-6 linked sialic acid, MAL II appears to bind sialic acid in an α2-3 linkage. The experiment was repeated 3 times and each analysis was of 10,000 gated events, representative graphs are shown in Figure 6.13. The control gated mean (±SD) was 30±2 and only Benzyl GalNAc appears to have an effect on cell surface sialic expression, causing a decrease as compared to control cells, with a gated mean of 23±2 (n=3, $p<0.005$) [Figure 6.12]. HT-29-MTX cells treated with Bafilomycin had a gated mean of 29±2, TNFα a gated mean of 30±2 and 5-CDP a gated mean of 30±2 (All n=3, and $p>0.05$).

Figure 6.12: Gated mean value of FACs analysis of MAL II binding

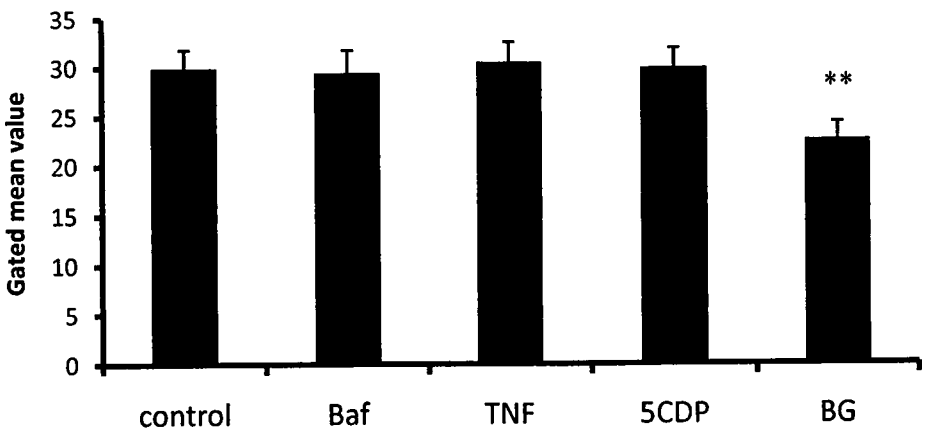


Figure 6.12: Gated mean values of HT-29-MTX cells which have been incubated with MAL II-FITC prior to FACS analysis. Benzyl GalNAc, $p< 0.005$ (**). All others not significant.

Figure 6.13: FACS analysis of MAL II binding after inhibitor treatment

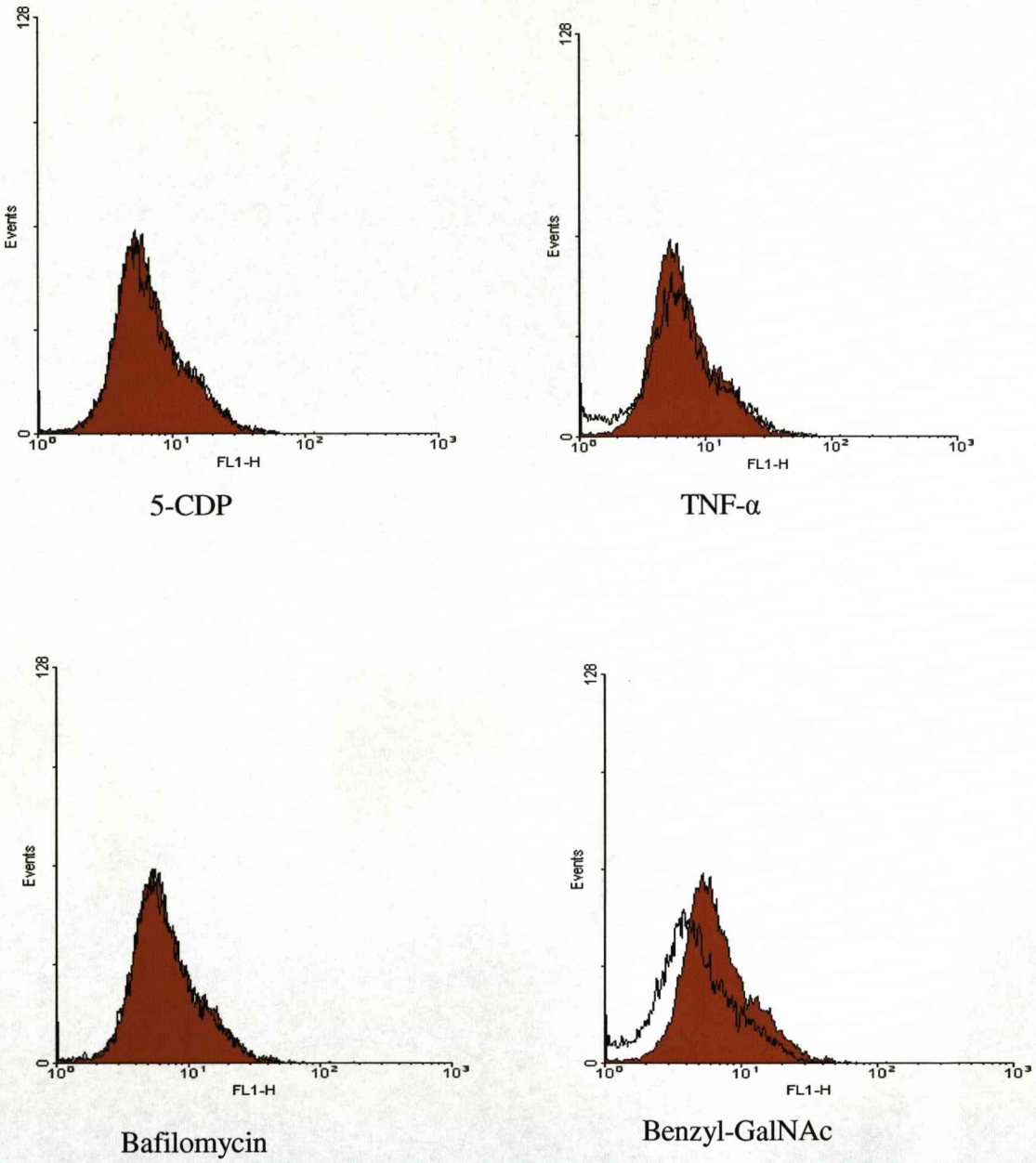


Figure 6.13: FACS analysis of HT-29 MTX cells that were treated with the *O*-glycosylation inhibitors for 48 h prior to incubation with MAL II-FITC conjugate. Only Benzyl GalNAc causes an increase of surface TF expression compared to control cells with a mean gated value of 23 (n=3, $p<0.005$)[ANOVA]. All other treatments had no significant effect.

The data from the FACS analysis implies that benzyl-GalNAc affected cell surface glycosylation with an increase in TF expression and a decrease in sialylation with a 48 h incubation with the inhibitor. It may be possible that a longer incubation period with the inhibitors may result in the other agents having an effect on cell surface glycosylation.

6.4.5 O-glycosylation inhibitor drugs shown no effect on reducing Hsp70 translocation into the nucleus

The effects of the *O*-glycosylation inhibitors on the expression of TF and sialic acid have then been established and this allows an investigation into the effect of these changes in glycosylation on NLS-dependent nuclear protein import by assessing the translocation of Hsp70 into the nucleus upon heat shock. *O*-glycosylation-inhibiting drugs benzyl GalNAc and 5-CDP and also bafilomycin, TNFα were used to treat HT-29 MTX cells for 48 h. A percentage of Hsp70 visualised in the cell nucleus compared to the cytoplasm was calculated, a representative image for each inhibitor is shown in Figures 6.14 and 6.15. For each experiment 10 cells at random were chosen, and the experiment was repeated 3 times. Control cells were shown to have a nuclear Hsp70 fluorescence of 43 ± 2 % [mean±SD] (n=30). None of the *O*-glycosylation inhibitors induced a significant variance from the control, bafilomycin = 42 ± 2% (n=30), TNFα = 42 ± 2 % (n=30), 5-CDP= 40 ± 2% (n=30), benzyl GalNAc = 40 ± 1% (n=30). The inter assay coefficient of variance for the experiments was 8.3% and the intra assay coefficient of variance was 4.1% [Figure 6.16].

HT-29-MTX cells were then heat treated at 42°C for 1 h to measure the translocation of Hsp70 from the cytoplasm into the nucleus. The control cells showed an increase in nuclear fluorescence with an average of 60 ± 2% [mean ± SD] (n=30) ($p<0.001$). The HT29-MTX cells that were treated with inhibitors showed no

significant variance from control cells, Bafilomycin = 61 ± 1% (n=30), TNFα = 60 ± 1% (n=30), 5-CDP= 61 ± 2% (n=30), Benzyl GalNAc = 59 ± 1% (n=30). The inter assay coefficient of variance for the experiments was 8.0% and the intra assay COV 3.0% [Figure 6.16].

Figure 6.14: Hsp70 nuclear localisation before heat treatment

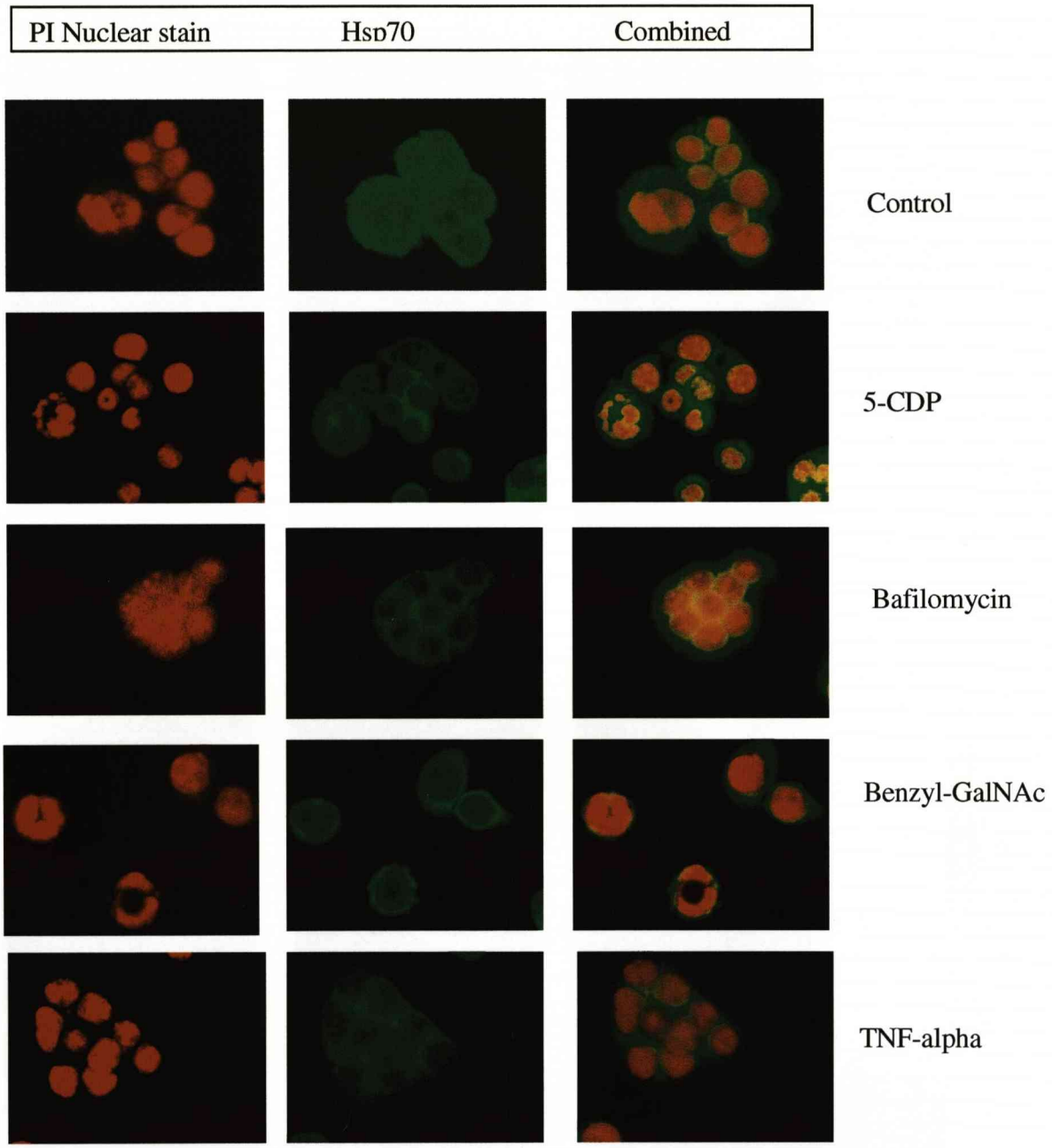


Figure 6.14: Representative images of Hsp70 immunohistochemistry captured with x40 objective. Hsp70 (green) and nuclear (red) in HT-29-MTX cells, showing localisation predominantly in the cytoplasm. The inhibitor treatments showed no significant change compared to control cells.

Figure 6.15: Hsp70 nuclear localisation after heat treatment

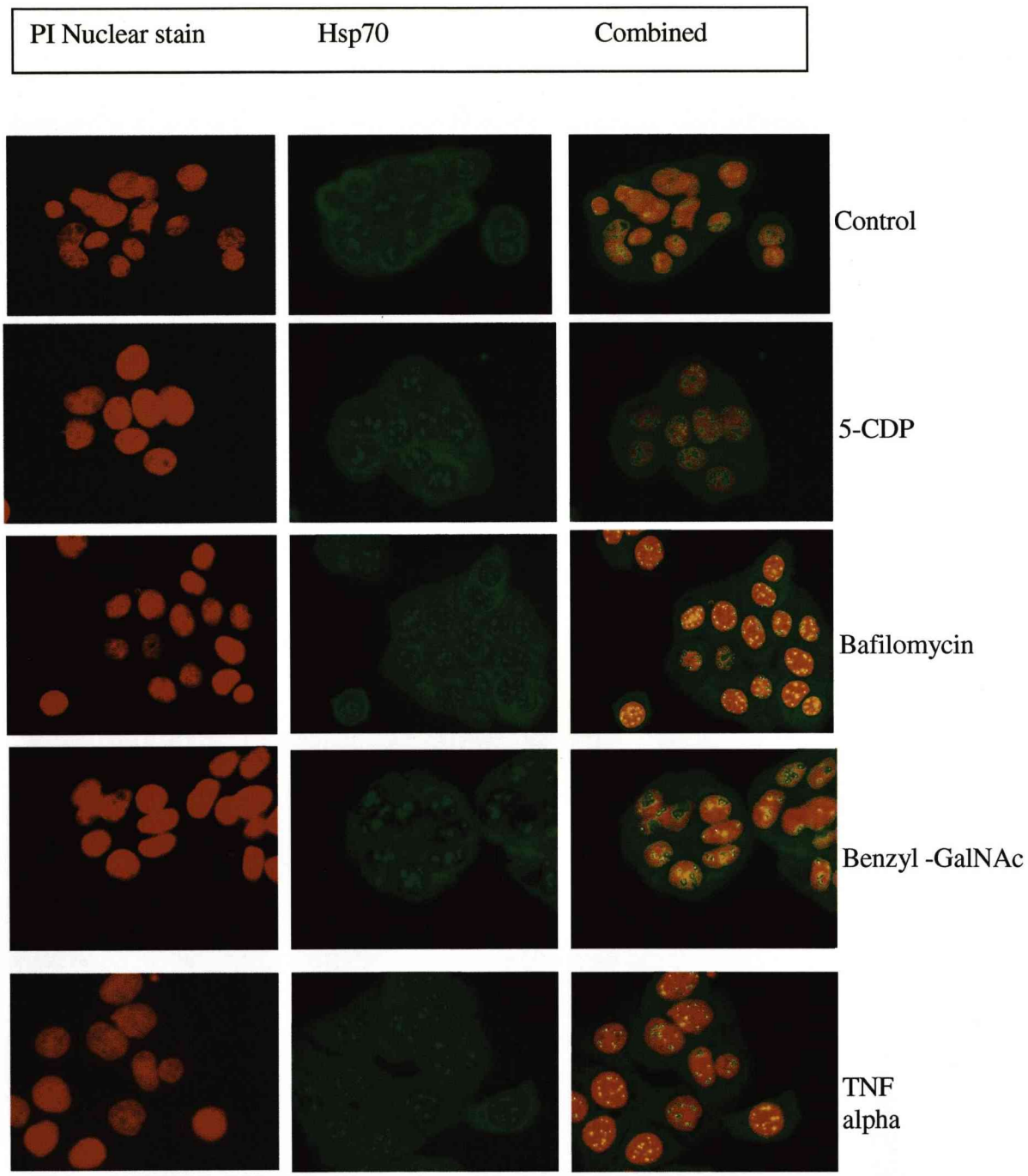


Figure 6.15: Representative images of Hsp70 immunohistochemistry captured x40 objective. Hsp70 (green) and nuclear (red) in HT-29-MTX cells, showing localisation in the nucleus after heat treatment for 1 h at 42°C. The inhibitor treatments showed no significant change compared to control cells.

Figure 6.16: Hsp70 nuclear localisation in HT-29 MTX cells after heat treatment.

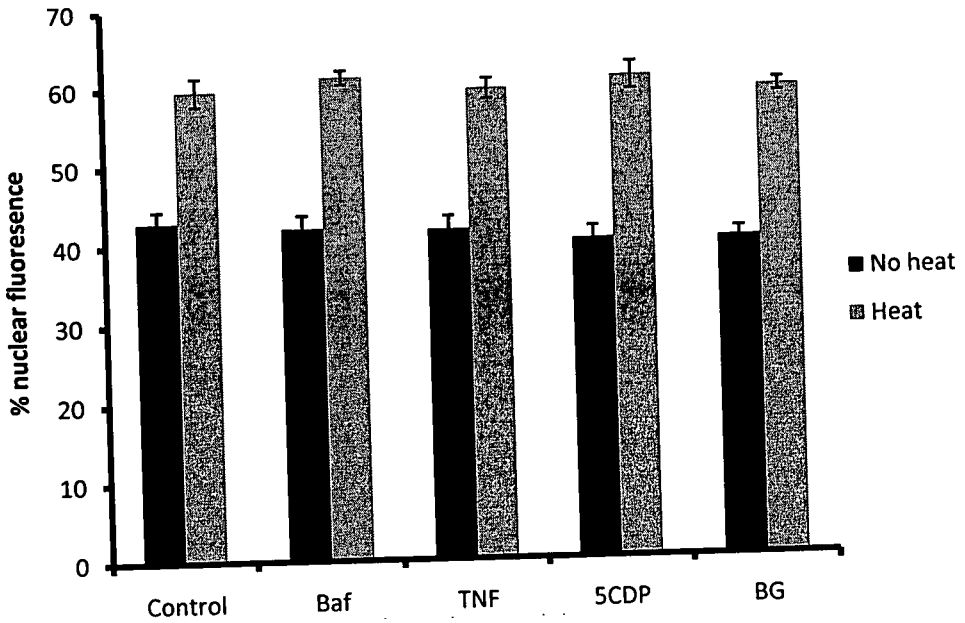


Figure 6.16: Nuclear fluorescence of control cells was increased upon heat treatment, average control= 43 ± 2% compared to average heat treated 60 ± 1.8% ($p<0.001$). None of the glycosylation inhibitors or TNFα have a significant effect compared to control. (BG=benzyl-GalNAc).

6.4.6 siRNA Galtransferase causes a reduction in TF expression and increase in Tn expression in HT-29 cells

This further experiment to uncover a possible role for the oncofetal antigen TF utilised siRNAs directed to the Galtransferase responsible for the formation of the TF antigen and again observed the effect on Hsp70 translocation following heat treatment. This experiment first establishes effectiveness of the siRNA Galtransferase on the reduction of TF antigen expression.

HT-29 cells were transfected with siRNA Galtransferase for 48h in a 96 well plate before direct lysis in slot blot lysis buffer and followed by slot blotting. Lectin blotting with either PNA for TF expression or VVA for Tn expression was quantified using Quantity One software (Bio-Rad; Hemel Hempstead, UK) [Figure 6.17]. The experiment was repeated 3 times and on each occasion in triplicate. The control and siRNA controls were found to be not statistically different from each other in any instance. TF decreased in response to siRNA Galtransferase by 21 ± 3% ($p<0.005$) compared to siRNA control. Tn expression increased in response to siRNA Galtransferase by 42 ± 3% ($p<0.001$) [Figures 6.18-6.20].

Figure 6.17: TF and Tn expression by HT-29 cells in response to siRNA Galtransferase transfection

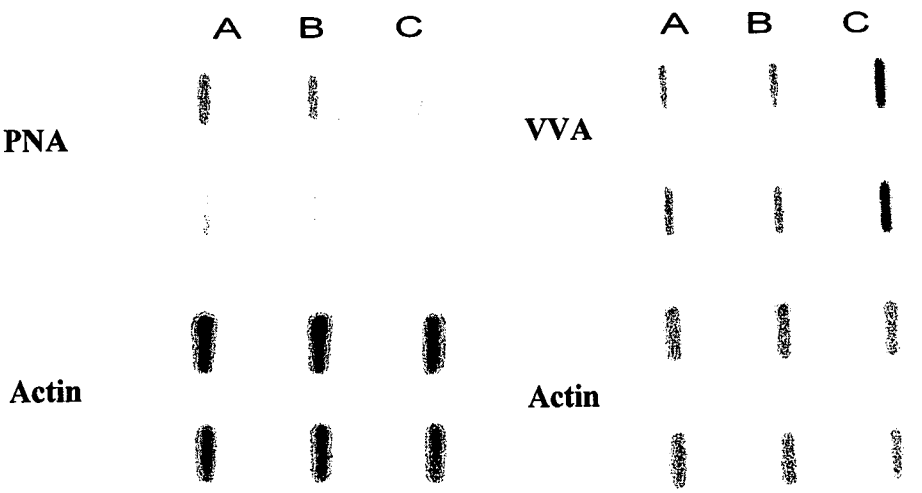


Figure 6.17: TF and Tn expression by HT-29 cells assessed by PNA and VVA lectin blotting respectively. Lane A: control, B: siRNA control, C: siRNA Galtransferase. Demonstrates the reduction of PNA binding and increase in VVA binding after siRNAGaltransferase incubation.

Figure 6.18: Reduction in TF expression in response to siRNA Galtransferase: quantative analysis

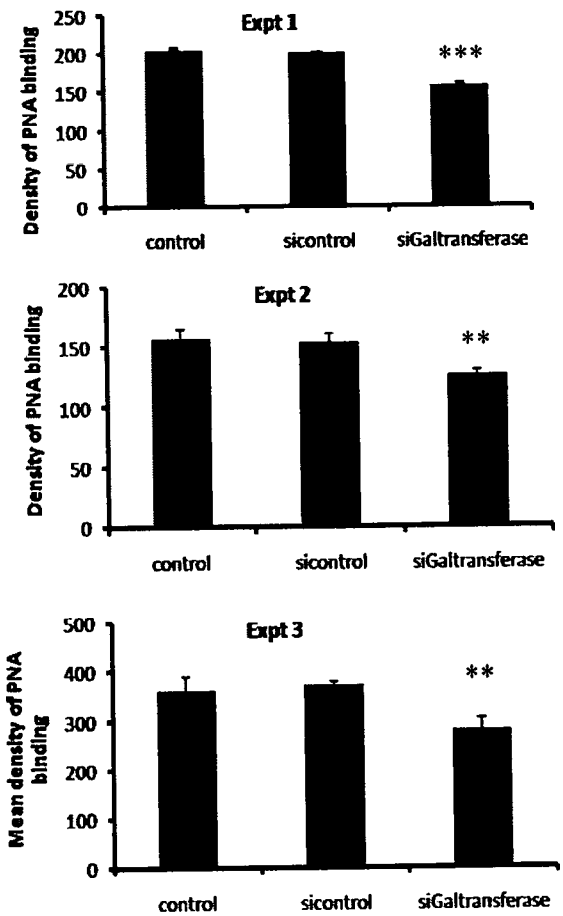


Figure 6.18: HT-29 cells were transfected with siRNA Galtransferase or siRNA control for 48 h in 96 well plates, prior to slotblotting and lectin analysis of TF expression with PNA lectin (arbitrary densiometric units). The experiment was repeated 3 times, on each occasion 3 separate wells were taken for analysis and for each experiment siRNA Galtransferase caused a significant decrease of TF expression, ($p<0.005$) for each experiment, n=3. [ANOVA].

Figure 6.19: increase in Tn expression binding after SiRNA Galtransferase treatment: quantative analysis

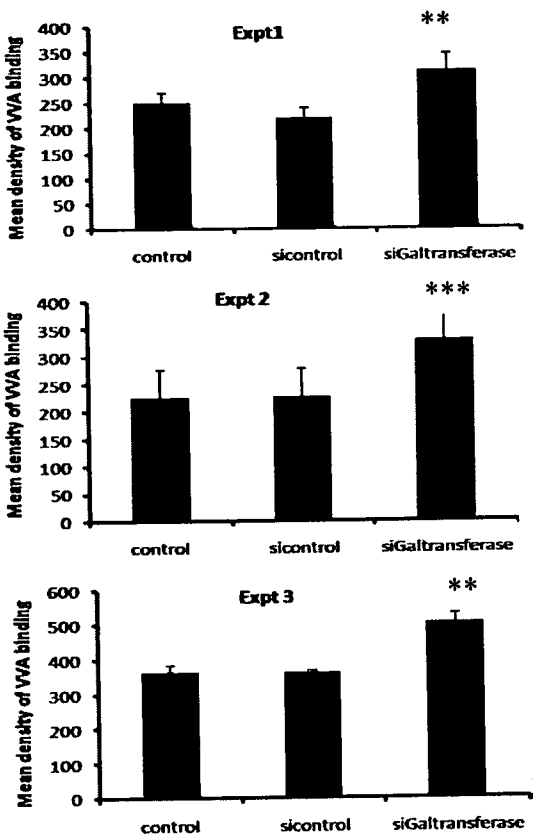


Figure 6.19: HT-29 cells were transfected with siRNA Galtransferase or siRNA control for 48 h in 96 well plates, prior to slotblotting and lectin analysis of Tn expression with VVA lectin (arbitrary densiometric units). The experiment was repeated 3 times, on each occasion 3 separate wells were taken for analysis and for each experiment siRNA Galtransferase caused a significant increase of Tn expression, ($p<0.005$) for each experiment, n=3. [ANOVA].

6.4.7 siRNA Galtransferase transfected cells show no reduction in Hsp70 translocation in to the nucleus upon heat treatment compared to controls

The effect of the siRNA Galtransferase upon NLS-dependent nuclear protein import was assessed by observing the translocation of Hsp70 into the nucleus upon heat shock. HT-29 cells were grown on cover slips and transfected with siRNA control or siRNA Galtransferase for 48 h. The localisation of Hsp70 after heat treatment was then assessed as an indication of NLS-dependent nuclear protein import, and the % nuclear fluorescence was calculated. The experiment was repeated 3 times, on each occasion 10 cells were selected at random (n=30) [Figure 6.21 and 6.22]. The control cells displayed a nuclear fluorescence of 42 ± 1%. The siRNA control transfected cells were similar to control cells with a nuclear fluorescence of 41 ± 1%. The siRNA Galtransferase transfected cells also were found not to be different from controls with a nuclear fluorescence of 40 ± 1%.

The percentage nuclear fluorescence of control cells after heat treatment was 61 ± 1%, significantly increased compared to non heat treated cells ($p<0.005$). The siRNA control treated cells displayed a similar nuclear fluorescence of 61 ± 1%, after heat treatment. Nuclear fluorescence of siRNA Galtransferase heat treated cells was also similar at 58 ± 1%. Thus no change in Hsp70 translocation was observed after siRNA Galtransferase transfection.

Figure 6.21: Hsp70 localisation after siRNA Galtransferase treatment

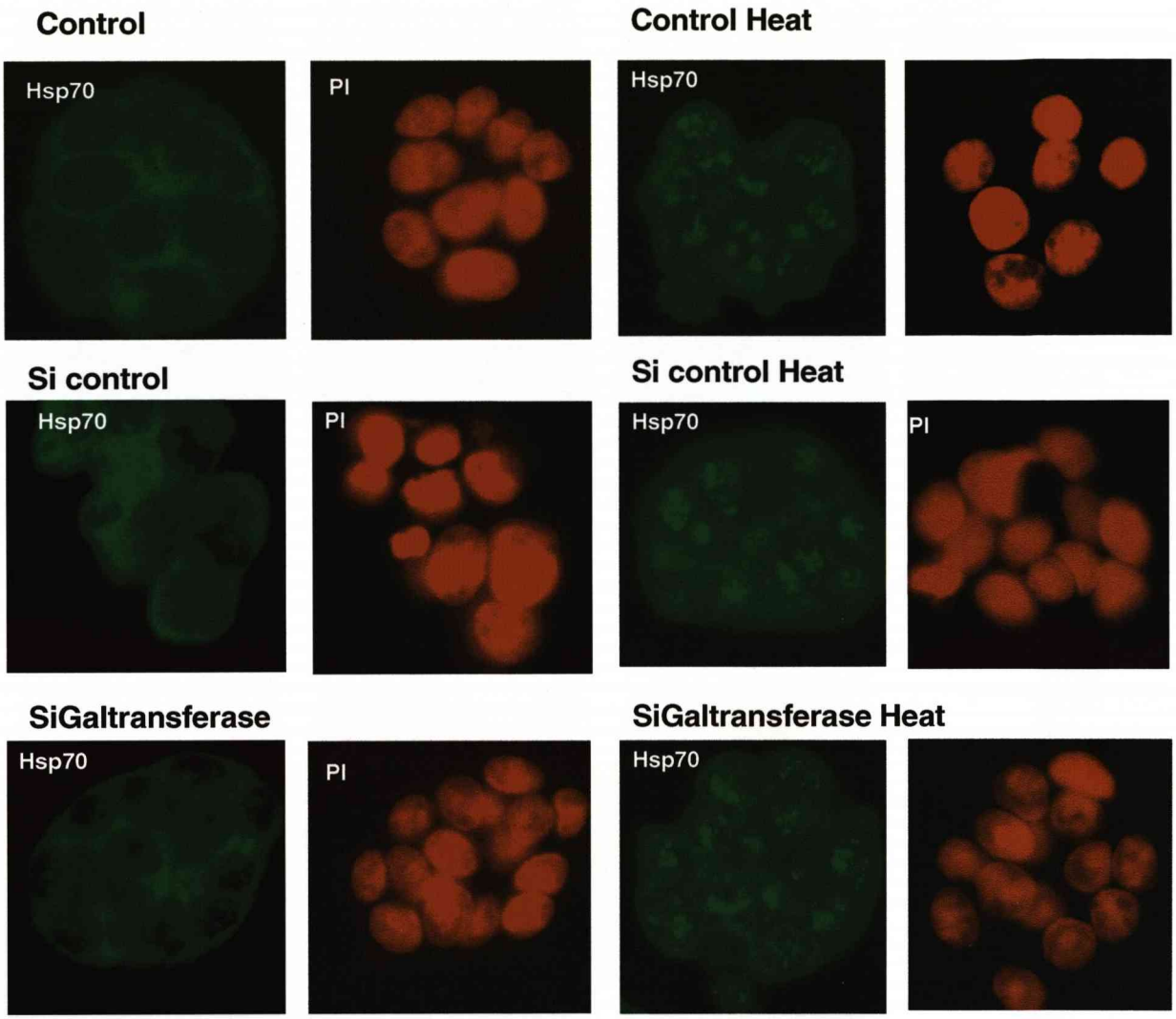


Figure 6.21: Hsp70 immunohistochemistry (x40 magnification). Hsp70 (green) and nuclear (red) localisation in HT-29-MTX cells after siRNA Galtransferase transfection and heat treatment. siRNA Galtransferase, although shown to cause a significant reduction in TF expression is shown not to have an effect on NLS-dependent nuclear protein import as assessed by nuclear import of Hsp70 in response to heat stress [Figure 6.21B].

Figure 6.21B. Nuclear fluorescence of Hsp70 with siRNA Galtransferase transfection

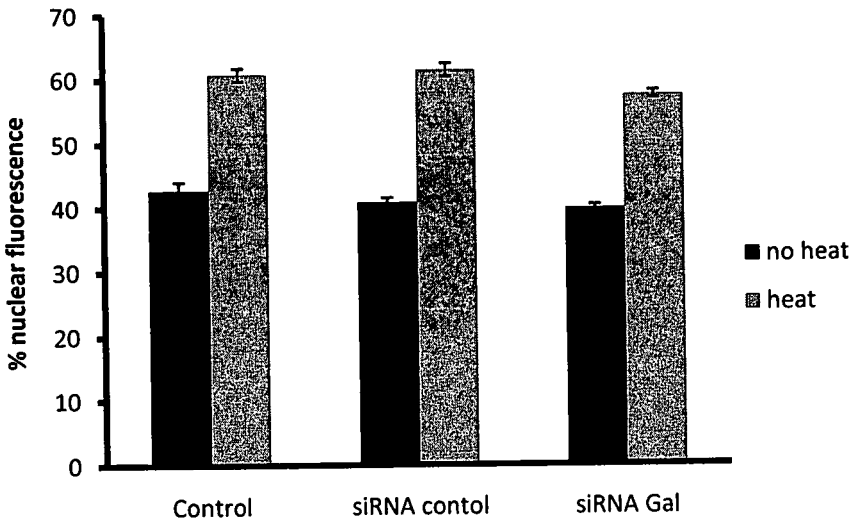


Figure 65.21B: HT-29 cells were grown on coverslips prior to Hsp70 immunohistochemistry. The percentage nuclear fluorescence was calculated. The siRNA Galtransferase transfected cells with nuclear fluorescence of 58 ± 1% after heat treatment demonstrated no difference to siRNA control cells after heat treatment with a nuclear fluorescence of 61 ± 1%

6.5 DISCUSSION

The oncofetal Thomsen-Friedenreich carbohydrate antigen is expressed in about 90% of all human carcinomas (Hanisch et al. 1997). There is a great interest in the TF antigen as a possible diagnostic marker in cancer detection and also in the understanding of the functional consequence of its expression by cellular glycoproteins. The demonstration in the previous chapter that a constitutively expressed cytoplasmic isoform of Orp150 expresses TF in its sialylated form and that this isoform of Orp150 is involved in NLS-dependent nuclear protein import, is intriguing and led to the investigation into the possible functional importance of TF in NLS-dependent nuclear protein import. Hsp70 translocation into the nucleus upon heat shock was utilised to determine the effect of the known *O*-glycosylation inhibitors and of siRNA Galtransferase on NLS-dependent nuclear protein import.

It was clearly demonstrated that bafilomycin, 5-CDP, TNFα and especially benzyl-GalNAc significantly increase cellular TF expression, determined by increased PNA binding. FACS analysis reveals that benzyl-GalNAc also increased the expression of cell surface TF, whereas the other inhibitors appeared not to have an effect. These results are consistent with the current understanding of the actions of these inhibitors from the literature and previous experiments in our laboratory. Densitometric analysis of MAL II binding also revealed that 5-CDP and benzyl-GalNAc caused a significant reduction of sialic acid content in the HT-29 MTX cells with benzyl-GalNAc also showing a cell surface reduction of sialic acid with FACS analysis. 5-CDP and benzyl-GalNAc have both been described previously to inhibit sialylation. It was initially expected that the FACS analysis would confirm the lectin blotting effects; however after 3 experiments it was apparent that there was a difference between the two techniques. FACS analysis upon fixed intact cells is

dependent upon the cell surface expression of glycans. The lack of effect upon glycosylation of the inhibitors by FACs analysis, with the exception of benzyl-GalNAc, compared to the lectin blotting technique could represent the difference between the total cellular glycosylation and the cell surface expression of glycans.

The translocation of Hsp70 into the nucleus after heat shock was unaffected by pre-incubation with the inhibitors. This indicates that either the sialyl-TF antigen is not involved in NLS-dependent nuclear protein import or that the inhibitors have no effect on glycosylation of the key proteins involved in NLS-dependent nuclear protein transport. In order investigate and specifically target the TF antigen, siRNA Galtransferase was utilised.

The novel use of siRNA to silence the gene responsible for the Core β1-3 Galtransferase is reported here. The investigation demonstrated a modest but statistically significant decrease in the TF antigen when analysed by PNA binding and this coincided with an increase in the Tn antigen. This reduction of TF however did not result in any alteration in the NLS-dependent nuclear import of Hsp70.

RNA silencing with siRNAs has increasingly been utilised for the specific suppression of a gene of interest, with a successful knockdown of protein expression seen at over 90% for the *O*-GlcNAc transferase (OGT) in 3T3 L1 adipocytes (Robinson et al. 2007). Compared to previous utilisation of siRNA Orp150, which gave sustainable reductions of >60%, the siRNA Galtransferase gave more varied results. Attempts to alter variables such as cell number and length of transfection aided the consistency of TF knock-down, but not the overall reduction in TF. In contrast to the other studied β-Galactosyltransferase families, which encompass many homologous genes, a complete investigation revealed the presence of only 1 Core β1-3 Galtransferase, responsible for the addition of Gal to GalNAc, which deems it unlikely

that any further human homologues will be identified (Ju et al. 2002). Therefore, it is intriguing that only a modest reduction of TF expression (a mean reduction of 21%) was observed with the siRNA Galtransferase transfection, as it would seem that a knock down of this key enzyme would cause a more significant reduction in TF with no other enzymes known to add galactose to GalNAc. The observed increase of Tn is an important positive control that confirms that reduced Gal addition to GalNAc has resulted in more unsubstituted GalNAc (Tn). One potential reason for a low knock-down of TF is the high abundance of Core β1-3 Galtransferase in the cell. It could also reflect the efficiency of Core β1-3 Galtransferase, and that only a small proportion of the enzyme is required for the addition of Gal to GalNAc. A number of attempts were made to immunoprecipitate the Orp150 protein and analyse the effects of the siRNA upon the Orp150 protein. Unfortunately the antibody was ineffective for this procedure.

Overall from these experiments it has been possible to demonstrate that TNFα, benzyl-GalNAc, 5-CDP, bafilomycin and siRNA Galtransferase all exert effects on the *O*-glycosylation processes of HT-29 cells with relation to TF expression. These global alterations in cellular glycosylation did not correlate with any alterations of NLS-dependent nuclear protein import. Further experiments could address more closely the role of the TF antigen on Orp150 if an antibody was available that would allow immunoprecipitation.

CHAPTER 7

EFFECTS OF BAFILOMYCIN AND ADHESIVE BACTERIA ON GOLGI ARRANGEMENT AND *O*-GLYCOSYLATION

7.1 HYPOTHESIS

Bacteria may interact with the colon epithelial cells to disrupt the Golgi apparatus and affect glycosylation.

AIMS

- To investigate the effect of a supernatant of a Crohn's disease mucosa-associated bacteria on the structure of the Golgi and TF expression in colon epithelial cells.
- Release of the pro-inflammatory cytokine, IL-8, to be measured as a positive control to monitor the response of epithelial cells to bacterial interactions

7.2 INTRODUCTION

7.2.1 Aberrant glycosylation in colonic disease

In colonic disease the increased expression of the TF antigen has been demonstrated following its specific cleavage by *O*-glycanase from mucins extracted from colon cancer and ulcerative colitis mucosal samples (Campbell et al. 1995), and the Tn antigen in its native and sialylated form (Brockhausen et al. 1998, Karlen et al. 1998) is seen in ulcerative colitis and in colonic adenomas (Itzkowitz et al. 1992). Another common glycosylation change seen in cancer is the more frequent occurrence of highly branched heavily sialylated glycoproteins (Warren et al. 1978). These alterations often affect Lewis antigens (Kim et al. 1986) known to be ligands which interact with various lectins and antibodies. In normal colonic mucosa type 1 and 2

chain extensions are formed, in colonic adenocarcinomas there is an increase in type 2 chains which are the precursor for the increased activities of α3-sialyltransferases seen in colorectal cancer, resulting in higher expression of sialyl-Lewis^x (SLe^x) in particular sialyl-dimeric lewis^x antigen which is correlated to poor prognosis. Colonic mucins in colitis can also be less sulphated (Brockhausen 2003) and colitis mucosal samples show lower sulphate incorporation compared to IBD controls when cultured *in vitro* (Raouf et al. 1992).

7.2.1.1 Mechanisms of altered glycosylation

In the normal colonic epithelium a broad range of *O*-glycans can be observed. One possible mechanism for the occurrence of truncated glycans in colonic disease is an alteration of the glycosyltransferase levels in the Golgi apparatus. A reduced level of Core 3 β3-*N*-acetylglucosamine (GlcNAc) is observed, allowing the GalNAc peptide to be acted upon by the Core 1 and 2 enzymes and leading to a higher prevalence of TF antigens. (Brockhausen et al. 2001). It has also been demonstrated that there is an elevated level of the transferase responsible for addition of sialic acid to this structure. Since ST6GalNAc-II is increased, leading to increased levels of sialyl-TF (Schneider et al. 2001). A high mucin type M-enzyme (C2Gnt) activity alongside leucocyte-type L-enzyme (C1GnT) converts the TF antigen into the Core 2 structure in the normal colonic tissue, however, in tumorigenic cells derived from human adenoma cells this C2GnT activity was diminished, but in some other human colon cancer cell lines such as HT-29 the activity was still high (Vavasseur et al. 1994, Vavasseur et al. 1995). Overall, however, it seems that C2GnT is up-regulated relative to C1GnT in most colon cancer tissues. This shift in relative activity would result in a decrease of Core 4 structures and an overall increase in Core 2 structures, which are the main bearers of SLe^x. However, a detailed study of the relative expressions of glycosyl-,

sialyl- and sulpho-transferases in colon cancer showed that although there are differences, these differences related poorly to the changes in the carbohydrate expression (Yang et al. 1994). With no straightforward correlation of enzymatic alterations and the occurrence of aberrant glycosylation this therefore, suggests that there are other mechanisms involved in disease-related changes in *O*-glycosylation.

7.2.1.2 Effect of pH on glycosylation and Golgi structure

The Golgi apparatus is the centre of the secretory pathway and is also responsible for the tight regulation of glycosylation [Figure 7.1]. Proteins on entering the *cis* face will pass through the Golgi encountering glycosyltransferases in a specific order in transit to the *trans* face. How the glycosyltransferases are localised is not fully understood, and the localisation of only a few specific glycosyltransferases is known, for example, the enzymes that synthesis core 1 and core 2 structures are seen mainly in the *cis*-Golgi (Roth et al. 1994, Rottger et al. 1998). Significantly the glycosyltransferases involved have been shown not to be segregated in separate compartments, but rather to form overlapping gradients (Rabouille et al. 1995), and there must be mechanisms in place to enable this distribution. Certain regions of glycosyltransferases have been shown to be critical for their localisation in the Golgi (Colley 1997). It has recently been demonstrated that Golgi-resident glycosyltransferases, which are type II integral membrane proteins have a short cytoplasmically exposed amino-acid tail, which plays a role in localisation and that coat protein complex I (COPI) vesicle mediated retrograde transport is also involved in their localisation (Tu et al. 2008). However, these cytoplasmic tails lack the known COPI binding motifs and it has been shown by Tu and colleagues that another protein involved with protein trafficking, Vps74p, promotes glycosyltransferase incorporation into COPI binding vesicles, which therefore maintains a steady-state localisation (Tu et

al. 2008). The identification of these targeting domains has uncovered further complexity and it is probable that there exist multiple mechanisms to ensure the correct localisation of glycosyltransferase. The understanding of the retention or positioning of glycosyltransferases against the flow of proteins moving through the Golgi requires an understanding of the movement of cargo across a Golgi stack. The mechanisms of cargo transport through the Golgi are still being investigated, but it is clear that the retention of glycosyltransferases is intimately linked to the organisation of Golgi membranes and control of anterograde and retrograde protein transport (Opat et al. 2001). It has been demonstrated that an alteration in Golgi pH can disrupt the traffic of proteins through the Golgi (Gustafson et al. 2000, Henkel et al. 1998, Wagner et al. 1986), and cause aberrant glycosylation of proteins resulting in changes that included an increase in expression of TF and Tn (Kellokumpu et al. 2002).

The Golgi contents are normally acidic and maintenance of this reduced pH is via vacuolar H^+-ATPases in the Golgi membrane (Glickman et al. 1983). The localisation of the specific glycosyltransferases, essential in the process of mucin type *O*-glycosylation, has been shown to be dependent on Golgi pH (Axelsson et al. 2001). In another investigation into the effect of pH on glycosylation, the pH was measured and found to be significantly more alkaline in HT-29 and SW-48 colon cancer cell lines compared to control, and only a 0.2pH increase in pH was needed to induce an increase in TF antigen expression in cells (Rivinoja et al. 2006). In addition, drug-induced alkalinisation of the Golgi by bafilomycin A1 or monensin in the goblet cell differentiated colonic cell line LS174T caused increased TF expression, as seen in colon cancer (Campbell et al. 2001). Such alterations in pH, by pH gradient dissipating drugs, disrupt the Golgi structure, as visualised by immuno-staining with Giantin (Kellokumpu et al. 2002)[Figure 7.2]. Giantin is a structural protein of about 350-400

KDa and its localisation, conservation, and physical properties suggest that it may participate in forming the intercisternal cross-bridges of the Golgi complex. The physical disruption of the Golgi is thought to prevent proteins that are undergoing glycosylation in meeting specific glycosyltransferases in the appropriate sequence to allow normal *O*-glycan structures to be formed.

Figure 7.1: The Golgi apparatus

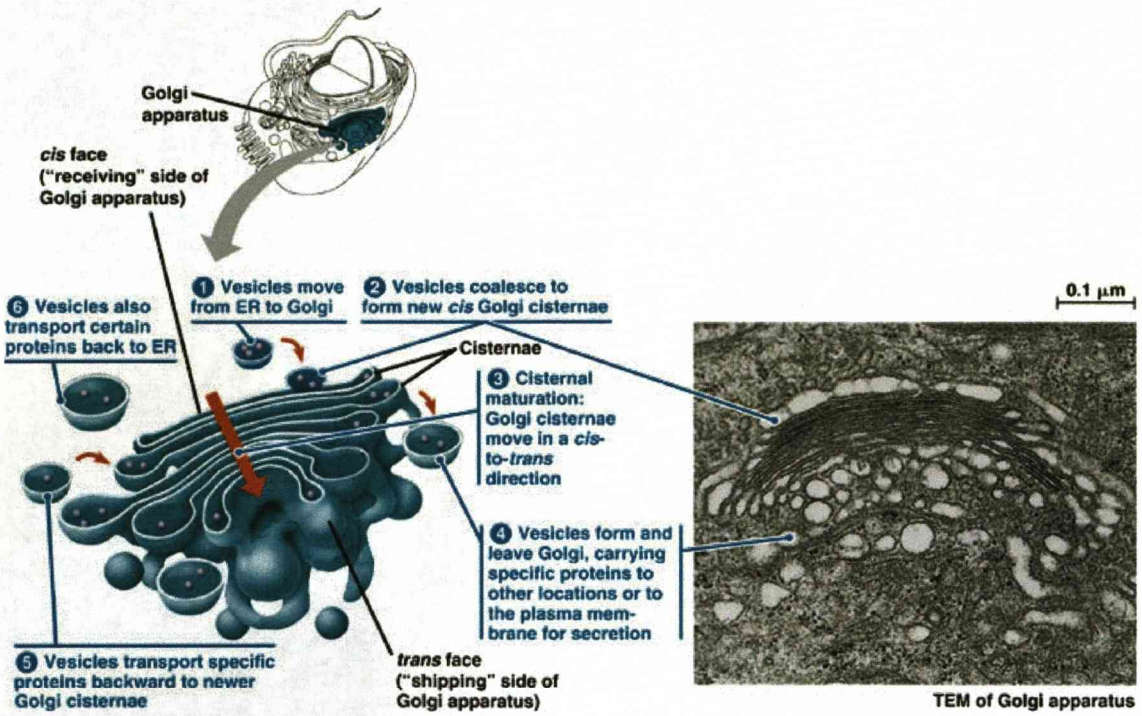


Figure 7.1: Transit through the Golgi apparatus (adapted from http://fig.cox.miami.edu)

How pH dissipation results in the structural disorganisation of the Golgi apparatus is not clear, but does suggest the possibility that by disrupting the flow through the Golgi disrupts not only the recycling and positioning of the

glycosyltransferases thereby affecting normal glycosylation, but also effects the structural organisation of the Golgi.

Figure 7.2: Disorganization of the Golgi apparatus in cultured NRK cells treated with pH gradient dissipating drugs

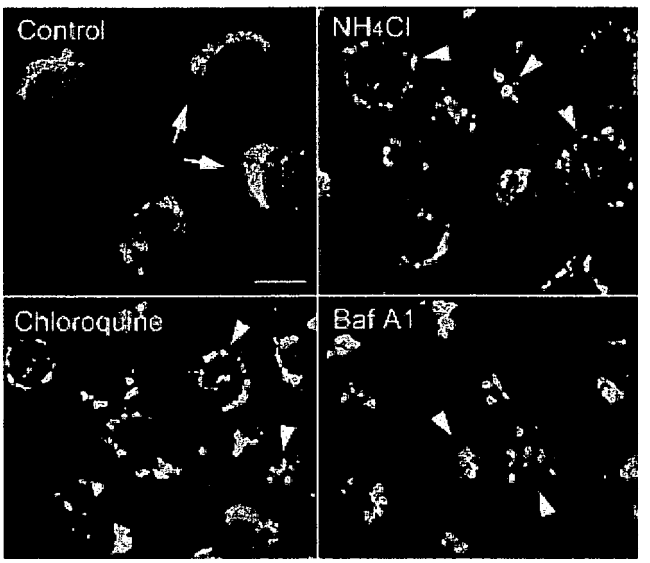


Figure 7.2: Anti-Giantin antibody staining. NRK cells were grown on coverslips and treated with the above dugs. The Golgi apparatus is fragmented and scattered near the nuclei in the drug-treated cells, but not in untreated cells (Kellokumpu et al. 2002).

7.2.2 Bacteria-epithelial interactions as a possible trigger for altered glycosylation

Previous studies from our group (Bodger et al. 2006) have shown that unaffected identical twins of IBD patients had increased TF expression on intracellular proteins in the colonic epithelium cells, despite the absence of histological inflammation. The fact that these changes were not seen in cells deeper in the crypts suggested that they are acquired rather than genetically determined. The glycosylation changes were found to correlate with immunohistological demonstration of NFκB

activation in the surface epithelium. In the absence of histological inflammation it seems a probable hypothesis that this NFκB activation may have resulted from bacteria-epithelial interaction, perhaps as a result of a genetically determined defect in the mucosal barrier. This has led to the experiments detailed here in which I have investigated directly the effect of bacteria-epithelial interactions on *O*-glycosylation in colon epithelial cells.

7.2.2.1 Colonic epithelium

The human intestine is colonised with between 500-1000 different bacterial species (Egert et al. 2006). The colonic epithelium is an effective physical barrier, protected by mucins and trefoil peptides secreted by goblet cells. The interactions of bacteria with the epithelium are complex and involve interactions between the bacterial surface determinants and the host epithelium receptors. Pathogens share similar mechanisms of interactions with the host but individual bacteria possess unique abilities to exploit the host processes. The epithelial glycocalyx is a layer of glycoconjugates expressed at the surface epithelium and these oligosaccharides can serve as receptors for microorganisms. The functional importance of altered intestinal epithelial glycosylation is still being uncovered but it could lead to the recruitment of bacteria to the epithelium.

The innate immune system is crucial in the defence against microbes of the gastrointestinal tract. It acts through a number of receptors present on the intestinal epithelial cells and macrophages which recognise microorganisms through unique prokaryotic molecular motifs and pattern recognition receptors (PRR), receptors include Toll like receptors (TLR) and the intra cellular Nod proteins. Eleven different TLR's have been identified in humans (Harris et al. 2006). These are able to recognise a range of bacterial and viral antigens such as lipopolysaccharide (TLR4), flagellin

(TLR5) and fatty acids (TLR2). Activation of the TLRs triggers a range of pro-inflammatory and anti-inflammatory responses. The location of the receptors appears to be important and it is known that the surface expression of TLRs, such as TLR2 and TLR4 in the gut epithelium is down-regulated (Akira et al. 2006). Where TLR's are expressed they can be relocated to intracellular compartments such as the Golgi apparatus. The TLR4 receptor is internalised and able to fully recognise internalised LPS (Hornef et al. 2003), the TLR5 receptor which recognises flagellin, is located on the basolateral aspect of intestinal epithelial cells (Gewirtz et al. 2006) allowing the recognition of any flagellin that crosses the epithelium. These mechanisms allow the host to recognise invading pathogens and also allow an interaction with the commensal bacteria.

7.2.2.2 Bacteria and inflammation

An aberrant mucosal response to intestinal bacteria is thought to be the trigger for the inflammatory bowel diseases (IBD) that consist chiefly of Crohn's disease and ulcerative colitis. Evidence from animal models of IBD suggests the requirement of commensal bacterial in order for inflammation to arise (Hoffmann et al. 2002). In IBD there is evidence for quantitative alterations in the mucosa-associated flora and the mucus-associated flora with evidence for a novel class of *E.coli* that lacks conventional pathogenicity genes (Darfeuille-Michaud et al. 1998). Swidsinski and colleagues showed that the colonic mucosa of patients with colorectal carcinoma, but not normal colonic mucosa has an increase in mucosa-associated *E.coli* (Swidsinski et al. 1998). Work from our laboratory has shown that both the mucosa associated *E.coli* from CD and colon cancer samples can induce the release of pro-inflammatory cytokines and that they do not have to adhere to the epithelial membrane to cause an inflammatory response (Martin et al. 2004). However, many of these *E.coli* express

haemagglutinins, which allow them to adhere to intestinal epithelial cell lines and subsequently cause IL-8 release, mediated by extracellular-regulated kinase (ERK) and p38 mitogen-activated protein kinase (MAPK) and inhibited by mesalazine (Subramanian et al. 2008). Numerous studies have now identified the presence of adherent and invasive *E.coli* in Crohn's disease and ulcerative colitis (Kotlowski et al. 2007) with similar *E.coli* observed in boxer dogs with granulomatous colitis (Simpson et al. 2006). The distribution of these bacteria in both inflamed and uninflamed mucosa suggests they are recruited even without initial inflammation and may be crucial in the pathogenesis of IBD. This study investigates the link between bacteria and aberrant glycosylation.

7.3 METHODS

7.3.1 Materials

Recombinant human IL-8 and TNFα were purchased from Peprotech EC (London; UK) and Sigma (Poole; UK) respectively. The mouse anti-human IL-8 monoclonal antibody (790A 28G2) and the mouse anti-human IL-8 monoclonal detection antibody (893C 4G2) were both purchased from Biosource (Invitrogen, Paisly, UK). Lipopolysaccharide (LPS) from *E.coli* 0127:B8 and Polymixin B were purchased from Sigma (Poole; UK). Human leucocyte elastase was purchased from Elastin Products (Owensville, Missouri, USA). The anti-Giantin rabbit monoclonal antibody was purchased from Bioquote (York; UK).

7.3.2 Bacterial culture and bacterial supernatant

The *E.coli* HM427 used in these experiments was previously isolated from colonic biopsies from a Crohn's disease patient (Martin et al. 2004). The common laboratory strain, *E.coli* K12, was used as a control bacteria due to its lack of virulence factors. Both were grown individually overnight on nutrient agar from stores kept at -20°C. The *E.coli* were harvested, washed three times with PBS and collected by gentle centrifugation at 70*g* for 1 min (1000rpm; MSE microcentaur, Sanyo Gallenkamp PLC, UK). The *E.coli* were then suspended in sterile water at an O.D of 0.125 at 550 nm equivalent to approximately $7x10^6$ *E. coli*/ml, and left for 24 h. A bacteria-free filtrate was achieved using a 0.2 μm pore diameter sterile filter and the resulting filtrate confirmed bacteria free by culture on Columbia agar. Fifty μl of the supernatant was added to 1ml of the DMEM to treat the HT-29 cells. An aliquot of the bacterial supernatant was treated with human sputum leukocyte elastase (0.25 μM in 0.05

sodium acetate, pH 5 containing NaCl, for 3 h at 37°C) to remove flagellin. LPS was removed by the addition of Polymixin B 10 μg/ml (3 h at 37 °C).

7.3.3 Outer membrane vesicle (OMV) purification from *E.coli*

OMVs were isolated by filtering the bacteria supernatants though a 0.45 μm filter and OMVs were collected by ultracentrifugation at 500,000 *g* for 1 h at 4°C (100,000 rpm; Beckman TL 100 Ultracentrifuge, Minnesota, USA). OMV pellets were resuspended in 10 mM Tris-HCL (pH 8.0)-150 mM NaCl, as described previously by Wai (Wai et al. 1995).

7.3.4 Isolation of flagella from HM427

Flagella from the mucosal Crohn's disease HM427 *E. coli* isolate was purified by the ammonium sulphate precipitation method. Bacteria grown overnight in Luria-Bertani broth with constant agitation at 37°C overnight were harvested in 100 ml of 10mM phosphate-buffered saline pH 7.4. Flagella were sheared by homogenization for 2 min followed by centrifugation at 8,000 *g* for 30 min. The supernatant containing sheared flagella was collected and the flagella separated from outer membrane proteins and other contaminants by precipitation with 60% ammonium sulphate for 18 h at 4°C. After centrifugation at 12,000 *g* for 30 min, the flagella-containing pellet was resuspended in PBS, and insoluble contaminants were removed by a similar centrifugation step. The supernatant was subjected to a second cycle of 20% ammonium sulphate precipitation. After dialysis against frequent changes of distilled water to remove excess salts, the purity of the preparations was monitored by SDS-PAGE and electron microscopy, as described in the methods chapter.

7.3.5 Treatment of HT-29 MTX cells with bacterial supernatant, LPS and flagella

HT-29 MTX cells are a homogeneous, mainly MUC5AC (normal gastric mucin) -secreting population of well-differentiated goblet cells that has been obtained by stepwise adaptation of HT-29 cells to 10^{-6} or 10^{-5} M methotrexate (MTX) (Lesuffleur et al. 1990). HT-29 MTX cells were seeded on to 24-well plates at a density of $1x10^5$ cells/well and cultured for 48 h at 37°C in a humidified atmosphere of 5% CO2, 95% air. Confluent HT-29 MTX cells were then infected, in triplicate, with 50 µl HM427 or K12 or prepared bacterial supernatant as described earlier for 10 days. Recombinant human TNF-α (2 ng/ml) was used as a positive control for IL-8 release compared to saline vehicle alone. In experiments examining the action of purified lipopolysaccharide (*E.coli* O127:B8, 1 µg/ml) was incubated with HT-29 MTX cells for 10 days. Polymixin B (10 µg/ml) was used to block LPS responses. To examine the effect of flagella, (1 µg/ml) was incubated with HT-29 MTX cells for 10 days, and 0.25 mM elastase used to block flagellin response.

The medium was collected to quantify IL-8 release by ELISA. The cells were prepared for immunohistochemistry with Giantin antibody to assess Golgi fragmentation. The cells were washed 3 times with PBS and fixed for 10 min in freshly prepared paraformaldehyde followed by 3 times with PBS washes. The fixed cells were then blocked with 300 µl of 5% goat serum (Dako; Glostrup, Denmark) for 1 h at room temperature, followed by the addition of anti Giantin antibody (Bioquote, York) 1:200 concentration for 2 h. The cells were then washed 2 times with PBS and then the FITC conjugated secondary antibody (Dako; Glostrup, Denmark) was added, concentration 1:500, for 1 h in the dark. The cells were then washed 3 times with PBS and the cover slips mounted on glass slides with a propidium iodide nuclear counter-stain mounting solution.

7.3.6 Enzyme-Linked ImmunoSorbent Assay (ELISA) for interleukin 8 (IL-8)

A ninety-six well microtitre plate was coated with 100 µl carbonate-bicarbonate buffer (pH 9.6) containing 1µg/ml mouse anti-human IL-8 monoclonal antibody (790A 28G2; Biosource, Belgium). Plates were incubated overnight at 4°C. Following three washes with PBS (pH 7.4) containing 0.1% v/v Tween 20, antibody-coated plates were blocked in buffer containing 1% w/v bovine serum albumin, for 2 h at room temperature. One hundred microliter aliquots of cell-free samples were assayed. Recombinant human IL-8 standards of known IL-8 concentration, in cell-lysis buffer and cell-free culture medium, were assayed in triplicate [Figure 7.3]. Fifty microliters of 0.4 µg/ml biotinylated mouse anti-human IL-8 monoclonal detection antibody (893C 4G2, Biosource, Belgium) was added to each well and incubated for 2 h at room temperature. After washing, 100 µl Extravidin-horseradish peroxidase conjugate (1:5000) (Sigma, Dorset, UK) was added to each well and the reaction was developed with O-phenylenediamine substrate reagent (Sigma, Dorset, UK) and was stopped with 4M H_2SO_4. Optical density was measured at 495 nm. The sample reading was read against the standard curve using FigSys software (Biosoft, Cambridge, UK) and data expressed as ng/ml IL-8 released. The inter assay coefficient of variation for the standards was 12% and the intra-assay coefficient of variation was 16%.

Figure 7.3: Representative IL-8 standard curve

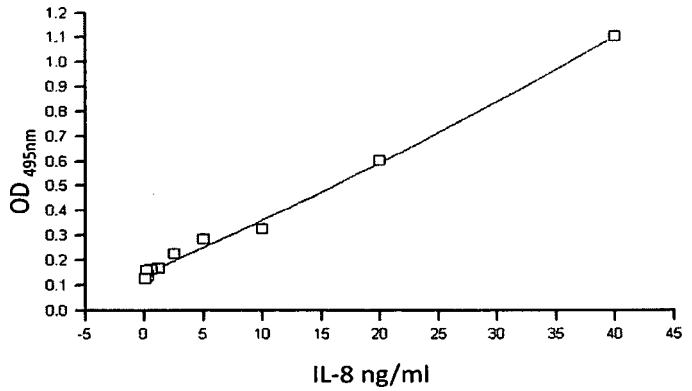


Figure 7.3: Recombinant human IL-8 standards of known IL-8 concentration, in cell-lysis buffer and cell-free culture medium, were assayed in triplicate, in order to create a standard curve to quantify HT-29 cell IL-8 release.

7.3.7 Quantification of Golgi fragmentation

HT-29 MTX cells were seeded at $1x10^5$ on coverslips and cultured for 15 days, followed by 10 days incubation with HM427 bacterial supernatant. Immunohistochemical staining for Giantin was performed on monolayers of cells grown on the coverslips and visualised using confocal microscopy. The experiment was performed in triplicate and for each replication 3 randomly selected fields of view were selected and a three slice Z stack of images captured by a confocal laser-scanning microscope equipped with 488-nm lasers (the acquired images were processed by LSM510 software version 3.2 SP2). The Z stack first image was taken from 1μm inside from the surface of the monolayer, and each subsequent image was taken 1μm apart. A ratio of the number of Golgi fragments per nucleus through the Z-stack was calculated by dividing each image through the Z-stack into quadrants, designated A,B,C,D [Figure 7.4]. Each quadrant was then analysed for the number of intact,

individual green stained Golgi fragments. The number of nuclei was also counted, discarding any which touched the outermost borders. The number of Golgi fragments and nuclei was averaged for each respective quadrant through the Z stack. This was done to allow a representation of the Golgi fragments on different levels through each cell. The quadrant averages were then collated to give an overall depiction of the number of Golgi fragments per nucleus across a 2 μm 'slice' of the HT-29 MTX monolayer.

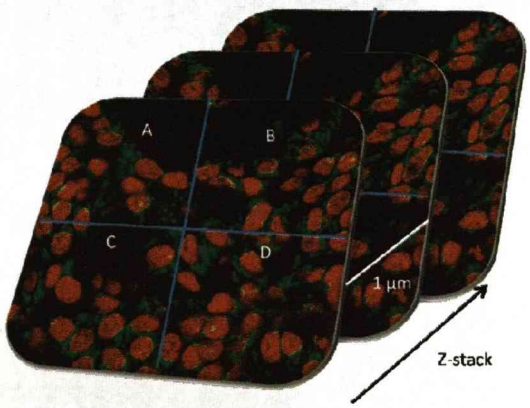


Figure 7.4: Z-stack

7.3.8 Analysis of Adenylate Kinase (AK) release into the medium

The Toxilight bioassay (Lonza, Basel; Switzerland) is a non-destructive bioluminescent cytotoxicity assay designed to measure toxicity in mammalian cell lines in culture.

To analyse the AK release in to the medium, first all the reagents were warmed to room temperature. The AK detection buffer was then reconstituted in assay buffer (Lonsa, Basel; Switzerland), 20 μl of the cell medium under analysis was then plated out in triplicate in a 96 well luminescence compatible plate to which 100 μl of the AK detection buffer was added. The plate was left for 5 min before reading in the luminometer. Total lysis of the remaining cells was achieved with a provided lysis buffer (Lonsa, Basel; Switzerland) to give a total AK amount in the well.

7.4 RESULTS

7.4.1 HM427 Bacterial supernatant causes an increase of TF expression in HT-29 MTX cells

This experiment investigates the effect of the bacterial supernatant upon glycosylation in HT-29 MTX cells, by specifically investigating the expression of TF after 1, 5 and 10 days incubation. HT-29 MTX cells were seeded at 1 $x10^5$ cells per/ml in 24 well plates and cultured for 15 days until post confluent as in previous experiments. Bacterial supernatant HM427 or K12, an OMV preparation or purified flagellin from HM427 was then added to the culture medium as described in the methods. The medium and bacterial supernatant was replaced every 24 h and the cells removed after 1, 5, or 10 days. Cells were washed 3 times with PBS before direct lysis with sample buffer, protein estimations were performed on identical wells to ensure equal loading on to the slot blot [Figure 7.5]. TF expression was quantified by densitometric analysis of the bands after PNA lectin blotting using Quantity One software (Bio-Rad; Hemel Hempstead, UK) [Figure 7.6]. The experiment was completed 4 times and on each occasion in duplicate. After 24h the mean PNA binding density of control HT-29 MTX cells was 1300 ± 200 ADU (arbitrary densometric units), this was not altered after any of the treatments. After 5 days the mean PNA binding of control cells increased to 1600 ± 300 ADU, incubation with the OMV preparation significantly increased the PNA binding density to 1800 ± 200 ADU ($p<0.005$)[ANOVA], none of the other treatments increased TF expression. After 10 days the control cell PNA binding density was 1400 ± 300 ADU, this was increased by incubation with the HM427 bacterial supernatant to 2300 ± 200 ADU ($p<0.005$), by the OMV preparation to 2000 ± 100 ADU ($p<0.005$) and by flagellin to 2000 ± 200

ADU ($p<0.005$), indicating that these three treatments after 10 days have increased the amount of TF expression of the HT-29 MTX cells.

Figure 7.5: Representative Slot blots of PNA binding after bacterial supernatant incubation

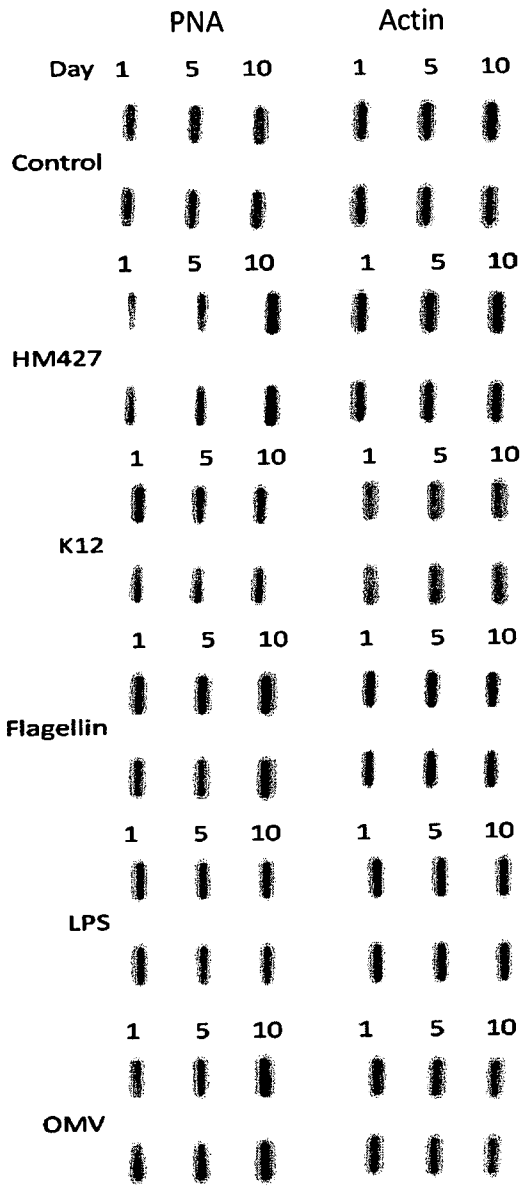


Figure 7.5: HT-29 MTX cells were seeded at 1 x10^5 cells per/ml in 24 well plates and cultured for 15 days until post confluent. Bacterial supernatant HM427 or K12, an OMV preparation or purified flagellin from HM427 was then added to the culture medium, the medium was replaced every 24 h complete with treatments, and the cells removed after 1, 5, or 10 days and subjected to slot blotting.

Figure 7.6: Mean density of PNA binding after incubation with bacterial supernatant HM427 or K12, OMV preparation and flagellin

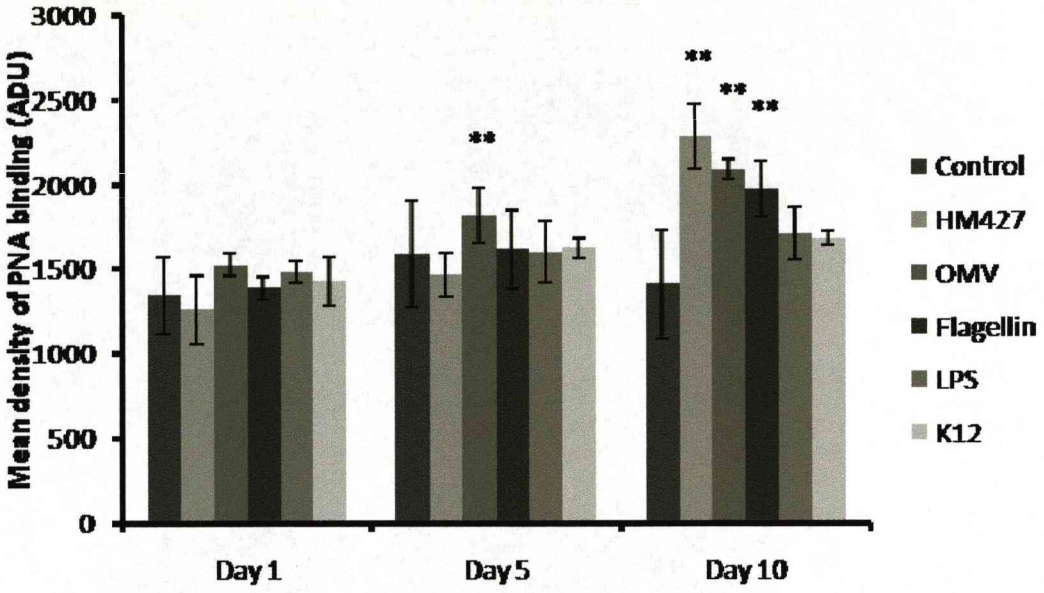
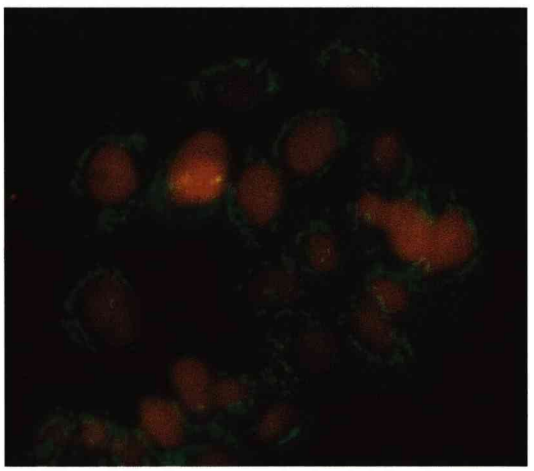


Figure 7.6: HT-29 MTX cells were seeded at 1 x10^5 cells per/ml in 24 well plates and cultured for 15 days until post confluent. Bacterial supernatant HM427 or K12, an OMV preparation or purified flagellin from HM427 was then added to the culture medium. After 10 days incubation HM427 bacterial supernatant, OMV and flagellin caused a significant increase in TF expression ($p<0.005$) compared to control. (Arbitrary densitometric units, ADU).

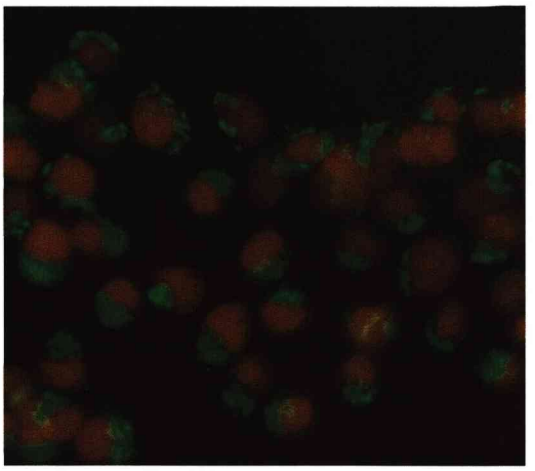
7.4.2 The cellular distribution of the Golgi apparatus is altered by Bafilomycin

The Golgi apparatus under normal cellular conditions is crescent shaped and perinuclear when viewed after immunohistochemical staining for the *cis*-Golgi structural protein, Giantin. Bafilomycin is an ATPase pump inhibitor causing alkalisation of the Golgi apparatus and also alterations in glycosylation. This investigation observes the Golgi structure after HT-29 MTX cells have been incubated with bafilomycin. HT-29 MTX cells were grown to 60% confluence on cover slips and then bafilomycin was added to the culture medium for 2 days at 100mM concentration. The cells were probed with anti-Giantin antibody, which in the control cells is displays a perinuclear crescent like shape. After the incubation with bafilomycin the Golgi appears more rounded and condensed [Figure 7.7]. The experiment was repeated on 2 separate occasions.

Figure 7.7: Representative immunohistochemistry. Golgi disorganisation shown by Giantin staining, after 2 day treatment with 100nm bafilomycin.



2 day Control



2 day treated with 100nM Bafilomycin

Figure 7.7. Anti-Giantin immunohistochemistry. HT-29 MTX cells were grown to 60% confluence prior to 48 h incubation with 100 nM bafilomycin. Immuno-stained with anti Giantin antibody, cells treated with bafilomycin have altered Golgi structure compared to control.

7.4.3 HT-29 MTX cells show increased MUC5AC expression from after 10 days of seeding

HT-29 MTX is a well differentiated cell line that forms a polarised goblet cell monolayer when post confluent; this makes it suitable for experiments involving the interaction with bacteria. It has been previously shown that the increased expression of MUC5AC accompanies the differentiation into goblet cells. HT-29 MTX cells were seeded at $1x10^5$ cells/ml and each day the MUC5AC secreted in the medium was calculated by immunoslotblotting and densitometric analysis of the bands [Figure 7.8 and 7.9]. The expression of MUC5AC secreted was seen initially to be extremely low with a mean binding less than <0.001, until day 10 where expression increased sharply to a mean density of 0.15 ($p<0.005$) [ANOVA] (n=3). From this experiment HT-29 MTX cells were then grown for 14 days prior to incubation with bacteria in all subsequent work.

Figure 7.8: Representative 0-15 day time course of MUC5AC expression

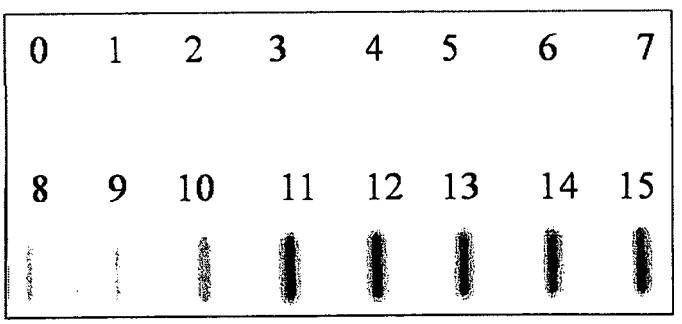


Figure 7.8: Anti-MUC5AC immunoblot. HT-29 MTX cells cultured for 8 days show expression of MUC5AC secreted into the medium, the expression increases until 11 days before plateau. The secretion of MUC5AC is a marker of differentiation into goblet cells.

Figure 7.9: Time course of MUC5AC expression

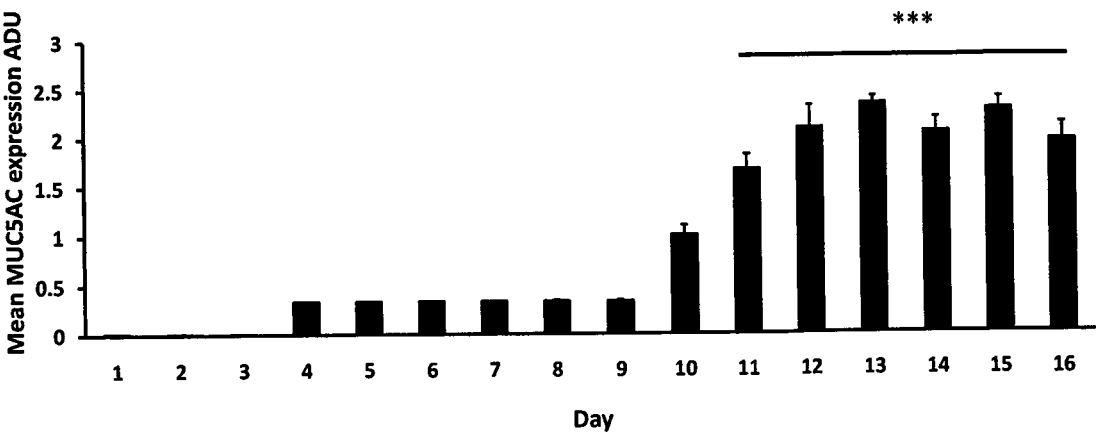


Figure 7.9: MUC5AC excretion in to medium is increased significantly from day 10 onwards. An increase occurs till day 12 when a plateau is observed. It has been previously shown that MUC5AC expression can be correlated with HT-29 MTX cell differentiation (Gouyer et al. 2001). The MUC5AC expression was measured in the medium and the amount was correlated to protein concentration of the lysed cells. MUC5AC protein was measured at a mean density of 0.09 ADU at day 9 (n=3) a significant increase at day 10 of a mean density of 0.15 ADU (n=3) ($p<0.005$), this significant increase was observed from day 10 onwards, indicating that the HT-29 MTX cells were differentiated.

7.4.4 Cellular distribution of the Golgi in sub confluent HT-29MTX cells is altered by 5 days incubation with mucosa-associated E.coli HM427 bacterial supernatant

Initial experiments focused on 24 h incubation with the bacterial supernatant on a confluent monolayer of HT-29 MTX cells. This experiment showed no visible alteration in the Golgi structure from the control perinuclear crescent shape. HT-29 MTX cells seeded at $1x10^5$cells/ml and cultured for 48 h were then incubated with HM427 bacterial supernatant for 5 days, before immunohistochemical staining with Giantin [Figure 7.10]; this led to the Golgi having a more fragmented appearance with no visual effects on cell health.

Figure 7.10: HT-29 MTX after 5 days incubation with bacterial supernatant

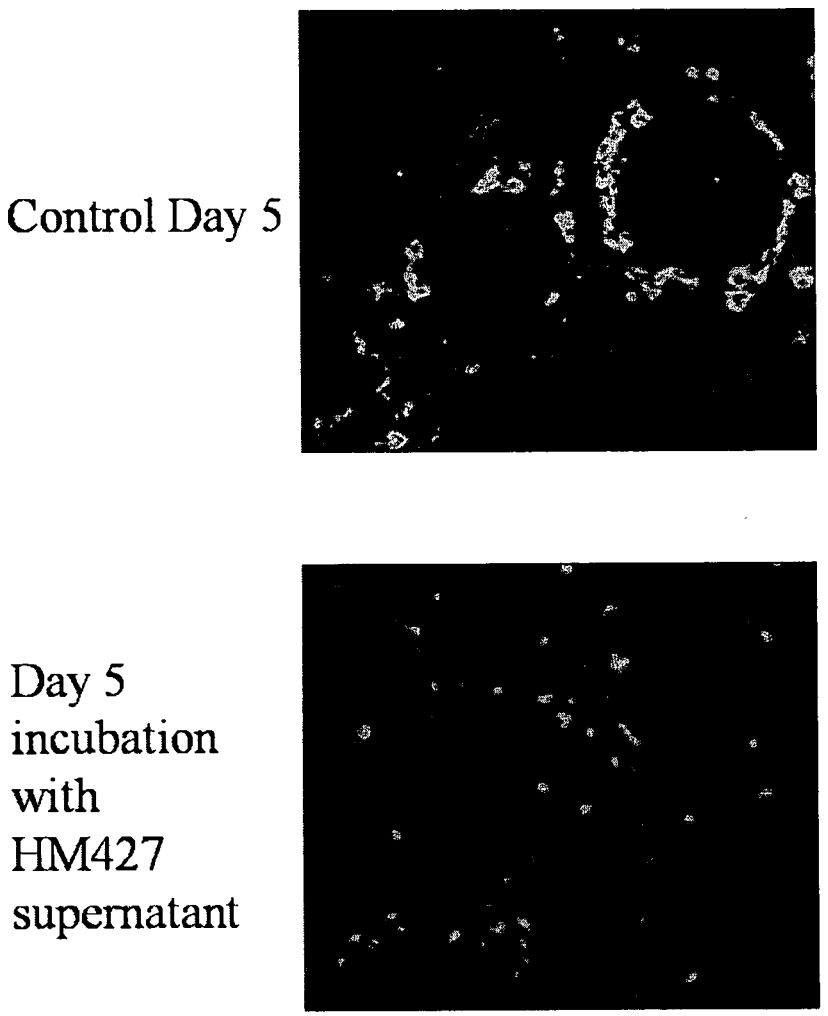


Figure 7.10: Anti-Giantin immunohistochemistry. HT-29 MTX cells were seeded at $1x10^5$ and cultured for 5 days on coverslips with HM427 bacterial supernatant. The green immunofluoresent stain shows Giantin. The control cells have a perinuclear crescent shape, compared to after the HM427 supernatant incubation where no perinuclear crescent shaped nuclei could be observed leading to a fragmented appearance of the Golgi.

7.4.5 Cellular distribution of the Golgi in post confluent, differentiated HT-29MTX cells is altered by 10 days incubation with mucosa-associated E.coli HM427 bacterial supernatant

The HT-29 MTX cells become differentiated from day 10 onwards with the secretion of MUC5AC into the cytoplasm. It was not possible to visualise successfully the Golgi apparatus of the cells in a confluent monolayer using conventional immuno-microscopy and so confocal microscopy was utilised. The Golgi apparatus has been shown to fragment in response to incubation with the HM427 bacterial supernatant in the previous experiments. In order to quantify this observed effect, the HT-29 MTX cells were again seeded at $1x10^5$ on coverslips and cultured for 15 days, followed by 10 days incubation with HM427 bacterial supernatant. Immunohistochemical staining with Giantin was performed on monolayers of cells grown on the coverslips and visualised using confocal microscopy. The effect of *E.coli* K12 supernatant was also investigated and the effects of the flagellin from HM427 and lipopolysaccharide (LPS) were also examined. Bafilomycin was used as a further control, as it has previously been shown to cause fragmentation of the Golgi (Kellokumpu et al. 2002) [Figure 7.7].

The experiment was performed in triplicate with each replication having 3 randomly selected fields of view were selected and a 3 slice Z stack of images captured [Figures 7.12-7.16], each beginning 1μm inside from the surface of the monolayer and with 1 μm space between each image. Each image was then subjected to an analysis of the number of Golgi fragments, as a ratio to the number of nuclei, which were counterstained with propidium iodide. The data from each Z stack were then collated and an average number of fragments per nucleus was then calculated.

The control HT-29 MTX monolayers have an average (±SD) 2.7±0.6 Golgi fragments per nucleus after 10 days. A 10 day incubation of the confluent monolayers

with HM42 causes an increase in the average Golgi fragments per nuclei to 4.5 ± 0.6, ($p<0.01$) [ANOVA], [Figure 7.17]. *E.coli* K12 bacterial supernatant caused a significant increase to 4.6 ± 0.8 ($p<0.01$) Golgi fragments per nucleus. The OMV preparation caused a significant increase in the average Golgi fragments per nucleus to 5.3 ± 1.3 ($p<0.01$), with the OMV free preparation having no significant effect. LPS and Flagellin caused no significant effect, bafilomycin after 2 day incubation caused a significant increase to 5.0 ± 1.0 ($p<0.01$) fragments of Golgi per nucleus.

Figure 7.12: Representative Giantin immunohistochemical images of HT-29 MTX cells after HM427 supernatant incubation.

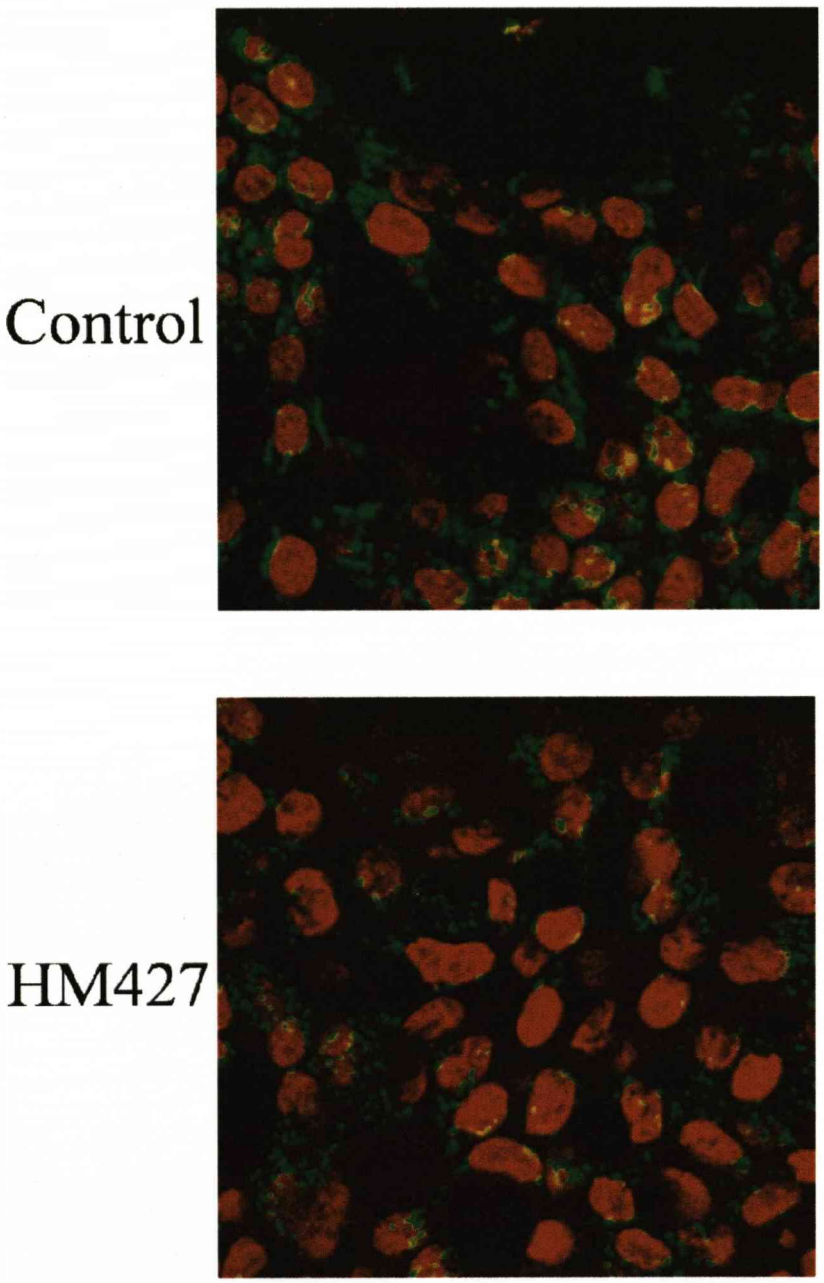


Figure 7.12: Anti Giantin immunohistochemical staining (green). HT-29 MTX cells were seeded at $1x10^5$ on coverslips and cultured for 15 days. The post confluent monolayers of cells were then incubated with HM427 bacterial supernatant which caused a fragmentation of the Golgi. Increasing the number of fragments per nucleus from an average of (±SD) 2.7 ± 0.6 to 4.5 ± 0.60.

Figure 7.13: Representative Giantin immunohistochemical images of HT-29 MTX cells after K12 supernatant incubation.

K12

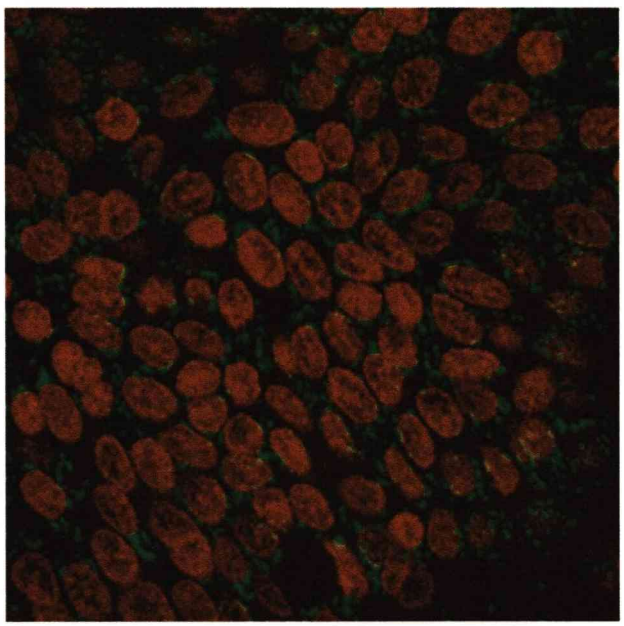
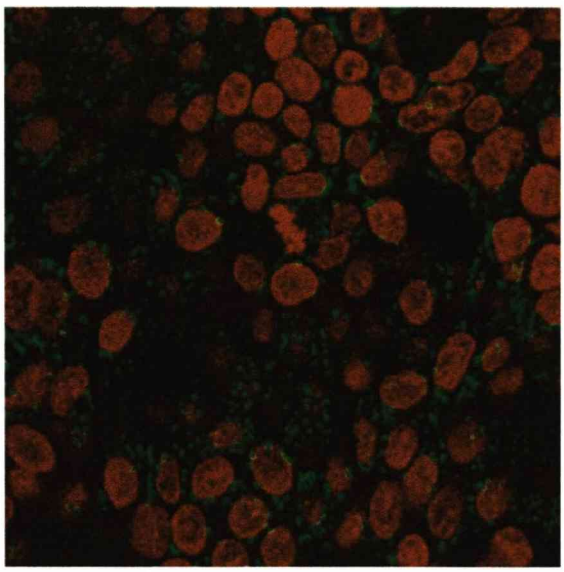


Figure 7.13: Anti Giantin immunohistochemical staining (green). HT-29 MTX cells were seeded at $1x10^5$ on coverslips and cultured for 15 days. The post confluent monolayers of cells were then incubated with K12 bacterial supernatant. K12 has a similar effect to HM427 in causing fragmentation of the Golgi, increasing the number of fragments per nucleus from an average of (±SD) 2.7 ± 0.5 to 4.6 ± 0.8 ($p<0.01$).

Figure 7.14: Representative Giantin immunohistochemical images of HT-29 MTX cells after incubation with outer membrane vesicle preparation.

OMV



OMV Free

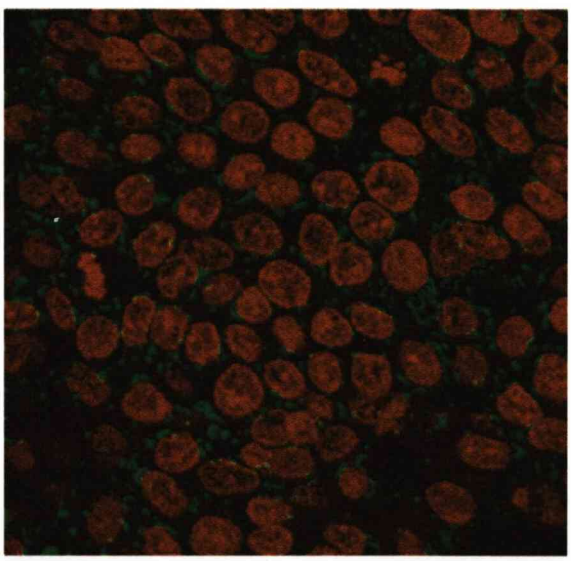


Figure 7.14: Anti Giantin immunohistochemical staining (green). HT-29 MTX cells were seeded at $1x10^5$ on coverslips and cultured for 15 days. The post confluent monolayers of cells were then incubated with OMV's, or a preparation with the OMV's removed. The OMV preparation caused fragmentation of the Golgi increasing the number of fragments per nucleus from 2.7 ± 0.5 to 4.3 ± 1.3 ($p<0.01$), the OMV free preparation had no effect.

Figure 7.15: Representative Giantin immunohistochemical images of HT-29 MTX cells after incubation with flagellin.

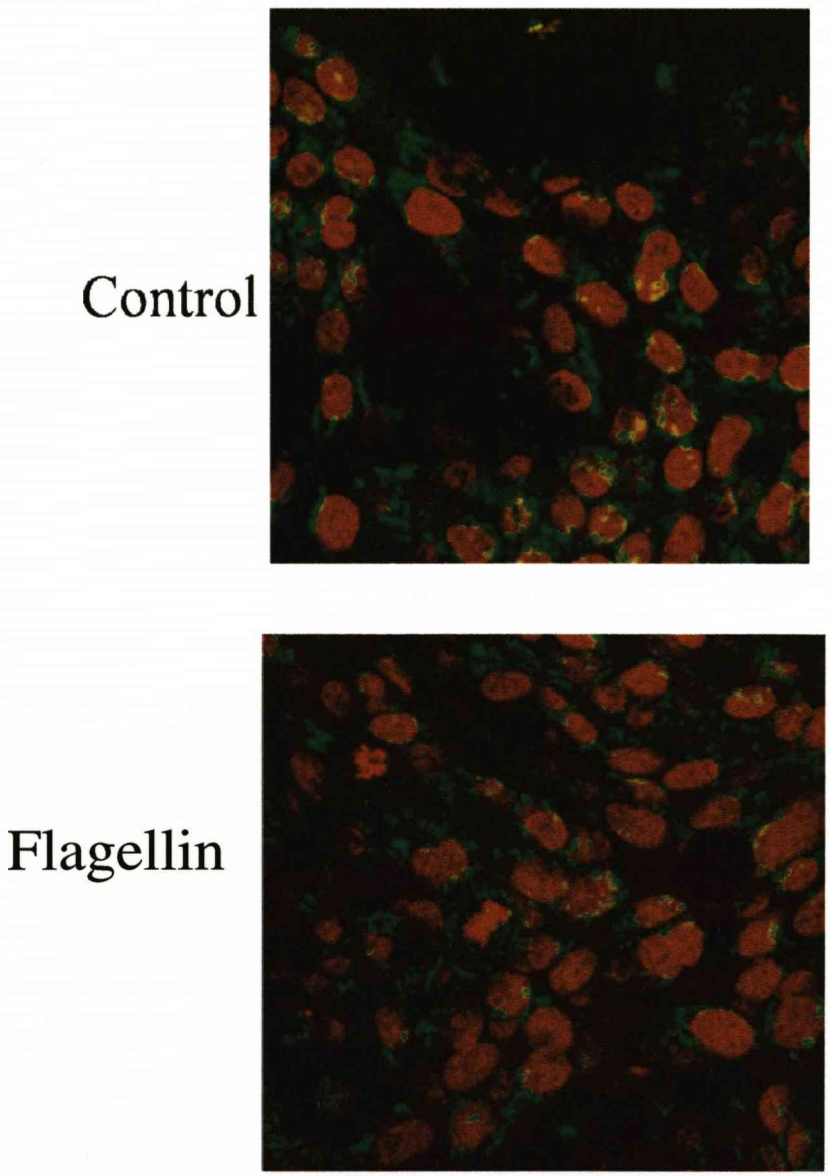


Figure 7.15: Anti Giantin immunohistochemical staining (green). HT-29 MTX cells were seeded at $1x10^5$ on coverslips and cultured for 15 days. The post confluent monolayers of cells were then incubated with flagellin which did not cause increased fragmentation of the Golgi.

Figure 7.16: Representative Giantin immunohistochemical images of HT-29 MTX cells after incubation with LPS

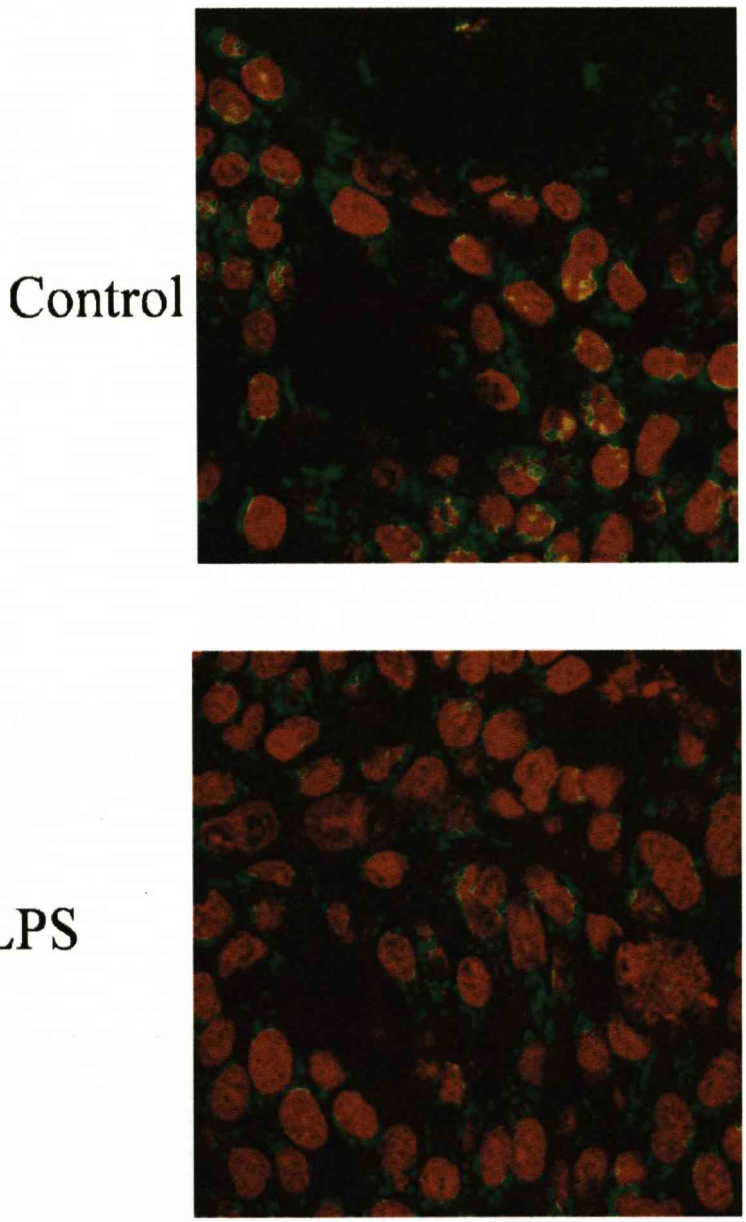


Figure 7.16: Anti Giantin immunohistochemical staining (green). HT-29 MTX cells were seeded at $1x10^5$ on coverslips and cultured for 15 days. The post confluent monolayers of cells were then incubated with LPS which did not cause increased fragmentation of the Golgi.

Figure 7.17: Representative Giantin immunohistochemical images of HT-29 MTX cells after bafilomycin treatment

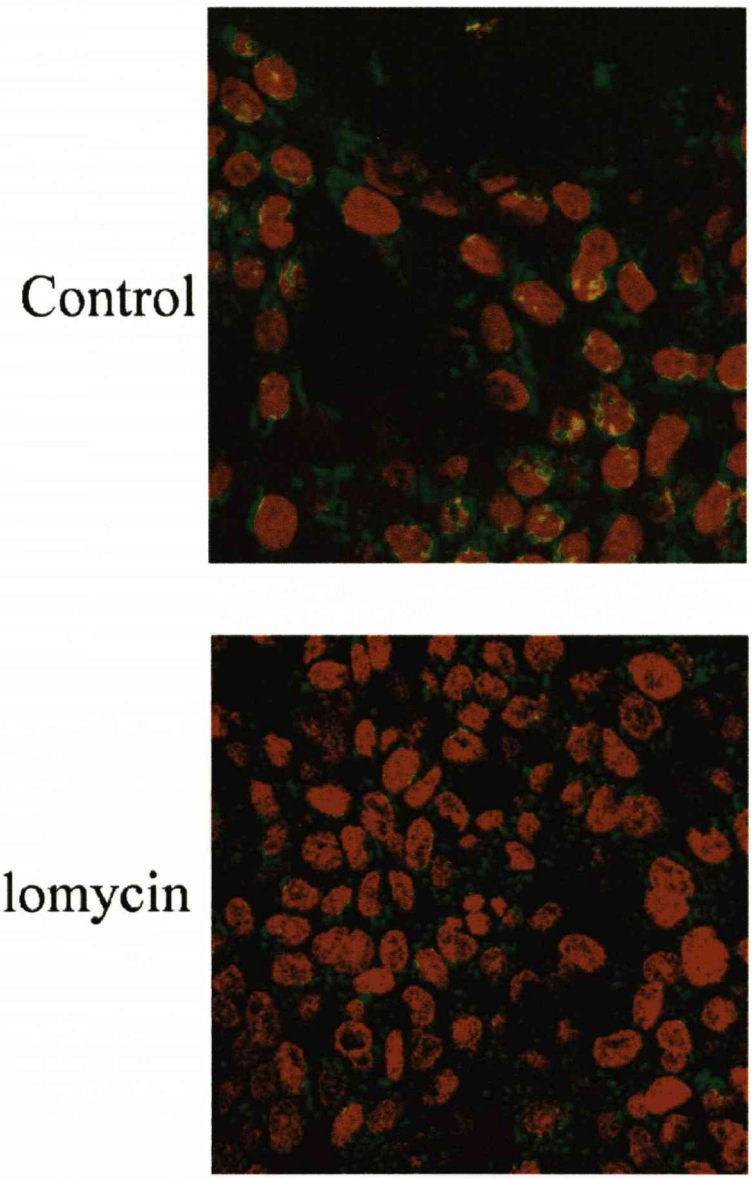


Figure 7.17: Anti Giantin immunohistochemical staining. HT-29 MTX cells were seeded at $1x10^5$ on coverslips and cultured for 15 days. The post confluent monolayers of cells were then incubated bafilomycin which caused increased fragmentation of the Golgi from an average 2.7 ± 0.5 fragments to 5.0 ± 1.0 ($p<0.01$)

Figure 7.18: Mean number of Golgi fragments per nuclei after incubation with bacterial supernatants

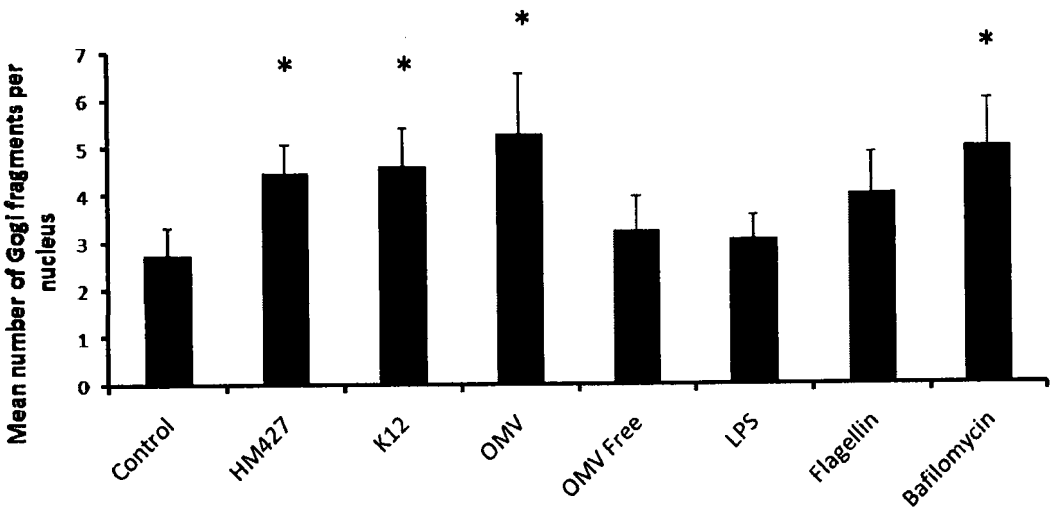


Figure 7.18: HT-29 MTX cells were seeded at $1x10^5$ on coverslips and cultured for 15 days. The post confluent monolayers of cells were then incubated with treatments for a further 10 days. The Golgi was visualised with an anti Giantin antibody using confocal microscopy, and the number of Golgi fragments calculated per nucleus as described in methods. HM427 and K12 both caused a significant increase in Golgi fragments (*) ($p<0.05$). OMV preparation and Bafilomycin control also caused a significant increase in the number of Golgi fragments per nucleus ($p<0.01$).

7.4.6 HM427 bacterial supernatant causes AK release in to medium

This study assesses the effect of the HM427 bacterial supernatant and other treatments on the health of the HT-29 MTX cells. Adenylate kinase (AK) is a protein that is released into the cell culture medium upon cell death. The enzyme phosphorylates ADP to ATP which can be quantitatively measured utilizing a bioluminescent assay. As the level of cytolysis increases the amount of AK released in to the culture medium also increases resulting in a higher emission of light from the assay (Lonza, Basel; Switzerland). The release of AK into the culture medium allows the kinetic monitoring of AK levels over time. A total lysis control was also employed to reveal the total AK in the HT-29 MTX cells.

The HT-29 MTX cells were seeded at $1x10^5$ cells/ml and cultured for a further 15 days. The post confluent monolayers were then incubated with the HM427 bacterial supernatant. Further treatments included a bacterial supernatant of *E.coli* K12, LPS purified from *E. coli* O127:B8, flagellin and an outer membrane vesicle preparation from HM427 and the *O*-glycosylation inhibitors bafilomycin, TNF α, benzyl-GalNAc and 5-CDP. The treatments were added to the DMEM at the concentrations outlined in the methods, which was replaced each 24 h complete with respective treatments and the medium collected for the AK bioluminescent assay on day 1, day 5 and day 10. The inhibitors were observed at day 1. The experiment was repeated 3 times and on each occasion in triplicate. After 1 day incubation the control cells gave an average (±SD) 30800 ± 350 toxilight RLU [Figure 7.18], cells that were incubated with HM427 bacterial supernatant had an average 34100 ± 200 RLU which was statistically significant ($p<0.005$) [ANOVA]. The K12 bacterial supernatant also caused a significant increase in AK release with an average reading of 34200 ± 200 RLU. The OMV preparation gave an average reading of 35800 ± 200 RLU ($p<0.005$) and the

OMV free preparation reading was not significant. Flagellin caused a significant reading of 35600 ± 200 RLU, the reading for LPS was not significant. Bafilomycin gave a larger reading of 54300 ± 3000 RLU ($p<0.001$) as did TNF alpha 42900 ± 3000 RLU, 5-CDP 41400 ± 3000 RLU and benzyl GalNAc 36200 ± 4400 RLU. The Total AK was greater than 100000 RLU.

After 5 days the control cells had an average reading of 32000 ± 1300 RLU, cells after incubation with HM427 bacterial supernatant gave an average reading of 39600 ± 500 RLU ($p<0.005$) [ANOVA]. HM427 and elastase gave a significant reading of 37200 ± 1100 RLU ($p<0.005$. The K12 bacterial supernatant caused no significant increase in the toxilight readings after 5 days incubation. The OMV preparation had an average reading of 42100 ± 1300 RLU ($p<0.005$) and the OMV free preparation gave no significant response. Flagellin gave a reading of 37100 ± 600 RLU ($p<0.005$) and LPS caused no significant response from control. After 10 Days the control cells gave an average reading of 27600 ± 500 RLU, this was increased by the incubation with HM427 bacterial supernatant to 34700 ± 1200 RLU ($p<0.005$). K12 bacterial supernatant gave an average reading of 32700 ± 800 RLU's ($p<0.005$). The OMV preparation gave a reading of 39300 ± 900 RLU ($p<0.005$) with the OMV free preparation not giving a significant response. Flagellin gave a reading of 39400 ± 1700 RLU ($p<0.005$) and combined incubation with elastase reduced this response to a non significant level of 29700 ± 1600 RLU. LPS did not give a significant response [Figure 7.18].

The data demonstrates that all treatments apart from OMV free and LPS cause modest toxicity to the cells with bafilomycin causing the most AK release.

Figure 7.19: AK released in to medium in 24 h after 1 day bacterial supernatant incubation

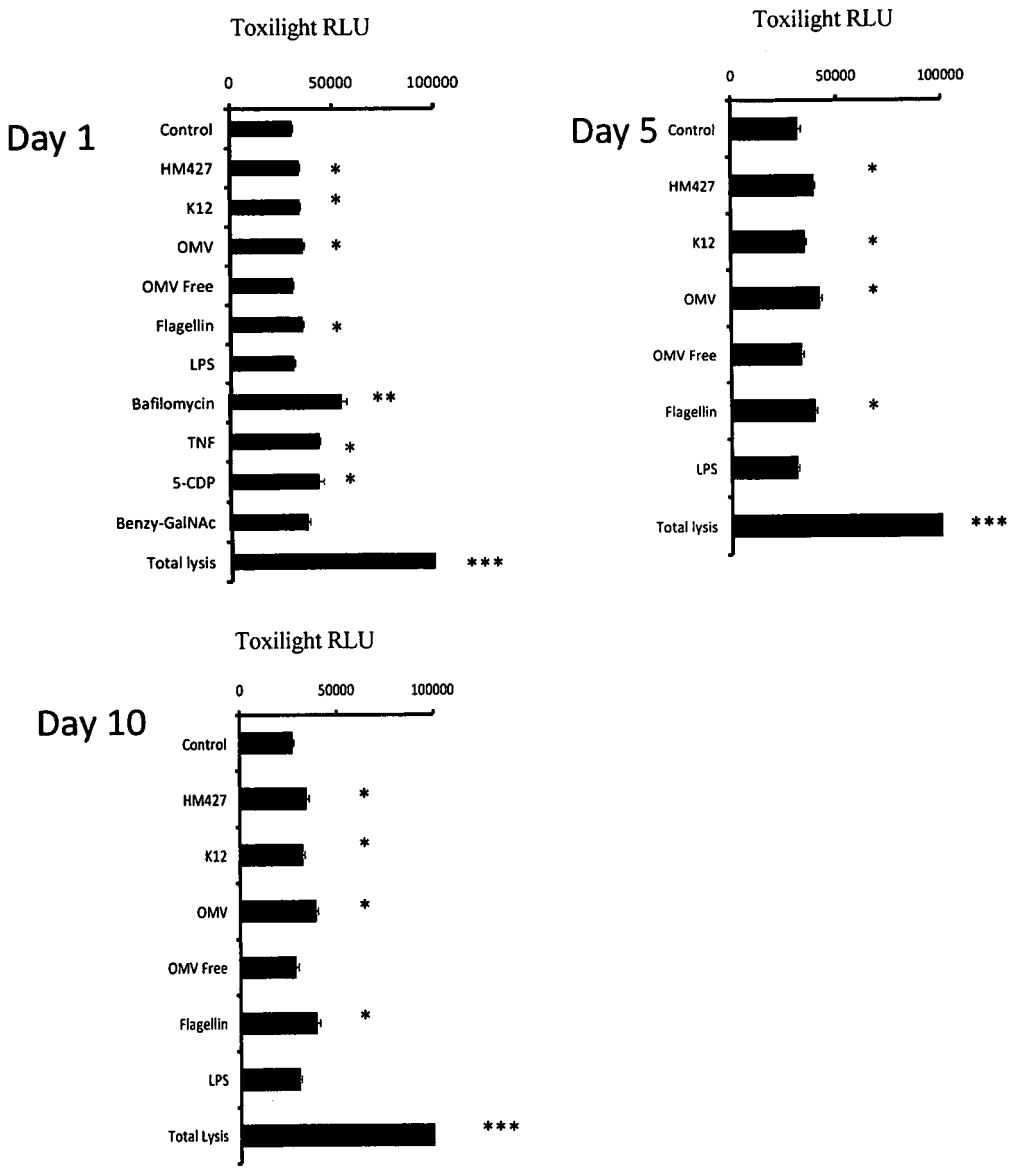


Figure 7.19: HT-29 MTX cells were seeded at $1x10^5$ cells/ml and cultured for 15 days until post confluent. The following treatments were then added to the culture medium: HM427 bacterial supernatant, bacterial supernatant of *E.coli* K12, LPS purified from *E. coli* O127:B8, flagellin from HM427, an OMV or OMV free preparation and the *O*-glycosylation inhibitors bafilomycin, TNF α, benzyl-GalNAc and 5-CDP. The medium was collected after 1 day and the AK content quantified by a bioluminescent assay (Lonza, Basel; Switzerland). Total AK release from the cells was achieved with toxilight lysis reagent (Lonza, Basel; Switzerland) (*=$p<0.01$, ***=$p<0.001$)

7.4.7 HM427 bacterial supernatant stimulates IL-8 release from HT-29 MTX cells

Previous experiments have shown that bacteria and bacterial supernatant can elicit IL-8 secretion from HT-29 cells (Subramanian et al. 2008), this IL-8 secretion occurs via a MAPK-dependent pathway. IL-8 response was therefore measured here as a positive control to indicate the physiological response to the bacterial supernatant.

This experiment observes amount of IL-8 secreted into the culture medium via an ELISA assay, stimulated by HM427 supernatant and K12 supernatant. Two of the active components of the bacterial supernatants are flagellin and LPS, the effects of both are also observed in this experiment. HT-29-MTX cells were seeded at $1x10^5$ cells/ml in 24 well plates and grown for 15 days until post confluent. The HT-29 MTX monolayers were then incubated with the HM427 bacterial supernatant, a bacterial supernatant of *E.coli* K12, LPS purified from *E. coli* O127:B8, flagellin from HM427 and an OMV or OMV free preparation. The culture medium was replaced every 24h and collected on day10 for analysis by ELISA for the amount of IL-8 secreted into the medium. A positive control of TNFα was utilised as a known stimulator of IL-8 secretion. The experiment was completed 3 times and on each occasion in triplicate.

After 10 days incubation the control cells released an average of 10 ± 2 ng/ml of IL-8 into the medium, this was increased by HM427 to 27 ± 5 ng/ml ($p<0.005$). K12 stimulated an IL-8 concentration in the medium of 16 ± 2 ng/ml ($p<0.01$). Flagellin caused an increase of IL-8 concentration to 23 ± 3 ng/ml ($p<0.005$). LPS caused an IL-8 concentration of 20 ± 3 ng/ml ($p<0.01$). TNF α stimulated an IL-8 concentration of 46 ± 6 ng/ml ($p<0.001$) [Figure 7.20].

Figure 7.20: IL-8 release into medium after 10 day incubation with bacterial supernatant and fractions

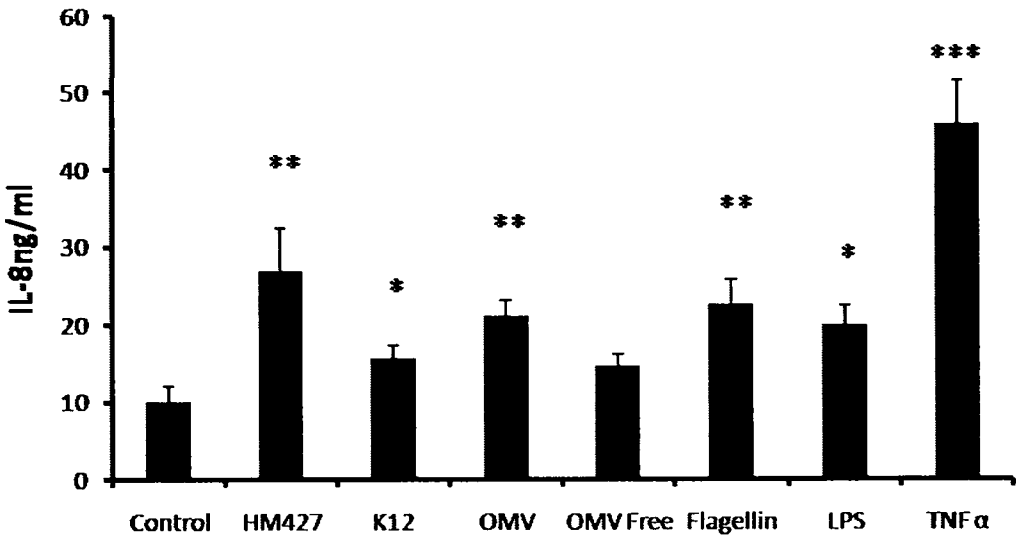


Figure 7.20: HT-29 MTX cells were seeded at $1x10^5$ cells/ml and cultured for 15 days until post confluent. The following treatments were then added to the culture medium: HM427 bacterial supernatant, a bacterial supernatant of *E.coli* K12, LPS purified from *E. coli* O127:B8, flagellin from HM427, an OMV or OMV free preparation and TNFα. The medium was replaced complete with treatments every after 24 h and the medium collected 10 days had the concentration of IL-8 secreted into the medium measured by an ELISA.

7.5 DISCUSSION

Altered glycosylation has long been associated with disease states, and the expression of TF, which is normally concealed by further modifications, has been demonstrated in about 90% of all human cancers (Hanisch et al. 1997). The increase of TF expression and Golgi fragmentation shown in these experiments in response to bacteria and bacterial components are in keeping with the hypothesis that intestinal mucosal glycosylation changes in inflammatory and malignant intestinal disease could occur, at least in part, as a result of increased interaction between bacteria and the epithelium. The colonic epithelium of inflammatory bowel disease patients, often has an increased number of immunocytes (*e.g.*, T lymphocytes, mast cells) as well as evidence of increased release of cytokines (Collins 2005), histamine, proteases, and prostaglandins (Barbara et al. 2004). These mediators could be responsible for intestinal dysfunction, as a consequence of a breakdown of the mucosal barrier, caused by a defect in the mucus, the glycocalyx, the tight junctions or a combination of all three. An increased permeability potentially exposes the mucosa to an increased challenge of luminal bacteria and dietary antigens that could promote and maintain mucosal immune activation. This seems likely to be part of the explanation for glycosylation changes seen in the non-inflamed surface epithelium in unaffected identical twins of IBD patients (Bodger et al. 2006).

From the present study it is not known by what mechanism the bacterial supernatant is mediating the effects seen and further experiments are required. The effect of the bacterial supernatant could reflect an interaction between released factors such as flagellin, cell wall peptidoglycans and lipopolysaccharide and their relevant toll-like receptors on the colonic epithelium. One or more of these factors may be responsible for the fragmentation of the Golgi apparatus and, therefore, a subsequent

disorganisation in mucin type *O*-glycosylation leading to the increased expression of TF. Such changes in *O*-glycosylation would also further damage the protective mucus and glycocalyx propagating the detrimental effects.

Bacteria adhere to the colonic mucosa through fimbrial or surface proteins known as adhesins (Soto et al. 1999), which act as lectins able to recognise the glycosyl motifs expressed on the cell surface glycoproteins. The aberrant glycosylation changes seen in ulcerative colitis and Crohn's disease, such as increased TF expression, have been demonstrated to be functionally important, allowing interaction with dietary lectins such as the peanut lectin (Ryder et al. 1998) and it, therefore, seems possible that microbial lectins may interact in a similar manner. There is increasing evidence that mucosa-associated bacteria may well be important in the pathogenesis of inflammatory bowel diseases, with increased numbers of *E.coli* reported to be adjacent to the ileal (Darfeuille-Michaud et al. 1998) and colonic mucosa (Martin et al. 2004, Swidsinski et al. 2002). These mucosa-associated bacteria lack conventional pathogenic genes, but are able to adhere and invade epithelial cell lines *in vitro*. *E.coli* account for a disproportionally high amount of the mucosa-associated bacteria found in inflammatory bowel diseases and colorectal cancers (Martin et al. 2004). However the effects seen in these experiments are unlikely to be specific to *E.coli*. *Streptococcus bovis* is another bacterium that has been associated with colon cancer (Tjalsma et al. 2006) and has also been shown to elicit a cytokine response *in vitro* in Caco-2 cells (Biarc et al. 2004).

Further evidence for the involvement of bacteria comes from the study of monozygotic twins, with the unaffected twin in the study possessing the same glycosylation alterations as the genetically identical twin, who was affected by inflammatory bowel disease (Bodger et al. 2006). This altered glycosylation was

predominantly observed in the surface epithelium, whereas genetic changes would be expected to originate from the stem cell compartments, affecting the whole crypts. Inspection of the mucosal biopsies revealed no histological inflammation but activation of NFκB at the surface epithelium. This suggests a possible interaction between the bacteria and epithelium for inducing these glycosylation changes rather than inflammation.

The overall picture from the present series of experiments reveals that the HM427 *E.coli* along with flagellin and outer membrane vesicles purified from HM427 *E.coli* can cause fragmentation of the Golgi and significantly increase the expression of TF on the HT-29 MTX cells. *E.coli* K12 supernatant also caused a significant increase in Golgi fragmentation, but not a significant increase in TF expression. The bacterial supernatants, flagellin and outermembrane vesicles all caused some amount of damage to the cells, as shown by adenylate kinase release into the medium and all stimulated cytokine interleukin-8 release.

The quantification of the Golgi fragmentation was complicated as the natural structure of the HT-29 MTX Golgi is slightly fragmented, therefore, any subsequent fragmentation is harder to quantify. In addition, the confocal images obtained give a 1-D perspective, which does not allow for the natural variations of shape of the Golgi which could cause the Golgi to appear to be more or less fragmented than in reality. To overcome this, 3 images were acquired over 3μm with a 1μm space between, the number of Golgi fragments were then averaged over these 3 images, however, there is still the possibility of error in this calculation. Further to this, the mean number of fragments were divided by the number of cells in the image, this was because the fragments counted could not be assigned to a particular cell in the image; this could be a further source of error.

Further experiments must be undertaken to support the results the demonstrate the fragmentation of the Golgi apparatus due to incubation with bacterial supernatant possibly by utilising other cell lines with more naturally complete Golgi. These experiments would be combined with an investigation into the effects of bacterial supernatant on the Golgi pH in intestinal epithelial cells. It is looking increasing likely that bacteria that interact with the colonic epithelium play a major role in the pathogenesis of colorectal cancer and inflammatory bowel disease and altered glycosylation may be one of the pathogenic mechanisms involved.

CHAPTER 8

SUMMARY OF THE MAIN FINDINGS IN THIS THESIS

- There exist two isoforms of Orp150. A Constitutive form localised in the cytoplasm and a heat stress inducible form is localised in the ER. A possible further third isoform is observed after 2-D electrophoresis.

- Constitutive Orp150 is bound by ABL/Jacalin indicating the likely presence of sialyl-TF.

- Analysis of Jacalin-lectin purified Orp150 by mass spectrometry did not reveal the presence of sialyl-TF but indicated the presence of 4 high mannose N-glycopeptides the most abundant of which were $Man_{5-8}GlcNAc_2$. One further glycopeptide was observed at m/z 747^{3+} indicating the presence of a single HexNAc.

- Depletion of Orp150 from HT-29 cells with siRNA causes reduced NLS-dependent nuclear protein import.

- The intra-cellular localisation of Ran is unaffected by reduced Orp150 protein expression induced by siRNA.

- ABL lectin affinity purification of a pure cytoplasmic fraction from colon epithelial cells yields several proteins implying the existence of several sialyl-TF expressing cytoplasmic proteins.

- ABL and Jacalin lectin both recognise cytoplasmic Hsp90.
- All of the *O*-glycosylation inhibitors tested increased TF expression, with benzyl-GalNAc having the greatest effect on increasing TF.
- Cell surface and whole cell sialylation was decreased by benzyl-GalNAc and 5-CDP.
- Inhibitors of *O*-glycosylation including benzyl-GalNAc and 5-CDP have no effect on Hsp70 translocation into the nucleus upon heat treatment.
- SiRNA Galtransferase caused a modest decrease of TF and more marked increase in Tn expression, but this had no effect on Hsp70 nuclear translocation in response to heat stress. Therefore, NLS-dependent nuclear protein import is probably not dependent on TF or sialyl-TF expression by Orp150 or other cytoplasmic proteins.
- Bafilomycin causes dispersion of the Golgi apparatus.
- 5 to 10 day incubation of confluent monolayers of HT-29 MTX cells with HM427 *E.coli* supernatant causes Golgi apparatus fragmentation.
- Incubation with an outer membrane vesicle preparation and flagellin from HM427 *E.coli* also causes Golgi apparatus fragmentation.
- Bacterial supernatant HM427, outer membrane vesicles and flagellin cause an increase of TF expression on HT-29 MTX cells.

CHAPTER 9

GENERAL DISCUSSION AND IMPLICATIONS FOR FUTURE STUDIES

The expression of sialyl-TF on a constitutive cytoplasmic isoform of oxygen regulated protein 150 (Orp150) is intriguing. Previous work by Yu *et al.* has demonstrated that the ability of ABL lectin to reversibly inhibit cellular proliferation is the result of ABL internalisation. The internal ligand for the lectin was shown to be Orp150 (Yu et al. 2002), thus implying the existence of sialyl-TF on Orp150. The work presented here further demonstrates that it is the cytoplasmic isoform of Orp150 that has a role in NLS-dependent nuclear protein transport by observing the translocation of Hsp70 into the nucleus upon heat shock. HT-29 colon cancer cells treated with siRNA to reduce Orp150 had a subsequently reduced Hsp70 translocation into the nucleus upon heat shock. This discovery adds evidence to support Orp150 as an essential protein in the NLS-dependent nuclear protein import mechanism [Figure 9.1]. Further evidence for the role of Orp150 in NLS-dependent nuclear protein import was uncovered when Orp150 was co-immunoprecipitated alongside Ran (Yu et al. 2002), which is a protein known to be essential in the NLS-dependent nuclear import of proteins. However, the intra-cellular localisation of Ran appears unaffected by a reduction of Orp150 by siRNA and this suggests that Orp150 is not involved in the translocation of Ran into and out of the nucleus.

The discovery of a constitutive isoform of Orp150 which expresses sialyl-TF is one of only 2 reports so far of a cytoplasmic protein expressing sialyl-TF, the other being alpha-synuclein (Shimura et al. 2001). The demonstration that Orp150 expresses sialyl-TF by lectin analysis, but not by mass spectrometry means further work must be continued to confirm this glycosylation. It is possible that *O*-glycans including sialyl-

TF might have been degraded during the preparation of Orp150 for mass spectrometric analysis. Another factor which might have affected mass spectrometry analysis is the low abundance of Orp150 isolated for analysis. Unfortunately immunoprecipitation of Orp150, which might have produced better yields was unsuccessful. Lectin affinity purification using TF recognising lectins did not yield enough protein to stain with coomassie and this hampered analysis. Evidence that Hsp90 also expresses sialyl-TF is presented in this thesis and the functional significance of this glycosylation also merits further exploration.

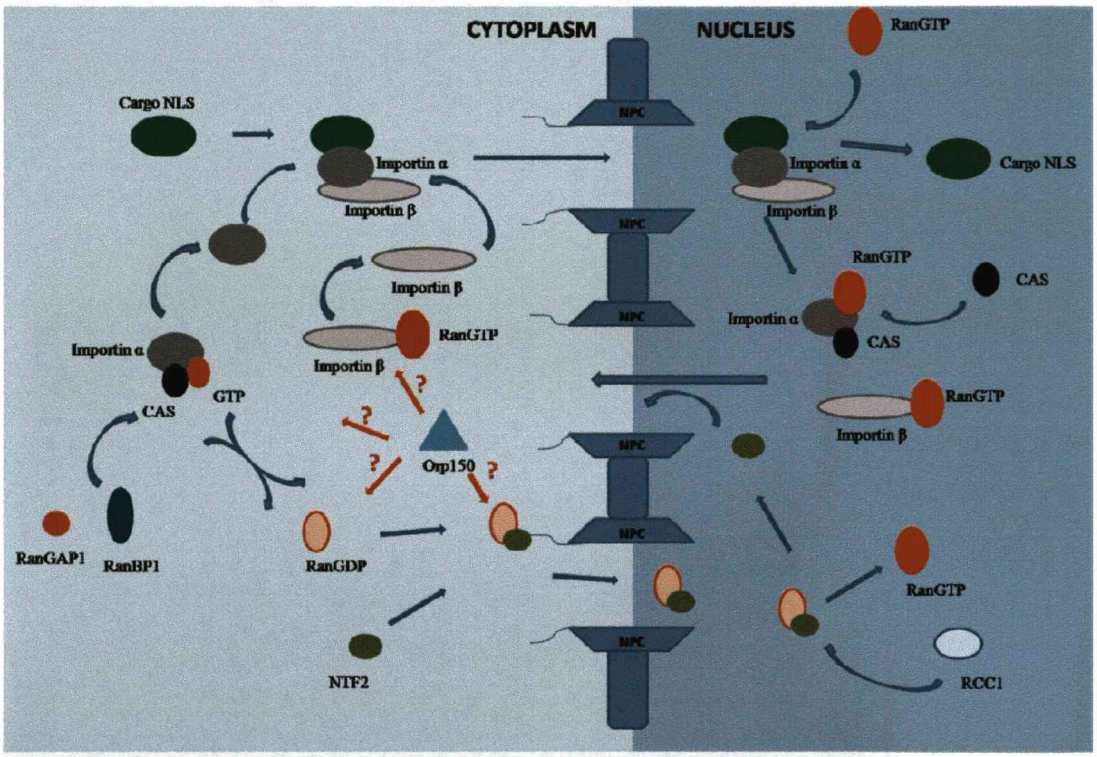


Figure 9.1: Hypothetical role of cytoplasmic constitutively expressed Orp150 in inhibiting NLS-dependent nuclear protein import.

ABL reversibly inhibited NLS-dependent nuclear protein import. The internal ligand was found to be a cytoplasmically localised isoform of Orp150 which was shown to be associated with Ran. The diagram indicates how Orp150 might inhibit NLS-dependent nuclear protein import by preventing the cycling of Ran GDP from the cytoplasm to the nucleus.

The functional importance of the sialyl-TF present on the cytoplasmic Orp150 protein remains unclear. Experiments utilising known *O*-glycosylation inhibitors demonstrated no significant change in NLS-dependent nuclear protein import. A limitation of the approach is that this will affect all cellular *O*-glycosylation and may therefore have other unforeseen effects. Direct demonstration of the effect of the inhibitors on the glycosylation of Orp150 was not achieved, as immunoprecipitation of the protein was not successful. A further experiment to address the role of sialyl-TF was made by using siRNA directed to the Galtransferase responsible for the addition of Gal to GalNAc. This is the first time this technology has been used to reduce the expression of this enzyme. The siRNA Galtransferase reduced the TF expression only modestly considering that only one transferase is responsible for the addition of Gal to GalNAc (Ju et al. 2002). With TF expression reduced Tn expression was observed to increase strongly. This observation confirms the down-regulation of the Galtransferase however it is not clear why the expression of the TF antigen was not more strongly diminished. Again, nuclear protein import was not affected.

There is a delicate relationship between the human intestine and the commensal bacteria and it has been shown that the normal bacterial flora is a prerequisite for the development of inflammation and inflammation-related colorectal tumours in animal models. Evidence that led to *H.pylori* becoming classified as a group 1 carcinogen in 1994 by the World Health Organization has triggered an interest into the association between epithelia-associated bacteria and colorectal cancer. The colonic mucosa of patients with colorectal carcinoma has an increase in mucosa-associated *E.coli*, and these *E.coli* were also present in non cancerous mucosa distant to the tumour (Martin et al. 2004, Swidsinski et al. 1998). In this thesis interaction between *E.coli* HM427 and colon epithelial cells has been shown to cause a significant

increase in the fragmentation of the Golgi. Similar fragmentation of the Golgi apparatus has previously been shown to be associated with aberrant glycosylation in colon cancer (Kellokumpu et al. 2002) and in this study an increase of TF expression was also observed. Truncated glycans such as the TF antigen could act as ligands to recruit further bacterial and dietary lectins which in turn have been shown to be able to exert a range of effects on cells such as the PNA lectin which increases cellular proliferation (Ryder et al. 1998). Unchecked mucosal proliferation and decreased mucosal apoptosis could increase the likelihood of the onset of cancer.

The incidence of colorectal cancer is about 28,000 cases per annum in the United Kingdom and it is the second most common cause of cancer-related deaths in the world (Evans et al. 2002). A diet high in vegetable consumption has long been associated with a protective role against colonic disease and cancer and high cereal fiber intake was also thought to be protective but lacks convincing evidence (Fuchs et al. 1999). There is a strong protective, effect from the consumption of brassicas, thought to stem from the isothiocyanates contain within them. Now it has been hypothesised that high galactose fiber contained in some vegetables may confer a protective effect (Evans et al. 2002). The glycosylation alterations observed in colon cancer and pre-cancer states, such as the increased expression the TF antigen, allows the epithelium to bind intraluminal galactose-binding lectins that would otherwise pass through the intestine. This has been successfully demonstrated with the peanut lectin which can cause colonic epithelial proliferation (Ryder et al. 1998). It is conceivable that bacteria or bacterial lectins could be acting in the same manner. An understanding of bacterial-epithelial interactions could lead to the design of inhibitors that act as a blockade to the lectin-carbohydrate interactions and this could have significant implication in treatment and prevention of colorectal cancer and inflammatory bowel

diseases. It could be an important mechanism for the protective effects of dietary fibre. The discovery that bacteria could be directly responsible for the disruption of the Golgi adds further to the understanding of the complex relationship between bacteria and the host in a disease state.

IMPLICATIONS FOR FUTURE STUDIES

This thesis has raised many interesting questions which require further investigation. The discovery of sialyl-TF on Orp150 should be further confirmed by mass spectrometry analysis. In addition, further work to observe the effects of *O*-glycosylation inhibitors in purified Orp150 may give insight not only into whether the expression of sialyl-TF was of functional importance, but also the mechanism by which it becomes expressed. If the glycosylation of Orp150 was not altered by either the inhibitors or the siRNA Galtransferase, this would hint at the possible presence of an additional Galtransferase, possibly with a cytoplasmic localisation. The analysis of *O*-glycosylated Hsp90 could be furthered by immunoprecipitation of the protein and further analysis by mass spectrometry to confirm the presence of sialyl-TF.

The possibility that disease-associated changes in intestinal glycosylation may be the result of bacterial epithelial interactions leading to Golgi fragmentation is intriguing. Further work is needed to elucidate the exact component of the bacterial supernatant responsible and to observe the effects of a range of bacteria on Golgi fragmentation. The fragmentation of the Golgi apparatus could also be examined in a cell line with a more naturally intact Golgi. Histological sections of inflammatory bowel disease affected tissue could be stained for Giantin and the structure of the Golgi and TF expression observed. The mechanism by which the bacterial supernatant causes

the effects also needs to be understood. The secreted factors in the bacterial supernatant such as flagellin and LPS are able to activate receptors. It has been shown that Toll like receptor 4 is expressed in the Golgi apparatus and LPS can activate NFκB when internalised (Hornef et al. 2003). It is possible that one of these factors is responsible for the disruption of the Golgi and future experiments may be able to elucidate why the Golgi apparatus becomes fragmented. There remains a great deal of interesting work to be done to elucidate the causes and consequences of altered *O*-glycosylation in disease states.

The principle conclusions from this study are:

1) Provision of further evidence that the cytoplasmic constitutive isoform of Orp150 is glycosylated with sialyl-TF and that the Orp150 protein is involved in NLS-dependent nuclear protein import.
2) Identification of another cytoplasmic protein (Hsp90) that expresses sialyl-TF.
3) Identification of Golgi disruption as a possible mechanism by which bacteria could cause aberrant glycosylation in colonic epithelial cells.

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APPENDIX 1

Components of cell culture medium and buffers

Dulbecco's Modified Eagle Medium (DMEM) (10X)

	mg/ml
Inorganic salts:	
$CaCL_2.2H_2O$	2640.0
$Fe(NO)_3.9H_2O$	1.0
KCl	4000.0
$MgSO_4.7H_2O$	2000.0
NaCl	6000.0
$Na_2H_2PO_4.H_2O$	1400.0
Other components	
D-glucose	4500.0
Hepes	170.0
Amino acids:	
L-Alanine	356.0
L-Asparagine	600.0
L-Arginine.HCL	840.0
L-Aspartic acid	530.0
L-Glutamic acid	590.0
Glycine	300.0
L-Histidine HCL.H_2O	420.0
L-Isoleucine	1050.0
L-Leucine	1050.0
L-Lysine HCL	1460.0
L-Methionine	300.0
L-Phenylalanine	660.0
L-Proline	460.0
L-serine	420.0
L-Threonine	950.0
L-Tryptophan	16.0
L-Tyrosine	72.0
L-Valine	940.0
Vitamins	
D-Ca pantothenate	40.0
Choline Chloride	40.0
Folic acid	40.0
i-Inositol	72.0
Nicotinamide	40.0
Pyridoxal HCL	40.0
Riboflavin	4.0
Thiamine HCL	40.0

Phosphate buffered saline (PBS):

	g/ml
$CaCl_2$	1.0
KCL	2.0
KH_2PO_4	2.0
$MgCl_2.6H_2O$	1.0
NaCL	80.0
$Na_2H_2PO_4.7H_2O$	21.6

SDS-PAGE buffers:

Running buffer:
25mM Tris
250 mM glycine, pH 8.3
0.1% SDS

Resolving buffer
1.5 M Tris, HCL to pH 8.8

Stacking buffer
1.0 Tris, N HCL to pH 6.8

SDS gel loading buffer (sample buffer)
70% Stacking buffer (4X)
20% Glycerol
10% Mercaptoethanol
4% SDS
0.01% Bromthymol

Destain buffer for commassie blue staining
10% Ethanol
10% Acetic acid

Blocking buffer for lectin/western blot

PBS
1% bovine serum albumin
0.5% Tween-20

Modified RIPA buffer

Tris-HCl: 50 mM, pH 7.4 ,
NP-40: 1%
Na-deoxycholate: 0.25%
NaCl: 150 mM
EDTA: 1 mM
PMSF: 1 mM
Aprotinin, leupeptin, pepstatin: 1 microgram/ml each
Na_3VO_4: 1 mM
NaF: 1 mM

2-D electrophoresis buffers

2-D lysis buffer
- 8M urea
- 2M thio urea
- 4% Chaps
- 1% DTT
- 5nM sodium vanadate
- 40mM Tris

Equilibration buffer
- 30% glycerol (v/v)
- 6M urea
- 2% SDS (w/v)
- 50ml 1.25M Tris-HCL to pH6.8 in distilled H_2O

Destain
- 50mM ACN (Acetonitrile)
- 50mM Ammonium bicarbonate (Ambic)

Rehydration buffer
- 50mM ammonium bicarbonate and trypsin (9µl ambic and 1µl trypsin stock, 100ng/µl)

APPENDIX 2

Average number of Golgi fragments per nucleus after HM427 supernatant incubation

		Average number of Nuclei	Average number of Golgi Fragments	Average Fragments/average nucleus per quadrant	Overall average
control 1	A	15.33	27.67	1.80	2.12±0.2
	B	12.33	25.67	2.08	
	C	12.00	28.33	2.36	
	D	14.00	31.67	2.26	
control 2	A	12.00	41.00	3.42	2.96±0.3
	B	15.67	45.33	2.89	
	C	14.33	41.33	2.88	
	D	18.33	49.00	2.67	
control 3	A	12.33	45.33	3.68	3.13±0.4
	B	9.67	31.67	3.28	
	C	18.67	53.33	2.86	
	D	13.67	37.33	2.73	
HM427 1	A	8.00	42.33	5.29	5.04±0.6
	B	9.33	44.33	4.75	
	C	8.00	46.00	5.75	
	D	12.33	54.00	4.38	
HM427 2	A	7.00	31.67	4.52	4.54±1.0
	B	12.00	51.00	4.25	
	C	8.33	29.33	3.52	
	D	8.33	49.00	5.88	
HM427 3	A	21.00	81.67	3.89	3.85±0.4
	B	20.00	80.00	4.00	
	C	19.33	63.33	3.28	
	D	17.67	75.33	4.26	

Average number of Golgi fragments per nucleus after K12 supernatant, LPS and Flagellin incubation

		Average number of Nuclei	Average number of Golgi Fragments	Average Fragments/average nucleus per quadrant	Overall average
K12 1	A	19.67	66.00	3.36	5.01±1.1
	B	13.00	70.67	5.44	
	C	15.33	90.67	5.91	
	D	16.00	85.00	5.31	
K12 2	A	15.67	54.33	3.47	3.72±0.7
	B	14.33	62.67	4.37	
	C	17.00	49.00	2.88	
	D	16.00	67.00	4.19	
K12 3	A	15.00	69.00	4.60	5.16±0.4
	B	12.33	65.33	5.30	
	C	12.67	68.33	5.39	
	D	12.33	66.00	5.35	
LPS 1	A	15.00	39.67	2.64	3.10±0.4
	B	13.67	50.67	3.71	
	C	14.00	42.67	3.05	
	D	13.67	41.33	3.02	
LPS 2	A	12.33	35.33	2.86	3.55±0.6
	B	13.00	45.67	3.51	
	C	10.00	43.00	4.30	
	D	11.33	40.33	3.56	
LPS 3	A	11.67	39.00	3.34	2.57±0.7
	B	15.00	42.67	2.84	
	C	18.67	30.67	1.64	
	D	14.00	34.33	2.45	
Flagellin 1	A	10.00	36.33	3.63	2.99±1.0
	B	14.33	36.00	2.51	
	C	18.67	52.00	2.79	
	D	10.33	31.33	3.03	
Flagellin 2	A	8.67	40.00	4.62	3.35±2.5
	B	4.67	12.00	2.57	
	C	8.67	27.00	3.12	
	D	8.33	26.00	3.12	
Flagellin 3	A	8.67	30.33	3.50	3.09±0.6
	B	12.33	39.67	3.22	
	C	10.67	36.67	3.44	
	D	12.00	26.67	2.22	

Average number of Golgi fragments per nucleus after OMV and Bafilomycin incubation

		Average number of Nuclei	Average number of Golgi Fragments	Average Fragments/average nucleus per quadrant	Overall average
OMV 1	A	18.00	64.00	3.56	4.18±0.5
	B	20.67	97.33	4.71	
	C	21.00	85.00	4.05	
	D	16.33	71.67	4.39	
OMV2	A	11.67	91.33	7.83	6.65±2.7
	B	25.00	105.33	4.21	
	C	21.33	98.00	4.59	
	D	8.33	83.00	9.96	
OMV3	A	12.33	55.00	4.46	5.09±0.6
	B	11.33	64.33	5.68	
	C	11.00	51.67	4.70	
	D	8.33	46.00	5.52	
OMV Free 1	A	15.00	70.67	4.71	3.92±0.6
	B	14.67	53.67	3.66	
	C	18.00	61.67	3.43	
	D	16.00	62.33	3.90	
OMV free2	A	12.00	30.00	2.50	3.32±0.8
	B	10.67	43.33	4.06	
	C	10.00	39.67	3.97	
	D	10.67	29.33	2.75	
OMV free 3	A	11.33	27.33	2.41	2.51±0.3
	B	10.67	29.67	2.78	
	C	12.67	27.67	2.18	
	D	12.33	32.67	2.65	
Bafilomycin 1	A	14.87	75.24	5.06	4.63±0.4
	B	15.23	70.24	4.61	
	C	16.78	69.23	4.13	
	D	15.28	72.17	4.72	
Bafilomycin 2	A	20.15	84.21	4.18	4.28±0.2
	B	20.87	86.14	4.13	
	C	23.67	100.78	4.26	
	D	21.59	98.27	4.55	
Bafilomycin 3	A	14.25	89.26	6.26	6.14±0.3
	B	17.54	84.66	4.83	
	C	12.36	89.75	7.26	
	D	13.77	85.32	6.20	

APPENDIX 3

LIST OF FIGURES

LIST OF TABLES

PUBLISHED WORK AND CONFERENCE PRESENTATIONS

Characterisation of epithelial IL-8 response to inflammatory bowel disease mucosal E. coli and its inhibition by mesalamine.
Subramanian S, Rhodes JM, Hart CA, Tam B, Roberts CL, Smith SL, Corkill JE, Winstanley C, Virji M and Campbell BJ.
Inflammatory Bowel diseases 2008 Feb;14(2):162-75

Golgi disorganisation as a consequence of bacteria-epithelial interaction
Benjamin A Tam, Campbell BJ, Lu Gang Yu, Jonathan M Rhodes
Poster presentation, Mucins conference Cambridge 2007

Characterisation of glycoforms of the stress-related Orp150 involved in NLS-dependent nuclear protein import
Benjamin A Tam, Lu Gang Yu, Jonathan M Rhodes
Poster presentation, Biochemical society conference, Glasgow 2007

PAPERS IN PROGRESS

Identification of glycosylated isoform of stress proteins Orp150 and Hsp90 in the cytoplasm: conformation of the role of Orp150 in NLS-dependent nuclear protein import
Benjamin A Tam, Deborah Ward, Lu Gang Yu, Jonathan M Rhodes

***O*-glycosylation changes in response to bacterial interaction with goblet-cell differentiated colon epithelial cells**
Benjamin A Tam, David Spiller, Barry Campbell, Lu Gang Yu, Jonathan M Rhodes

My candle burns at both ends;
It will not last the night;
But ah, my foes, and oh, my friends--
It gives a lovely light!

First Fig by Edna St. Vincent Millay