INTEGRIN α3β1:
CANCER-ASSOCIATED GLYCANS
AND COLON CANCER

A Thesis Submitted to the College of
Graduate Studies and Research
in Partial Fulfillment of the Requirements
for the Degree of Doctor of Philosophy
in the Department of Biochemistry
University of Saskatchewan

By

Nicole Lesley Prokopishyn

Fall 1997

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College of Graduate Studies and Research
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Submitted in partial fulfillment
of the requirements for the

DEGREE OF DOCTOR OF PHILOSOPHY
by
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University of Saskatchewan

Fall 1997

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INTEGRIN α3β1: CANCER-ASSOCIATED GLYCANS AND COLON CANCER

Cancer arises from the accumulation of cellular changes which ultimately give cancer cells the ability to spread throughout the body or metastasize. Cancer cells display alterations to their cell surface carbohydrates, such as increased synthesis of β1-6 branched Asn-linked glycans. Glycans have been implicated in cell adhesion and migration; hence changes to glycans may confer properties crucial to metastasis. Antibodies were generated which detect glycoproteins bearing β1-6 branched Asn-linked glycans which may be vital in colon cancer. MAb 3A7 detects an epitope containing blood group A (GalNAcα1-3) or B (Galα1-3) structures on type 2 chains (-Galβ1-4GlcNAc) which is expressed at high levels in colon tumors. As well, mAb 3A7 detects a glycoprotein species of 140 kDa (gp140) which is differentially expressed in human colon cancer cell lines. gp140 was isolated and used to produce monoclonal antibodies which detect the polypeptide portion of gp140. Analyses demonstrated that 3A7-immunoreactive gp140 corresponds to α3β1 integrin, a cell adhesion molecule. Integrin α3β1 is a major carrier of cancer-related glycans, including β1-6 branched Asn-linked glycans, type 2 chain repeats and the 3A7 epitope. The 3A7 epitope is located primarily on the β1-6 branch of Asn-linked oligosaccharides. Analysis of colon cancer cell lines revealed that expression of α3 integrin subunit, rather than glycosyltransferase levels, appears to regulate cell surface expression of the 3A7 epitope. Integrin α3β1 contributes to the adhesion and migration of colon cancer cells to extracellular matrix proteins. These data suggest that α3β1 integrin and perhaps its glycan moiety, including the 3A7 epitope, contribute to colon cancer spread.
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ABSTRACT

Colon cancer arises from the gradual accumulation of several genetic and biochemical changes in cells. Ultimately, these changes give cancer cells the ability to spread throughout the body or metastasize. Cancer cells display a variety of alterations to their cell surface carbohydrates. Cell surface glycoconjugates have been implicated in the adhesion, migration and invasion of cells, suggesting that changes to these structures may confer properties necessary for tumor cell metastasis. One such alteration is increased expression of β1-6 branched Asn-linked oligosaccharides on glycoproteins, which has been linked to the metastatic potential of cells. Hybridoma technology was used to generate monoclonal antibodies which detect glycoproteins bearing β1-6 branched Asn-linked oligosaccharides which may be important in colon cancer. MAb 3A7 was selected for further study because it detected an epitope expressed at high levels in rat and human colon tumors. In addition, expression of the epitope defined by mAb 3A7 was shown to be developmentally-regulated in rat intestine. Thus, mAb 3A7 detected an oncodevelopmentally-regulated determinant in colon. As well, mAb 3A7 detects a major glycoprotein species of 140 kDa (gp140) which is differentially expressed in human colon cancer cell lines. MAb 3A7 recognizes an epitope containing blood group A (GalNACα1-3Galβ-) or B (Galα1-3Galβ-R) structures exclusively on type 2 chains (Galβ1-4GlcNAc). 3A7-immunoreactive gp140 was isolated from the human colon cancer cell line, HT29, by lectin affinity and gel filtration chromatography. Partially purified gp140 was used to generate monoclonal antibodies which detect the polypeptide portion of gp140, namely mAbs 7A8, 7B11, 8C7 and 8H7. Immunological, molecular and biochemical analyses were used to demonstrate that the 3A7-immunoreactive gp140 corresponds to α3β1 integrin, a cell surface adhesion molecule which mediates cell-cell and cell-extracellular matrix interactions. Analysis of α3β1 integrin expression in human
colon carcinoma cell lines revealed that this glycoprotein is a major target for the addition of several cancer-associated carbohydrate structures, including β1-6 branched Asn-linked oligosaccharides, poly-N-acetyllactosamine (type 2 chain repeats) and the 3A7 epitope. Significantly, the 3A7 epitope appears to be located primarily on the β1-6 branch of Asn-linked oligosaccharides on α3β1 integrin. Analysis of a panel of blood group A, AB and B positive human colon carcinoma cell lines revealed that expression of α3 integrin subunit, rather than glycosyltransferase levels, appears to regulate cell surface expression of the 3A7 epitope in colon cancer cell lines. Finally, α3β1 integrin expressed by human colon cancer cells contributes to the adhesion and migration of cells toward extracellular matrix proteins. These data suggest that α3β1 integrin and perhaps its glycan moiety, including the 3A7 epitope, contribute to colon cancer progression.
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DEDICATION

To Mom, Dad, RLee and Reneé.
Not bad for someone who couldn't pass kindergarten.

To Kim for slurpees and waterfalls.

To Barb for waiving the nickel fee.
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<tr>
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<tr>
<td>AP</td>
<td>alkaline phosphatase</td>
</tr>
<tr>
<td>APC</td>
<td>adenomatous polyposis coli</td>
</tr>
<tr>
<td>Asn</td>
<td>asparagine</td>
</tr>
<tr>
<td>A transferase</td>
<td>α1-3 N-acetylgalactosaminyltransferase</td>
</tr>
<tr>
<td>β1-6 branch</td>
<td>[GlcNAcβ1-6Manα1-6Manβ]</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>BCIP</td>
<td>5-bromo-4-chloro-3-indolyl-phosphate-toluidine</td>
</tr>
<tr>
<td>B transferase</td>
<td>α1-3 galactosyltransferase</td>
</tr>
<tr>
<td>BzaGalNAc</td>
<td>benzyl-α-N-acetyl-D-galactosamine</td>
</tr>
<tr>
<td>BzaGlcNAc</td>
<td>benzyl-α-N-acetyl-D-glucosamine</td>
</tr>
<tr>
<td>C-I</td>
<td>collagen type I</td>
</tr>
<tr>
<td>C-IV</td>
<td>collagen type IV</td>
</tr>
<tr>
<td>CEA</td>
<td>carcinoembryonic antigen</td>
</tr>
<tr>
<td>CHAPS</td>
<td>3-[(3-Cholamidopropyl)-dimethylammonio]-1-propane-sulfonate</td>
</tr>
<tr>
<td>CHO</td>
<td>chinese hamster ovary</td>
</tr>
<tr>
<td>CMF-PBS</td>
<td>calcium and magnesium free-phosphate buffered saline</td>
</tr>
<tr>
<td>Core 1 transferase</td>
<td>β1-3 galactosyltransferase</td>
</tr>
<tr>
<td>Core 2 transferase</td>
<td>β1-6 N-acetylgalactosaminyltransferase</td>
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<td>DAB</td>
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<td>DBA</td>
<td>dolichos biflorus agglutinin</td>
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<td>DCC</td>
<td>deleted in colon carcinoma</td>
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<td>DMEM</td>
<td>Dulbecco’s minimum essential medium</td>
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<td>datura stramonium agglutinin</td>
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<tr>
<td>ECM</td>
<td>extracellular matrix</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>EGF</td>
<td>epithelial growth factor</td>
</tr>
<tr>
<td>EIA</td>
<td>enzyme-linked solid phase immunosorbent assay</td>
</tr>
<tr>
<td>ELAM-1</td>
<td>endothelial leukocyte adhesion molecule</td>
</tr>
<tr>
<td>FAP</td>
<td>familial adenomatous polyposis</td>
</tr>
<tr>
<td>Fb</td>
<td>fibrinogen</td>
</tr>
<tr>
<td>FBS</td>
<td>fetal bovine serum</td>
</tr>
<tr>
<td>FITC</td>
<td>fluorescein isothiocyanate</td>
</tr>
<tr>
<td>Fn</td>
<td>fibronectin</td>
</tr>
<tr>
<td>Fuc</td>
<td>L-fucose</td>
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<tr>
<td>α1-2Fuc T</td>
<td>α1-2 fucosyltransferase</td>
</tr>
<tr>
<td>GDP</td>
<td>guanidine diphosphate</td>
</tr>
<tr>
<td>Glc</td>
<td>glucose</td>
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<tr>
<td>GlcNAc</td>
<td>N-acetylgalactosamine</td>
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<tr>
<td>Gal</td>
<td>galactose</td>
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<tr>
<td>Gal T</td>
<td>galactosyltransferase</td>
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<tr>
<td>GalNAc</td>
<td>N-acetylgalactosamine</td>
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<tr>
<td>GnT</td>
<td>N-acetylgalactosaminyltransferase</td>
</tr>
<tr>
<td>G proteins</td>
<td>GTP-binding proteins</td>
</tr>
<tr>
<td>gp140</td>
<td>the 140 kDa glycoprotein detected by mAb 3A7</td>
</tr>
<tr>
<td>HAT</td>
<td>hypoxanthine, aminopterin, thymidine</td>
</tr>
<tr>
<td>Hb</td>
<td>hybridoma</td>
</tr>
<tr>
<td>HNPCC</td>
<td>hereditary non-polyposis colorectal cancer</td>
</tr>
</tbody>
</table>
HPA  helix pomatia agglutinin
HPV  human papillomaviruses
HT   hypoxanthine, thymidine
IF   immunofluorescence
Ig   immunoglobulin
IP   immunoprecipitation
lamp lysosomal membrane associated protein
LEA  lysopersium esculentum agglutinin (tomato lectin)
Le\(\text{a}\) Lewis A
Le\(\text{x}\) Lewis X
Ln-1 laminin-1
Ln-5 laminin-5
L-PHA leucophytohemagglutinin
mAb monoclonal antibody
Man mannose
MAP mitogen-activated protein
\(\alpha\)-mm methyl-\(\alpha\)-D manno pyranoside
MMP matrix metalloproteinase
MMT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide: thiazolyl blue
n.a. not available
Na\(\text{N}_3\) sodium azide
NBT p-nitroblue tetrazidium chloride
N-CAM neural cell-adhesion molecule
N-glycans asparagine-linked oligosaccharides
NGS normal goat serum
NMS normal mouse serum
O-glycan serine/threonine-linked oligosaccharides
PBS phosphate buffered saline
PDGF platelet-derived growth factor
PMSF phenylmethylsulfonylfluoride
P-P-Dol pyrophosphatidolichol
RER rough endoplasmic reticulum
RGD arginine-glycine-aspartate
RSV Rous sarcoma virus
SDS-PAGE sodium dodecyl sulfate-polyacrylamide gel electrophoresis
Se Secretor
Ser serine
SF media serum free media
Sialyl T sialyltransferase
SW480-E epithelial-type subpopulation of the SW480 cell line
SW480-R round-type subpopulation of the SW480 cell line
TBS Tris-buffered saline
TE trypsin-EDTA
TGF transforming growth factor
Thr threonine
TIMP tissue inhibitor of matrix metalloproteinases
TMN tumor, lymph node metastasis, distant metastasis classification
UDP uridine diphosphate
uPA urokinase plasminogen activator
VLA very late-acting antigen
WGA wheat germ agglutinin, tritium vulgaris
1.0 INTRODUCTION

Cancer affects each one of us -- whether it is a personal fight from within or a struggle to accept its destruction in others. Perhaps the most disconcerting aspect of the disease is that cancer spawns from a betrayal of our own bodies; a betrayal which manipulates the very mechanisms that sustain us. The 'cure for cancer' remains elusive due to the complexity of the disease, and will only come with an understanding of how and why this cellular mutiny occurs.

The research presented in this thesis is a valuable piece in the world-wide effort to solve the puzzle of cancer. This study focuses on a unique carbohydrate determinant present on cell surface glycoproteins which appears to be important in the progression and spread of colonic neoplasia. The literature survey presented is not meant to be an all-encompassing review of cancer and the changes that occur, but rather a survey of significant knowledge pertaining to the involvement of cell surface glycoconjugates in cellular interactions and cancer. As well, the information presented is intended to highlight concepts crucial to the objectives and studies described in this text.

2.0 LITERATURE SURVEY

2.1 Cancer.

Cancer is the leading cause of death in the developing world, resulting in nearly six million deaths worldwide each year (Rennie and Rusting, 1996). Cancer begins with the formation of a primary tumor (abnormal cellular mass) but most often continues with the spread of tumor cells throughout the body -- that is, metastasis. It is the metastatic spread of tumor cells that eludes current treatment and is therefore the most lethal aspect of cancer. Notably, surgical removal of localized tumors, followed by subsequent
radiation treatment and chemotherapy have significantly improved survival rates; still, the outlook for patients with distant metastatic spread is bleak (Murphy et al., 1995). Cancers can afflict most of the cell types within the body. The most common human cancers are carcinomas, which are cancers of the epithelium. Epithelial cells constitute the cell layers which line the body cavities. A unique set of properties and behaviors accompanies each type of cancer; however, the past decades of research have uncovered a number of general principles that govern the initiation, progression, and spread of most cancers.

2.1.1 Cancer initiation and progression.

Cancer begins with the transformation of a single cell, usually by mutation, which results in uncontrolled cell proliferation. Transformation is normally a result of mutations caused by chemical or physical agents or replication errors. However, alterations in gene expression can also induce cellular transformation (Copper, 1995). A number of environmental agents are suspected carcinogens. In addition to causing DNA damage, many of these carcinogens also promote inappropriate DNA synthesis and cell division and thus support transformation events (Hsu et al., 1987). Chemical carcinogens act as mutagenic agents through interaction with DNA. These chemicals, which include occupational and social carcinogens, such as asbestos, pesticides, and cigarette smoke are strongly linked to a variety of human cancers (Shubik, 1995; Trichopoulos et al., 1996). Chemical carcinogens frequently require activation by cellular metabolic pathways in organs such as the liver. For instance, the polycyclic compounds (e.g. Aflatoxin β1) require activation by the enzyme complex cytochrome P450-associated mixed function oxidases (MFO) before they become active carcinogens (Miller, 1994). This need for activation of certain carcinogens often results in induction of tumors in only select organs. Physical carcinogens also cause transformation by mutating DNA. Specifically, ultraviolet (UV) B rays cause more than 90 percent of skin cancers, including the most
fatal form, melanoma (Trichopoulos et al., 1996). As well, individuals afflicted with the genetic disorder xeroderma pigmentosum are unable to repair UV ray-induced thymine dimers which lead to DNA mutation and ultimately cancer (Cleaver, 1986). Environmental infectious agents, especially DNA viruses, appear to initiate tumor formation by influencing the expression of specific genes, and have been implicated in a variety of human cancers (Mueller, 1995). For example, hepatitis B virus infection is associated with liver cancer (Maynard, 1990), and human papillomaviruses (HPV) type 16 and 18 are involved in the onset of cervical cancer (Park et al., 1995). Interestingly, recent studies have suggested that Helicobacter pylori, a bacterium linked to stomach ulcer formation, may be involved in the onset of stomach cancer (Munoz, 1994). Long-term exposure of cells to chemical and physical carcinogens, as well as exposure to a number of promoting agents including hormones and chemical accelerants, promote transformation by increasing the rate of DNA synthesis and cell division, thereby ensuring passage of genetic mutations to future cell generations.

Transformation of cells, and ultimately cancer, occur when genes regulating cell growth and proliferation are aberrantly expressed. The proteins encoded by cellular protooncogenes and tumor suppressor genes meticulously regulate normal cell function to ensure balanced growth and division. In general, protooncogenes encode proteins which stimulate cell growth and division, whereas tumor suppressor genes encode proteins which inhibit cell proliferation (Cooper, 1995). Recent studies have suggested that these definitions are too restrictive, since many tumor suppressor genes appear to prevent cancer formation by maintaining appropriate cell-cell and cell-substratum interactions (Hesketh, 1995). Mutations in both protooncogenes and tumor suppressor genes and subsequent over-activation of oncogenes (the carcinogenic form of protooncogenes) or inactivation of tumor suppressor genes result in unbalanced growth, deregulated proliferation and inappropriate function of cells. The role of oncogenes and tumor suppressor genes has been well established in a variety of human cancers (Klein and
Klein, 1985). In the majority of human tumors, alterations to a number of these genes are required for malignancy.

The proteins encoded by protooncogenes and tumor suppressor genes include growth factors and their receptors, cytoplasmic signal transduction molecules and transcription factors (Cooper, 1995). For instance, hyper-activation of the ras oncogenes due to point mutation is commonly observed in human tumors (reviewed in Barbacid 1987). The ras family of genes encode GTP-binding proteins (G proteins) involved in growth factor stimulated signal transduction pathways (Hall, 1994). Mutant ras genes encode a protein that is continually active regardless of growth factor signalling, leading to unregulated cell division. Similarly, increased activity of c-src, a tyrosine kinase, is observed in a variety of human tumors including colon and breast carcinomas (Wang et al., 1991). Studies suggest that overactivation of c-src confers anchorage-independent growth properties on tumor cells by aberrant tyrosine phosphorylation of specific proteins involved in these processes. Moreover, the p53 gene encodes a nuclear phosphoprotein that regulates passage of cells through the cell cycle and induces abnormal cells into programmed cell death (i.e. apoptosis) (Levine et al., 1991). Mutant p53 protein is unable to stop abnormal cells from progressing through the cell cycle, nor is it able to induce cells into apoptosis. Therefore, tumor cells with mutant or inactive p53 continue to divide even though they are clearly abnormal (Knudson, 1993). Mutations to the p53 gene are the most common genetic alterations in human cancers (Levine et al., 1991).

Anomalous expression of growth control genes leads to rapid and uncontrolled proliferation and the formation of a benign tumor mass or primary tumor. In addition, this rapid and unchecked cell division is thought to increase genetic instability and generate the characteristic aneuploid nature of many tumor cells (Nowell, 1986). Tumor cells experience increased growth rates and metabolism, increased DNA and protein synthesis, and altered intracellular and extracellular properties. With increasing tumor size there is an increased demand for oxygen and nutrients (Folkman, 1986). This
demand is met by vascularization of the tumor by the process of angiogenesis (formation of new blood vessels). Tumor cells secrete angiogenic factors, such as vascular cell endothelial growth factor (VEGF) and angiogenin which stimulate the growth of blood vessels (Folkman and Klagsburn, 1987). The blood supply produced by new vessel formation not only supplies nutrients to the growing tumor, but also provides an entry route into the circulation for metastasizing cells. As tumor growth progresses, some of the cells within the tumor acquire the ability to emigrate out of the primary tumor, enter the circulation and spread to distant sites. Studies suggest that only a subpopulation of the cells within a tumor acquire all of the properties required for metastasis (Fidler and Hart, 1982); however, the exact size of this subpopulation is not known (Liotta et al., 1991; Kerbel, 1990).

2.1.2 Metastatic spread of tumor cells.

The process of metastasis is a multi-step process which requires dynamic changes to the adhesiveness, motility and invasive potential of tumor cells. The formation of metastases requires primary tumor growth, extensive vascularization, local invasion of cells out of the primary tumor and into the circulation, detachment of cells and embolization of tumor cells in the circulation, survival in the circulation, arrest in capillary beds of a secondary organ, extravasation or invasion into the new tissue and proliferation within that tissue to form a secondary tumor (Figure 2.1) (reviewed in Fidler, 1991).

Metastasis requires a breakdown in the mechanisms that maintain the integrity of tissues and keep cells within a specific tissue. Normal cells are kept in place by precise cell-cell and cell-extracellular matrix adhesions (Ruoslahti, 1996). Cells possess a unique set or pattern of receptors and ligands on their surface that through specific interactions guarantee that a cell remains within a tissue and functions appropriately. The presence of incorrect receptors, or the loss of receptors and/or ligands in normal cells result in cell detachment and death (Gumbiner and Yamada, 1995). Tumor cells, having overcome the
Figure 2.1: Steps in the Formation of Metastases. Tumor cells must be able to complete all these steps in order to form metastases at distant sites. Adapted from Fidler, 1991.
restraints of anchorage-dependent cell growth, move freely throughout the body. As well, tumor cells are capable of interacting with and adhering to a variety of different cell types, including endothelial cells and cells of the organs to which they spread. It appears that tumor cells execute the latter feat by altering the pattern of cell adhesion receptors and ligands on their surface (Nicolson, 1988; Yeatman and Nicolson, 1993).

In addition to dynamic cellular interactions, tumor cells must be able to break through the extracellular matrix (ECM) unit that encases epithelial tissues (Yurchenco and Schittny, 1990) (Figure 2.2A). Epithelial tissues are maintained on a continuous basement membrane and an underlying interstitial stroma. The basement membrane is composed of the basal lamina which is a dense matrix of adhesive ECM glycoproteins including collagen type IV, laminin, fibronectin, proteoglycans, entactin and perlecan (Mosher et al., 1992), and the reticular lamina which contains fibrillar collagens (Gumbiner, 1996). The interstitial stroma contains stromal cells, fibroblasts, myofibroblasts and loose connective tissue (Liotta et al., 1991). A cancer cell must be able to invade through or penetrate this ECM barrier and the subendothelial basement membrane in order to enter the circulation (Barsky et al., 1983). Tumor cell invasion of host tissues seems to require the combined effort of a number of mechanisms including mechanical pressure produced by the neoplasm, cell attachment to ECM components, degradation of the ECM and tumor cell motility (Fidler, 1991). Liotta and associates (1991) proposed that invasion of tumor cells through the basement membrane involves three basic steps: attachment, matrix dissolution, and migration (Figure 2.2B). Tumor cells bind to components of the basement membrane through specific cell surface receptors, both integrin and non-integrin. Tumor cells often display atypical levels and types of receptors (Section 2.3.2). Following attachment, tumor cells secrete (or induce host cells to secrete) proteolytic enzymes, including serine-, thiol- and metalloproteinases, which form a 'zone of proteolysis' at the invading edge of the tumor cells (Brown et al., 1990). Indeed, metastatic cells not only secrete irregular matrices, but also
Figure 2.2: Migration and Invasion of Cells. Cancer cells must be able to migrate out of the primary tumor and invade through a number of different extracellular matrices (ECM) in order to metastasize. A: Composition and structure of intestinal epithelial tissues including the underlying basement membrane (BM) and connective tissue. B: A cartoon depicting the migration/invasion of a cell involving the forward movement of the cell through a zone of proteolysis created by matrix proteinases.
elevated levels of proteolytic enzymes and decreased levels of proteolytic enzyme inhibitors (refer to Section 2.2.2). The tumor cell migrates or moves through the zone of proteolysis made in the basement membrane and stroma. This migration is achieved by the continuous making of attachments at the leading edge and breaking of attachments at the lagging edge of the cell (Lauffenburger and Horwitz, 1996) (Figure 2.2B). The invasive tumor cell enters the circulation by penetration of either a lymphatic vessel or a blood vessel.

Cancer cells within the circulation must be able to survive turbulent forces, evade the immune system and form cell-cell contacts at extravasation sites (Fidler, 1991). Studies indicate that cancer cells maximize their chances of survival in the blood by forming aggregates or tumor emboli by adhering to each other and to blood-borne host cells (Gasic, 1984). Tumor emboli are transported to the microcirculation where the metastatic cells interact with endothelial cells lining the blood vessels. This adhesive interaction seems to involve events akin to events witnessed in platelet aggregation during inflammation (Honn and Tang, 1992). Specifically, tumor cells often express the carbohydrate epitopes sialyl Lewis X (Le$^X$) and sialyl Lewis A (Le$^a$) which act as ligands for endothelial leukocyte adhesion molecules (ELAM-1) on endothelial cells (Phillips et al., 1990; Berg et al., 1991). Tumor cell attachment is followed by extravasation into the tissue by mechanisms similar to invasion, followed by proliferation of cells to form a secondary tumor (Fidler, 1991).

Metastasis is often an organ-specific process with cancers preferentially metastasizing to certain organs (reviewed in Rusiano and Burger, 1992). For instance, carcinomas originating in the colon traditionally spread to the liver (Beahrs, 1991). The patterns of metastasis are a consequence of a variety of host and metastatic cell factors including anatomical factors (Ewing, 1928; Arguello et al., 1991) and patterns of cell adhesion receptors and ligands (Nicolson, 1988; Pauli et al., 1990). Notably, expression of certain gangliosides has been implicated in organ-specific metastasis (Coulomber and
Furthermore, studies have suggested that the pattern of integrin receptors is indicative of metastatic spread to specific organs (Yeatman and Nicolson, 1993).

As seen from the above description of metastasis, interactions between cancer cells, the ECM, and host cells are critical in a number of the events in this process. It seems likely that genes encoding or regulating the expression of proteins involved in adhesion events would be critical to the metastatic phenotype. Many studies have implicated altered expression of a variety of adhesion molecules in metastasis (refer to Section 2.3). Other genes associated with metastasis are genes regulating the histocompatibility antigen, which may influence tumor cell immune resistance (Wallich et al., 1985), HER-2/neu oncogene in breast cancer carcinoma (Slamon et al., 1987) and nm23 tumor suppressor gene (Steeg et al., 1988). Identification of other metastasis associated genes and their mechanisms of action will be required to develop strategies to combat metastasis.

2.1.3 Colon Cancer

Colorectal cancer is the second leading cause of cancer-related death in the Western world, resulting in nearly 150,000 deaths annually in North America (Parker et al., 1996; Boring et al., 1991). Studies suggest that approximately 15% of colorectal cancers are inherited, however the cause of the remaining 85% of colorectal cancers is not clearly understood (Greenwald, 1992). Like all forms of cancer, colon cancer is initiated by genetic mutation, and progresses due to a variety of genetic and epigenetic changes until the tumor reaches a metastatic state. Often, surgical removal of primary tumors of the colon is accompanied by a poor prognosis because metastasis has occurred prior to tumor detection and treatment. In cases where the colon cancer has spread to distant sites, the five year survival rate for patients is a dismal 5-6%. These alarming statistics
highlight the need for a better understanding of colon cancer, especially the metastasis of colon cancer.

2.1.3.1 Colon Cancer Staging.

Colon cancer, like most cancers, is a multi-step disease beginning with the transformation of an epithelial cell, abnormal growth and cellular dysplasia. If left untreated, these tumors continue to proliferate and progress until they become invasive tumors, i.e. carcinomas. The stages of colon cancer progression have been well documented both clinically and pathologically. A number of classification or staging systems have been developed to facilitate the identification and prognosis of cancers (Figure 2.3). Cancers can be assessed using the tumor (T), lymph node metastasis (N) and distant metastasis (M) symbols established by the TNM Committee of the International Union Against Cancer (1989) which encompasses the extent to which a tumor has invaded into the tissue and the presence of metastatic lesions. The TMN classification for cancers of the colon and rectum has been modified to correspond with the more commonly used Astler-Coller modified Duke’s classification system (Astler and Coller, 1954) (Figure 2.3). As depicted in Figure 2.3, Duke’s B tumors correspond to tumors invading into or through the muscularis propria without regional lymph node or distant metastasis involvement and Duke’s C tumors correspond to tumors with regional lymph node involvement. Tumors with distant metastasis to the liver or other major organs are classified as Duke’s D (Beahrs, 1992). Samples, both human and rodent, used in the studies described within this thesis are classified according to the Astler-Coller modified Duke’s classification system. All human colon carcinoma cell lines have been converted to the Duke’s classification system.
Figure 2.3: Staging of colon cancer. Extent of invasion into the underlying tissue and involvement of metastases to the lymph node and distant organs (including liver and lung) are represented for tumors of differing histopathological grades. Astler-Coller modified Duke's Staging, Grading and TMN Classification are represented. Adapted from Cancer Principles and Practice of Oncology, 1993.

2.1.3.2 Susceptibility Factors.

The specific cause of colorectal cancer has not been established. Rather, a variety of environmental, dietary and genetic factors appear to contribute to the disease. There is very little definitive evidence linking environmental agents to colorectal cancer (Levin, 1992). Still, lifestyle and dietary factors appear to be very important in determining susceptibility to colorectal cancer.

A number of studies have demonstrated a positive correlation between sedentary lifestyle and colorectal cancer (Gerhardsson -de Verdict et al., 1990). Furthermore, dietary studies in humans and rodent colorectal cancer models suggest a protective effect of high fibre and low fat diets in the disease (Willett et al., 1990). Epidemiological studies of Asian immigrants to the United States provided the strongest evidence linking diet to colorectal cancer risk (Whittemore et al., 1990). Chinese immigrants have a higher...
risk of colon cancer with increased time spent in North America, likely due to increased exposure to diets rich in saturated fatty acids.

Dietary fibre appears to have a protective effect through reduction of the interaction of ingested carcinogens and/or promoters with the intestinal epithelial cells (Levin, 1992). Interestingly, specific dietary fats (e.g. omega-3 fatty acid), acetylsalicylic acid (Aspirin) and red wine are believed to protect against colorectal cancer. Although diet clearly plays an important role in colorectal cancer and the progression of the disease, genetic factors, both inherited and sporadic, appear to be key to the initiation and progression of the disease.

2.1.3.3 Genetic factors associated with colon cancer.

Certain families have a predisposition to colorectal cancer due to germ-line mutations in tumor suppressor genes associated with the initiation and progression of the disease (Knudson, 1995). Approximately 15% of colorectal cancers are inherited. The three inherited syndromes that predispose individuals to colon cancer are familial adenomatous polyposis (FAP) (Ahnen, 1990), hereditary non-polyposis colorectal cancer (HNPCC) (Ahnen, 1990) and Lynch's Syndrome (Knudson, 1995). FAP is a rare syndrome, characterized by the formation of thousands of adenomatous polyps in the colon. With this great number of polyps, the chances of progression to malignancy is increased and cancer develops around average age 43. HNPCC accounts for about 5-10% of the colorectal cancer cases and is characterized by the formation of a small number of polyps that once formed, readily become malignant. Patients inflicted with Lynch's syndrome have an increased incidence of a variety of cancers including colorectal and ovarian. The other 85% of colon cancer cases are termed sporadic cancers and although they are linked to specific genetic alterations, these genetic alterations are not a result of germ-line mutations, rather they are the result of spontaneous genetic events.
Studies of both inherited and sporadic tumors of various stages have linked a number of known tumor suppressor genes and oncogenes to colorectal cancer. In fact, genes associated with the inherited forms of colon cancer also appear to play a role in the formation of sporadic colon cancer. Studies by Vogelstein and associates have demonstrated that colon carcinomas have at least seven individual genetic mutations, and that perhaps even more alterations are required for invasion or metastasis (reviewed in Kinzler and Vogelstein, 1996; Fearon and Vogelstein, 1990). Figure 2.4 depicts the stages of colon cancer and known genetic mutations associated with progression to each stage. The increased presence of certain mutations at specific stages (e.g. increased ras mutations in adenomas as compared to polyps) suggests that not only accumulation of mutations, but also the order in which these mutations occur is important in neoplasia (Kinzler and Vogelstein, 1996).

Mutation(s) to the APC(adenomatous polyposis coli) gene locus resulting in the translation of a truncated dysfunctional protein, is believed to be the earliest event in colon cancer (Kinzler and Vogelstein, 1996). Patients suffering from the genetic disorder FAP carry a germ-line mutation in this gene and thus a predisposition to colon tumors; however, a second mutation is required for the onset of the disease. Greater than 85% of sporadic colon carcinomas possess mutations in the APC gene. Therefore, this gene appears to be important in both inherited and sporadic forms of the disease (Kinzler and Vogelstein, 1996). Recent studies suggest that the APC protein acts as a 'gate-keeper' or it inhibits the initiation of tumors by controlling colonic cell proliferation. Levels of APC protein are higher in cells migrating up through the colonic crypts, suggesting that it may be involved in differentiation and control of renewing cell populations within the colon (Miyashiro et al., 1995). Interestingly, a number of proteins have been shown to associate with the APC protein, including a homologue of the Drosophila tumor suppressor gene discs large (DLG) (Matsumine et al., 1996) and β-catenin (Rubinfeld et al., 1993; Su et al., 1993). The catenins are proteins which associate with the cadherin
Figure 2.4: Colon Cancer Progression and the Genetic Events Associated with this Process. The stages of colon cancer and genetic events believed to be involved in progression to each stage are depicted. Adapted from Kinzler and Vogelstein, 1996.
family of receptors and are believed to be involved in cell adhesion and signal transduction (Takeichi, 1993). Mutant APC proteins in colon tumors lack at least one of the two catenin binding domains, suggesting that interaction between β-catenin and APC is important in preventing colonic neoplasia (Korinek et al., 1997; Morin et al., 1997).

Other tumor suppressor genes that have been linked to colon cancer include DCC (deleted in colon carcinoma) and p53. These genes are lost or mutated in greater than 80% of colorectal carcinomas (Fearon and Vogelstein, 1990). Both genes appear to be involved in growth regulation. Specifically, the DCC gene product shares homology with the neural cell-adhesion molecule N-CAM and other cell surface adhesion molecules and is thought to be involved in self-recognition of colonic epithelial cells (Fearon et al., 1990). Inactivation of the DCC gene may aid in migration and invasion of colon cancer cells. Mutant forms of p53 are believed to give tumor cells a selective growth advantage by allowing abnormal cells passage through the cell cycle (Knudson, 1993).

As well, colorectal tumors from HNPCC patients and about 15% of sporadic colon tumors exhibit inactivation of the DNA mismatch repair genes, hMLH1, hMSH2 and MSH3 (reviewed in Kinzler and Vogelstein, 1996). With these genes inactive, cancer cells can allow mutations to go unrepaired and accumulate. Mutations to these genes are not associated with the onset of neoplasia but rather with the acceleration of its progression (Figure 2.4).

Oncogenes, especially ras, are also critical in colon carcinoma development. The K-ras gene is mutated or missing in over 50% of colon carcinomas. Since at least 50% of adenomas carry ras mutations, it is believed that this event occurs relatively early in the cancer process (Figure 2.4) (Vogelstein et al., 1988). Ras mutations lead to overstimulation of the mitogen-activated protein (MAP) kinase cascade and subsequent aberrant cell proliferation and growth (Hall, 1994).

In addition to these mutation events, it has been postulated that a number of other events are required for acquisition of a metastatic phenotype in colon cancer. There is no
definitive evidence linking the known metastatic suppressor genes to the metastatic spread of colon cancer cells but it appears likely that there are specific genes involved in this step that are yet to be discovered. As well, it appears that DNA methylation and other epigenetic events are potentially important in colon neoplasia (Fearon and Vogelstein, 1990).

2.2 Biochemical alterations in cancer cells.

Neoplastic and transformed cells display numerous biochemical irregularities in addition to and as a result of the genetic changes described above. Tumor cells exhibit altered protein synthesis, altered response to and synthesis of bio-active molecules and cytoskeletal changes. As well, tumor cells display numerous changes to their exterior including anomalous extracellular matrices, altered cell associated carbohydrate structures and abnormal expression and structure of molecules involved in cellular interactions (Nicolson, 1987). Since one of the main requirements for invasion and metastasis of tumor cells is altered cell-cell and cell-matrix interactions, it is not surprising that many of the changes to tumor cells are seen in cell surface properties that influence these interactions. The vast array of biochemical differences in cancer cells has been reviewed elsewhere (Nicolson, 1987). Hence, this section will be primarily confined to documenting irregularities to cell associated carbohydrates and cellular adhesion modulators in cancer cells.

2.2.1 Cytoskeletal changes.

Cancer cells frequently display aberrant cytoskeletal organization and interactions. The intracellular matrix or cytoskeleton consists of actin, tubulin and intermediate filament proteins which form a mesh involved in the maintenance of cell shape, spreading, division, signalling and compartmentalization (Segall and Gerisch, 1989). Interactions between the cytoplasmic domains of cell surface receptors and the intracellular matrix
components transmit signals from the external milieu of the cell to the interior. As well, these interactions are often essential for receptor function. For example, CD44-hyaluronic acid binding requires interaction between the CD44 receptor and the cytoskeletal protein, ankyrin (Bourguignon et al., 1992). Disorganization of the cytoskeleton and detachment of the cytoskeleton from the plasma membrane following neoplastic transformation, lead to altered cell morphology, inappropriate signalling and disrupted cell recognition and adhesion. In addition, it has been postulated that anomalous glycosylation in cancer cells is in part due to inappropriate compartmentalization and localization of proteins due to disorganized cytoskeleton (Dennis, 1992).

2.2.2 Synthesis and degradation of the extracellular matrix.

As described in Section 2.1.2 and depicted in Figure 2.2B metastasis requires not only interactions with ECM components, but also breakdown of that matrix. The attachment of cells to the ECM is critical to the maintenance of normal tissue integrity. In fact, each tissue resides on a basement membrane that is composed of a unique set of components (Gumbiner and Yamada, 1995). Therefore, any factors that influence the composition and structure of these matrices can influence cell function. It is not surprising that tumor cells secrete altered matrices and produce molecules to modify the ECM surrounding the cells.

Recently, Ohtaka et al. (1996), have demonstrated that rectal adenocarcinoma cell lines migrate and proliferate more effectively on laminin matrices as compared to collagen type I matrices. As well, many human cancer cells secrete matrices deficient in fibronectin (Huang and Chakrabarty, 1994). The absence of fibronectin within matrices appears to increase the motility and invasive potential of tumor cells. In contrast, the deposition of high levels of specific ECM components by certain cancer cells is thought to
accentuate the proliferation of these cells. For example, certain human colon carcinoma cell lines secrete high levels of laminin-5 (Pyke et al., 1995).

Invasive tumor cells move through the ECM by degrading limited areas of the matrix (Liotta and Stetler-Stevenson, 1991). Proteinases including aspartic, cysteine, serine and metallo-proteinases are capable of degrading ECM components and as expected, cancer cells display abnormalities in the activity of many of these proteinases. Antibodies that inhibit the activity of plasminogen activator (uPA), a serine proteinase, can block invasion of melanoma cells in vivo, suggesting that this proteinase is involved in metastasis (Mohanam et al., 1993). The matrix metalloproteinase (MMP) gene family, including interstitial collagenases, type IV collagenases (gelatinases), stromelysins, matrilysins and the newly characterized membrane-bound MMPs are associated with metastatic potential (Duffy, 1992). Specifically, colon carcinoma cells secrete higher levels of matrilysins (Yamamoto et al., 1994) and gelatinase A (Levy et al., 1991) compared to normal colonic mucosa. Moreover, invasive cells have impaired synthesis and secretion of endogenous proteinase inhibitors. Cells normally secrete proteinase inhibitors such as the TIMPs (tissue inhibitors of matrix metalloproteinases) which control the degree of ECM dissolution. The TIMPs (e.g. TIMP-1 and TIMP-2) form 1:1 complexes with active MMPs and inhibit the action of these enzymes. Indeed, serine proteinase inhibitors, TIMP-1 and TIMP-2 can block tumor cell invasion in vitro (Mignatti, et al., 1986; De Clerck et al., 1992). As well, up-regulation of TIMP-1 in melanoma cells can suppress their metastatic ability (Khokha et al., 1992).

Recently, studies have demonstrated that cell-ECM interactions can influence the expression and secretion of MMPs by tumor cells (reviewed in Heino, 1996). For example, α2β1 integrin-collagen I binding increases the synthesis of MMP-1 by sarcoma cell lines in three dimensional collagen gels (Riikonen et al., 1995) and vitronectin-αvβ3 integrin binding activates MMP-2 in melanoma cells (Seftor et al., 1992).
2.2.3 The synthesis of and response to growth factors.

Transformed cells in culture and tumor cells in vivo typically display a decreased requirement for growth factors due to 'autocrine' secretion of growth factors and activation of growth factor independent proliferation pathways (Kerbel, 1993). For instance, over-expression of transforming growth factor (TGF) α in benign colon tumor cell lines increases their tumorigenicity (Howell et al., 1993). In addition, cells from more advanced or aggressive tumors often have decreased sensitivity to growth inhibitors (Kerbel, 1993). For example, human colon carcinoma cells are less sensitive to TGFβ, an inhibitor of normal colon epithelial cell proliferation, than pre-malignant adenoma cells (Hague et al., 1993). As well, insulin-like growth factors appear to be involved in the transformation and metastasis of colon cancer cells (Singh and Rubin, 1993).

Recent studies have suggested that cell-ECM interactions are closely linked to the growth factor responsiveness of cells (Yamada and Miyamoto, 1995). Growth factor-mediated intracellular signaling and integrin-mediated signaling share many common factors, suggesting that these two processes can influence each other. Indeed, stimulation with platelet-derived growth factor can lead to clustering of integrin receptors and activation of ECM binding (Miyamoto et al., 1995). Thus, irregularities in either growth factor response or adhesion events could have profound effects on cancer cells.

2.2.4 Changes to cell surface carbohydrates.

Some of the most consistent biochemical changes displayed by cancer cells are altered expression and structure of cell surface carbohydrates. Aberrant lectin binding properties of transformed cells provided the first evidence linking irregular glycosylation to cancer (Smets and van Beek, 1984). Lectins are plant and animal proteins which recognize and bind specific carbohydrate structures (Goldstein and Hayes, 1978). Anomalies in oligosaccharide structure are not surprising, since it is now known that carbohydrates play critical roles in many of the cell-cell and cell-matrix interactions
necessary for cancer cell survival, invasion and spread. Furthermore, carbohydrate structure synthesis is under strict developmental and tissue-specific regulation (Kobata and Takasaki, 1992). Indeed, changes to cell surface carbohydrates in cancer cells often mimic embryonic or fetal patterns of carbohydrate expression (Feizi, 1985). Alterations to the carbohydrates and/or the macromolecules carrying these carbohydrates appear to have dramatic effects on cells and their properties.

2.2.4.1 The structure and function of cell surface oligosaccharides.

Oligosaccharides are synthesized by the action of glycosyltransferases which transfer monosaccharides from nucleotide and lipid sugar donors to glycan chains in specific linkages (Kleene and Berger, 1993). Carbohydrate residues are added to specific sites on polypeptide chains or lipid molecules to form different classes of glycoconjugates. Although the core structures of these glycan moieties are distinct, many of the extension and termination carbohydrate structures are common to many classes of glycoconjugates.

The glycoconjugates are essential to a number of biological functions including blood clotting, lubrication, structural support, immunological protection, hormone activation and recognition, storage of bio-active molecules, cell-cell and cell-matrix interactions and adhesion (reviewed in Varki, 1993). On the cell surface, oligosaccharides are ideal structures for recognition and adhesion events since they are large molecules with the potential for vast variation. In fact, both carbohydrate-protein and carbohydrate-carbohydrate interactions are central to cell-cell, cell-matrix and cell-pathogen recognition (Liotta, 1992). For instance, recognition of endothelial cells in leukocyte migration is the result of interaction between the selectin family of receptors (refer to Section 2.3.2.1) and sialylated-Le^x and -Le^a carbohydrate ligands (refer to Section 2.2.4.6.2.1) (Phillips et al., 1990). As well, stage-specific embryonic antigen
(SSEA) is involved in compaction of the embryo at the 16-cell stage through LeX-LeX interactions (Eggens et al., 1989).

As well, the presence of oligosaccharides on macromolecules can significantly influence the structure, function and activity of the molecule. For example, deglycosylated human β chorionic gonadotrophin hormone is able to bind its receptor but this binding does not transmit an intracellular signal (Chen et al., 1982). As well, the addition of oligosaccharide is a key factor in the biosynthesis and processing of many glycoproteins, especially cell surface molecules. Indeed, O-linked glycosylation is essential to Artic fish antifreeze glycoprotein function and glycosylation is necessary for surface expression of the Lutropin receptor (Davies and Hew, 1990).

Thus it appears that oligosaccharides are essential not only for cell adhesion and cell-cell recognition, but also for the appropriate functioning of many cell surface receptors. Irregular expression and/or structure of glycans in cancer cells could influence cellular function in numerous ways. A complete and detailed review of glycosylation and their functional significance in cancer cells is beyond the scope of this survey. However, a description of glycosylation of glycolipids and glycoproteins and highlights of some changes to glycan structure in cancer is necessary.

### 2.2.4.2 Alterations to glycosphingolipids.

Glycosphingolipids or glycolipids are formed by covalent linkage of a carbohydrate residue to ceramide and subsequent sequential addition of monosaccharides in specific linkages to the growing core structures (Figure 2.5). Glycolipids and their glycan moieties are pivotal not only to cellular recognition and adhesion events, but also to signal transduction processes (reviewed in Hakomori, 1990; Hakomori, 1996).

There are a variety of changes to cell surface glycolipids and their glycan moieties in cancerous cells, including incomplete synthesis of normal carbohydrate chains with subsequent precursor accumulation and synthesis of neoglycolipids due to the activation
Figure 2.5: Glycosphingolipid Structure. A: Typical glycolipid structure includes sugar residue linked to a hydrophobic ceramide component. B: Typical structures of some glycolipids.

of new glycosyltransferases (reviewed in Hakomori, 1985a). These different structures lead to the formation of tumor-associated carbohydrate markers; however, the majority of these alteration are not tumor specific, rather they appear selectively in normal situations during development or in specific tissues. A variety of human cancers, especially those derived from gastrointestinal, lung and mammary tissues, display aberrant accumulation of many fucosylated and sialylated glycolipid structures (Hakomori et al., 1984). The glycan moiety of these fucose-containing glycolipids, which include the ABH and Lewis blood group determinants, are related to structures present on complex carbohydrate chains conjugated to proteins and will be discussed in Section 2.2.4.6. Atypical glycolipid synthesis has also been linked to the metastatic potential of tumor cells (reviewed in Hakomori, 1996). For instance, non or weakly -metastatic mutants of MDAY-2 mouse tumor cells have a decreased total ganglioside content (Laferté et al., 1987). As well, highly metastatic rat mammary adenocarcinoma cells express a isoglobotetraosylceramide on their surface, whereas non-metastatic cell populations of these adenocarcinoma lack this glycolipid on their surface (Carlsen et al., 1990; 1993).
2.2.4.3 Variation to O-linked oligosaccharides.

Cancer cells also exhibit a vast array of unusual carbohydrate structures covalently linked to polypeptide chains. Carbohydrates are linked to polypeptides at both serine/threonine (Ser/Thr) and asparagine (Asn) residues to form O-linked and Asn-linked oligosaccharides, respectively. Alterations specific to O-linked and Asn-linked oligosaccharides, as well as modifications common to both glycan types are frequently exhibited by cancer cells.

2.2.4.3.1 Synthesis of O-linked oligosaccharides.

O-linked oligosaccharides or glycans include all glycans having an N-acetylgalactosamine (GalNAc) linkage to a serine or threonine of a polypeptide chain (reviewed in Schachter and Brockhausen, 1992). These glycans are a heterogeneous collection of carbohydrate structures that are typically present in high quantities on mucin or mucin-like glycoproteins (Section 2.3.1). Moreover, they appear on a variety of other glycoproteins, including those that possess Asn-linked oligosaccharides (Kim, 1992).

O-linked oligosaccharide synthesis is initiated in the cis-Golgi apparatus with the transfer of GalNAc from UDP-GalNAc to a Ser/Thr residue of a completed polypeptide chain by the enzyme UDP-GalNAc:polypeptide α-N-acetylgalactosaminyl transferase (polypeptide GalNAc transferase (EC 2.4.1.41)) (Figure 2.6A) (Wang et al., 1992). The close proximity of a number of proline residues to the Ser/Thr site is required for effective transfer by this enzyme (Briand et al., 1981). Following the addition of GalNAc to the Ser/Thr residue, a variety of other monosaccharides including galactose (Gal), sialic acid (SA), N-acetylgalactosamine (GlcNAc) and L-fucose (Fuc) are added to the growing oligosaccharide chain in specific linkages by individual glycosyltransferases.

O-linked glycans are extremely diverse in structure and size but can be classified into five subclasses or core structures (Figure 2.6B) (Schachter and Brockhausen, 1992). Core 1 and 2 O-linked glycans predominate in most tissues, although core 3 and 4
structures are relatively abundant on intestinal mucins (Dennis, 1993). Core 5 O-linked glycans have only been detected in rectal carcinomas (Kurosaka et al, 1983). The individual glycosyltransferases responsible for synthesis of the core structures are represented in Figure 2.6B. These glycosyltransferases are expressed in a tissue specific, developmentally-regulated manner (reviewed in Schachter and Brockhausen, 1992).

Further diversity of O-glycan structure is achieved through addition of carbohydrate residues to form backbone structures, including type 1 and type 2 chains, which are subsequently terminated at their non-reducing ends by specific antigenic
determinants, such as ABH and Lewis blood group antigens. These antigens are common to O-linked glycans, Asn-linked oligosaccharides and glycolipids and will thus be discussed in Section 2.2.4.5 and 2.2.4.6.

The precise function of O-glycans on mucins and other glycoproteins is not known. However, the presence of a large number of compact O-glycans on a polypeptide appears to protect and stabilize the polypeptide backbone. As well, the presence of a number of O-linked glycans produces unique patches of determinants that may be involved in specific interactions (Hart, 1992). Interestingly, removal of O-linked glycans from ZP3, the sperm receptor on mouse eggs, destroys its activity, suggesting that the O-glycans are essential for sperm binding and receptor function (Bliel and Wassarman, 1988).

2.2.4.3.2 Changes to O-linked oligosaccharides in cancer.

Incomplete or truncated O-linked oligosaccharides are commonly observed in malignancy. In general, mucins from cancer cells contain significantly less carbohydrate than their normal counterparts (Kim et al., 1974). This decreased carbohydrate content has been linked to decreased chain number and/or length which is often a result of the decreased activity of glycosyltransferases involved in O-linked glycan elongation (Bolerd and Deshmukh, 1990). In colon cancers, premature termination of O-glycans appears to result from decreased activity of core 2 β6-GlcNAc transferase and Iβ6 GlcNAc transferase (Yang et al., 1994) (refer to Figure 2.6B). This incomplete glycosylation uncovers structures on the cell surface that are normally hidden or masked. For example, increased presence of T and Tn antigens, Galβ1-3GalNAc-O and GalNAc-O, respectively, are commonly detected on human colon and breast carcinomas (Springer, 1984; Itzkowitz et al., 1989). Furthermore, an increased ratio of Tn/T antigen in breast carcinomas has been associated with less differentiated and more malignant tumors and decreased patient survival (Leathem and Brooks, 1987). Increased expression of the T
antigen in colon cancer patients can be explained in part by increased core 1 \( \beta3 \) galactosyltransferase activity and a concomitant decrease of core 3 \( \beta3 \)-GlcNAc transferase activity (Yang et al., 1994).

Certain tumor types undergo alterations in the class of core O-linked glycans synthesized. For example, there is an increase in core 2 O-linked glycans with the structure SA\( \alpha2 \)-3Gal\( \beta1 \)-3(SA\( \alpha2 \)-3Gal\( \beta1 \)-4GlcNAc\( \beta \)\-6)GalNAc- in leukemic cells compared to normal T lymphocytes, which express O-glycans of the structure SA\( \alpha2 \)-3Gal\( \beta1 \)-3(SA\( \alpha2 \)-6GalNAc-) (Saitoh et al., 1991). As well, increased activity of core 2 \( \beta6 \)-GlcNAc transferase in transformed rodent cells results in elevated levels of poly-N-acetyllactosaminyl structures (Gal\( \beta1 \)-4GlcNAc\( \beta \)\-6) on core 2 O-linked glycans (Yousefi et al, 1991; Fukuda et al, 1986).

2.2.4.4 Changes to asparagine-linked oligosaccharides in cancer.

Aberrant expression and structure of asparagine-linked oligosaccharides is frequently seen in malignant cells. The basic structure and biosynthesis of Asn-linked oligosaccharides is unique to this class of glycoconjugates. Still, the peripheral oligosaccharide structures added to the non-reducing termini of Asn-linked glycans are similar to those displayed on O-linked oligosaccharides and glycolipids.

2.2.4.4.1 Biosynthesis of Asn-linked oligosaccharides.

Asn-linked oligosaccharides, also called N-glycans, consist of three general types: high mannose-type, which contain mannose (Man) and GlcNAc residues, complex-type, which have a trimannnosyl core and substituted GlcNAc residues, and hybrid-type, which possess both high mannose-type and complex-type properties (Figure 2.7).

N-glycans, unlike O-glycans, are synthesized by transfer of a tetradecasaccharide from a lipid-linked precursor to an asparagine residue of a newly synthesized polypeptide chain (Figure 2.8). Sequential trimming and addition of monosaccharides ultimately
Figure 2.7: Asparagine-linked oligosaccharide structures. Typical High-Mannose, Hybrid- and Complex- Type asparagine (Asn)-linked structures are shown. N-acetylglucosamine (GlcNAc) (■), mannose (○), galactose (▲), N-acetyleneuraminic acid (sialic acid) (◇) and fucose (▼).

produces the complete N-glycan (Li et al., 1978; reviewed in Kornfeld and Kornfeld, 1985). The lipid-linked precusor, Glc3Man9GlcNAc2 pyrophosphoryldolichol (P-P-Dol), is synthesized in the rough endoplasmic reticulum (RER) by sequential addition of sugars to dolichyl-pyrophosphate by membrane-bound glycosyltransferases (reviewed in Kobata and Takasaki, 1992). Glucose and GlcNAc are transferred from nucleotide sugar donors and mannose is transferred from a dolichol donor. The oligosaccharide is transferred to an asparagine residue with the sequence Asn-X-Ser/Thr, where X can be any amino acid except proline, by the enzyme dolichyl-pyrophosphorylglucosyltransferase (Das and Heath, 1980). Efficient transfer of the precursor requires an accessible Asn-X-Ser/Thr sequence and full glucosylation of the precursor (Spiro et al., 1983).

Following transfer, the oligosaccharide is trimmed and processed, as depicted in Figure 2.8 (Kornfeld and Kornfeld, 1985; Kobata and Takasaki, 1992). Glucosidase I and II remove the α1-2 and α1-3 linked glucose residues, respectively, and α-mannosidase removes one or more mannose residues. Removal of these residues is required for transport of glycoproteins out of the RER and to the Golgi apparatus. In the cis Golgi, α-mannosidase I removes an α1-2 linked mannose, yielding high mannose-
Figure 2.8: Biosynthesis of Asn-linked oligosaccharides. Structures of carbohydrate intermediates and the glycosidas and glycosyltransferases involved in processing are depicted. Site of action of commonly used glycosylation inhibitors are shown with red arrows. Glucose (●), mannose (♦), N-acetylglucosamine (GlcNAc) (■), galactose (▲), fucose (▼) and N-acetyleneuraminic acid (sialic acid) (♦). Adapted from Kornfeld and Kornfeld, 1985.
type glycans (Figures 2.7). Transport of the glycoprotein to the medial Golgi is followed by addition of a GlcNAc residue by N-acetylglucosaminyltransferase (GnT) I, which produces a hybrid-type glycan (Figure 2.7). Removal of mannose by α-mannosidase II and subsequent addition of GlcNAc residues by GnT II, GnT IV and GnT V produce multi-antennary complex type N-glycans (Figure 2.9). Bisected complex-type structures are produced by the action of GnT III (Figure 2.9). In the trans Golgi, galactose, sialic acid and fucose are added to the oligosaccharide structure to produce mature complex type N-glycans (Figure 2.7). Moreover, within the Golgi apparatus these glycoproteins are often modified by the addition of O-linked oligosaccharides (Kornfeld and Kornfeld, 1985). The newly synthesized glycoprotein is transported from the Golgi apparatus to its destination via vesicles.

\[
\begin{align*}
\text{GnT VI} & \quad \text{GlcNAc\(\beta_1\)-4} \\
\text{GnT V} & \quad \text{GlcNAc\(\beta_1\)-5} \\
\text{GnT II} & \quad \text{GlcNAc\(\beta_1\)-2Man\(\alpha_1\)-6} \\
\text{GnT III} & \quad \text{GlcNAc\(\beta_1\)-4Man\(\beta_1\)-4-R} \\
\text{GnT I} & \quad \text{GlcNAc\(\beta_1\)-2Man\(\alpha_1\)-3} \\
\text{GnT IV} & \quad \text{GlcNAc\(\beta_1\)-4}
\end{align*}
\]

Figure 2.9: Branching of complex type Asn-linked oligosaccharides. Structure and linkages of GlcNAc branches are shown on the right and N-acetylglucosaminyltransferases (GnT) responsible for initiating synthesis of these branches are shown on the left (bold).

2.2.4.4.2 Regulation of glycosylation.

The presence of oligosaccharide structures is cell specific and developmentally regulated (Varki 1993). The synthesis of oligosaccharides is dependent on the activity and availability of glycosyltransferases within the cell. Moreover, glycosylation can be influenced by nucleotide sugar and acceptor availability, the type of cations present, subcellular organization and localization of the glycosyltransferases (Kobata and Takasaki, 1992), and competition between glycosyltransferases for a common substrate.
(Schachter and Brockhausen, 1992). Indeed, glycan synthesis is a meticulously regulated process.

In general, glycosyltransferases add a specific carbohydrate residue in a specific linkage to the oligosaccharide chain, i.e. these enzymes follow a 'one linkage-one glycosyltransferase rule' (Schachter, 1986). Thus, the presence of individual glycosyltransferases ultimately determines the synthesis of specific oligosaccharide structures. In addition, two or more glycosyltransferases often compete for the same acceptor (reviewed in Kornfeld and Kornfeld, 1985; Schachter and Brockhausen, 1992). For example, α2-6 sialyltransferase and α1-3 fucosyltransferase act on the same substrate, Galβ1-4GlcNAc. If α1-3 fucosyltransferase acts on this substrate first, to produce Galβ1-4(Fucα1-3)GlcNAc, then α2-6 sialyltransferase cannot transfer sialic acid to the galactose (Paulson et al., 1978). Similarly, α1-3 fucosyltransferase cannot transfer fucose to the structure SAα2-6Galβ1-4GlcNAc. Likewise, substitution of core 1 structures with sialic acid, GlcNAc or fucose arrests or slows down the synthesis of core 2 structures (Kuhns et al., 1993).

Furthermore, certain monosaccharides must be added in a specific order. For instance, the addition of GlcNAc in a β1-6 linkage to GalNAc, forming core 2 and core 4 O-linked glycans, can only occur after the addition of galactose or GlcNAc in a β1-3 linkage (the 'three-before-six rule'). These enzyme specificities are likely due to the three dimensional structures generated by the glycans (Schachter, 1986).

As well, addition of certain oligosaccharide structures appears to be dependent on the presence of defined substrates, be they peripheral sugar residues, branches, or proteins. For example, α2-6 sialyltransferase from rat liver specifically transfers sialic acid to Galβ1-4GlcNAc chains and not Galβ1-3GlcNAc chains (Wlasichuk et al., 1993). Therefore, the presence of sialylated structures in this cell type is dependent on the presence of specific oligosaccharide substrates. Likewise, many structures are preferentially added to specific branches of complex O-linked and Asn-linked...
oligosaccharides. The preferred substrate of rat liver β1-4 galactosyltransferase is the GlcNAc residue on the Manα1-3 branch of a GlcNAcβ1-2Manα1-6(GlcNAcβ1-2Manα1-3)Manβ1-4GlcNAcβ1-4(Fucα1-6)GlcNAc-Asn structure over the Manα1-6 branch (Paguet et al., 1984). In the case of GlcNAcβ1-6(GlcNAcβ1-2)Man structures, the preferred substrate of β1-4 galactosyltransferase is the GlcNAc on the β1-6 branch (Blanken et al., 1984). The presence and/or availability of these substrates influences the degree and rate of galactosylation of N-glycans. Similarly, the β1-6 branch of complex type oligosaccharides is the preferential branch for substitution with poly-N-acetyllactosamine structures (Section 2.2.4.5) (van den Eijnden et al., 1988).

Recently, studies have suggested that the synthesis of O-linked and Asn-linked oligosaccharides depends on the structure of the polypeptide substrate. In rat colon, the action of core 1 β3 galactosyltransferase and core 3 β3 GlcNAc transferase is influenced by the peptide moiety (Brockhausen et al., 1990). As well, it has been postulated that accessibility and structure of Asn-linked oligosaccharide chains influences GnT V enzyme action (Do et al., 1994).

2.2.4.4.3 Cancer associated changes to Asn-linked oligosaccharides.

The most common overall alteration to Asn-linked oligosaccharides in neoplasia is increased presence of larger N-glycans. For example, baby hamster kidney (BHK) cells transformed with Rous sarcoma virus (RSV) have a higher percentage of large molecular weight glycopeptides (Smets, 1984). Originally, Warren et al. (1972), proposed that the increased size of Asn-linked oligosaccharides was a result of increased sialylation due to enhanced sialyltransferase activity. However, studies have demonstrated that increased branching and addition of poly-N-acetyllactosamine structures (Galβ1-4GlcNAc)n are major contributors to the increased molecular weight of N-glycans in transformed cells (reviewed in Dennis, 1992; Yamamoto et al., 1984; Yamashita et al., 1984). Interestingly, in addition to larger, highly branched N-glycans, malignant cells often
display incomplete or truncated N-glycan structures. For example, RSV-transformed chick embryo fibroblasts produce glycoproteins enriched in highly branched complex-type oligosaccharides and truncated high mannose-type oligosaccharides (Hunt and Wright, 1985). Studies speculate that the presence of these diametrically opposite patterns of glycosylation in malignant cells is a consequence of inappropriate compartmentalization of glycosyltransferases and sugar nucleotides, as well as aberrant sorting and localization of glycoproteins due to cytoskeletal disruptions (Kelly, 1990). Malignancy is often associated with irregular microtubular organization and disrupted cellular organization.

Modifications to the branching of Asn-linked oligosaccharides is typically observed in transformed and tumor cells. Indeed, the expression of most of the branching enzymes, including GnT III, IV, V and VI is tissue-specific and developmentally regulated (refer to Figure 2.9 for structures synthesized by these enzymes) (Schachter, 1986). Notably, small intestine possesses high levels of GnT V activity, whereas the large intestine has significantly lower levels (Perng et al., 1994). Re-expression or increased expression of these enzymes is often observed in cancer cells and results in the biosynthesis of atypical N-glycan structures. For example, hepatoma cells display neoexpression of GnT III which catalyzes the biosynthesis of bisected N-glycans. Bisected Asn-linked oligosaccharides are normally only expressed in hemopoietic and kidney cells (Nurasimhan et al., 1988; Yamashita et al., 1983). Similarly, patients with hepatocarcinomas typically contain transferrin in their serum that have increased tri-, tetra- and penta-antennary N-glycans due to expression of GnT IV and GnT VI (Yamashita et al., 1983). The most notable modification in neoplastic and metastatic cells is the increased synthesis of the β1-6 branched Asn-linked oligosaccharides due to increased activity of GnT V (Dennis et al., 1987).
2.2.4.4.4 Expression of β1-6 branched Asn-linked oligosaccharides and malignancy.

Many studies have demonstrated that the increased size of N-glycans in transformed cells is a result of extensive [GlcNAcβ1-6Manα1-6Manβ] (β1-6) branching and subsequent extension of this branch (reviewed in Dennis, 1992) (Figure 2.9). The presence of the β1-6 branch in Asn-linked oligosaccharides can be detected with the lectin leucophytohemagglutinin (L-PHA). Increased β1-6 branching has been detected in a variety of transformed rodent tumor cells (Takashai et al., 1980; Pierce and Arango, 1986; Collard et al., 1985) and human carcinomas including breast (Dennis and Laferté, 1989) and colon (Fernandes et al., 1991). Moreover, increased β1-6 branching has been directly correlated with invasion and metastasis in a variety of murine and human cancer models (Dennis et al., 1987; Debray et al., 1986; Lu et al., 1994). Indeed, overexpression of GnT V, the enzyme which initiates synthesis of the β1-6 branch, leads to increased invasion in vitro and enhanced metastasis in vivo of a variety of tumor cell types (Yagel et al., 1989; Demetriou et al., 1995).

Studies of glycosylation mutants selected from metastatic tumor cells lines have provided evidence linking β1-6 branched Asn-linked oligosaccharides to metastasis (reviewed in Dennis et al., 1989). Specifically, mutants were selected from the highly metastatic murine lymphoma cell line, MDAY-D2, based on their resistance to specific lectins. Plant lectins are capable of cross-linking to specific oligosaccharide structures on the cell surface and in some cases, induce cell death (Stanley, 1983). Mutants that are resistant to these lectins do not possess the structures to which these lectins bind, due to specific defects in glycosylation (Stanley, 1983). MDAY-D2 mutants resistant to L-PHA-mediated killing, due to reduced levels of the β1-6 branch, were no longer metastatic (Dennis et al., 1987). These mutants have diminished GnT V activity and thus cannot synthesize the β1-6 branch of Asn-linked oligosaccharides.
As well, treatment of cells with swainsonine, which inhibits processing of Asn-linked oligosaccharides prior to initiation of the β1-6 branch and diverts synthesis towards hybrid-type structures (Tulsiani et al., 1982), inhibits experimental and spontaneous metastases (Humphries et al., 1986; Dennis, 1986). B16F10 melanoma cells grown in the presence of swainsonine have reduced organ colonization potential when injected into mice (Dennis, 1986). As well, the ability of ras transformed NIH 3T3 cells to form lung tumors after intravenous injection into nude mice is significantly reduced if the cells are L-PHA resistant (Lu et al., 1994). These findings attest that the β1-6 branch plays a critical role in the invasive and metastatic potential of tumor cells. As well, these data imply that inhibition of the synthesis of this branch may significantly reduce metastasis in vivo. Indeed, the usefulness of swainsonine treatment is currently being addressed in clinical trials (Dennis, 1992).

Notably, swainsonine treatment of tumor cells increases the adhesion of many cells to basement membranes while inhibiting their invasion through membranes in vitro (Yagel et al., 1989). As well, CHO and MDAY-D2 glycosylation mutants that are defective in GnT V, called Lec4 and KBL-1, respectively, have reduced metastatic potential and show increased TIMP transcript levels (Korczak and Dennis, 1993). These data suggest that tumor cells with decreased β1-6 branching may be less metastatic due to increased expression of inhibitors of ECM degradation. Furthermore, the presence of the β1-6 branch on specific glycoproteins (refer to Section 2.3.3) may influence the function and activity of these glycoproteins.

As well, studies have demonstrated that the β1-6 branch is the preferential site for addition of many cancer-related carbohydrate structures, including poly-N-acetyllactosamine structures (refer to Section 2.2.4.5). Clearly, the β1-6 branch plays a key role in tumor invasion and metastasis, however it is not clear whether it is the branch itself or modifications to this branch which are responsible for these effects.
2.2.4.5 Type 1 and type 2 chains.

There are two main core oligosaccharide structures found within mature O-linked and Asn-linked oligosaccharides. These include type 1 chains (Galβ1-3GlcNAcβ1-3-) and type 2 chains (Galβ1-4GlcNAcβ1-3-) which can be extended and further modified with the I (branched) and i (linear) blood group antigens (Figure 2.10). Repeating type 2 chains are termed poly-N-acetyllactosamine structures. These chains can be further modified by Lewis and ABH blood group determinants, as described below. Synthesis of type 1 and type 2 chains requires the sequential activities of either β1-3 or β1-4 galactosyltransferase and β1-3 N-acetylglucosaminyltransferase (β1-3 GnT) (Holmes, 1988; Piller and Cartron, 1983). β1-3 GnT (i) is an oncodevelopmentally regulated enzyme that controls the synthesis of type 2 chains and this enzyme is likely distinct from the β1-3 GnT responsible for synthesis of type 1 chains (Holmes, 1988). Holmes et al. (1987) have demonstrated that β1-3 GnT (i) activity is elevated in colon carcinomas and results in increased synthesis of poly-N-acetyllactosamine. Furthermore, β1-3 GnT (i) preferentially substitutes the GlcNAc on the [GlcNAcβ1-6Manα1-6Manβ] branch of Asn-linked oligosaccharides providing evidence that poly-N-acetyllactosamine is preferentially added to the β1-6 branch of these glycans (van den Eijnden et al., 1988). In fact, increased poly-N-acetyllactosamine content is directly linked to increased β1-6 branching of Asn-linked oligosaccharides in thyroid carcinoma cells (Yamamato et al., 1984). As well, increased synthesis of poly-N-acetyllactosamine on the β1-6 branch of Asn-linked oligosaccharides is responsible for a significant amount of the increased molecular weight of Asn-linked glycans in metastatic cell lines (Yousefi et al., 1991; Fukuda, 1992). Thus, the synthesis of the β1-6 branch is implicated in controlling type 2 chain or poly-N-acetyllactosamine synthesis in cells. Interestingly, addition of the β1-6 branch and subsequent synthesis of poly-N-acetyllactosamine occurs on a select number of glycoproteins in human cancer cell lines (refer to Section 2.3.3).
**type 1**
Galβ1-3GlcNAcβ-R

**type 2**
Galβ1-4GlcNAcβ-R

**poly-N-acetyllactosamine (type 2 repeats):**

- Linear:
  \[ \text{Galβ1-} (4\text{GlcNAcβ1-3Galβ1-})_n4\text{GlcNAc-} \]
- Branched:
  \[ \begin{array}{c}
  \text{I} \\
  \text{Galβ1-4GlcNAcβ1} \\
  \text{Galβ1-} (4\text{GlcNAcβ1-3Galβ1-})_n4\text{GlcNAc-}
  \end{array} \]

**Figure 2.10:** Backbone structures common to O-linked and Asn-linked oligosaccharides. Both type 1 and type 2 chains modify oligosaccharides. Type 2 chain repeats are the basis of the blood group i (linear) and I (branched) structures.

### 2.2.4.6 Alterations in terminal glycosylation.

In addition to alterations in the branching and core structure of Asn-linked and O-linked oligosaccharides, tumor cells display a variety of alterations to the non-reducing ends of oligosaccharides. The sugar residues and antigenic determinants present at the terminal ends of oligosaccharides are often common to many classes of glycoconjugates. Changes in the level and patterns of sialic acid and blood group determinants are often associated with neoplasia (reviewed in Hakomori, 1989; Dennis, 1992). Although these structures normally occur on a variety of glycoconjugates, the changes in tumor cells are often restricted to a specific macromolecule or class of molecules, suggesting that these alterations are not mere random events but rather highly controlled processes.

#### 2.2.4.6.1 Sialylation

Increased sialylation has been shown to contribute to the metastatic phenotype of tumor cells. For instance, the extent of sialylation of glycoconjugates correlates with the lung colonization potential of murine tumor cell lines (Yogeeswaran and Salk, 1981). Wheat-germ agglutinin (WGA) resistant mutants of lymphoma, melanoma and leukemia cell lines, which have decreased levels of sialic acid, display reduced metastatic potential.
in experimental and spontaneous metastasis models (Dennis, 1986; Tao and Burger, 1977; Benedetto et al., 1989). Furthermore, revertants of the lymphoma and melanoma WGA resistant mutants not only display wild-type sialylation patterns, but also increased metastatic potential (Ishikawa et al., 1988; Dennis and Laferté, 1986; Finne et al., 1989). The form of sialic acid does not appear to influence the metastatic potential of tumor cells, since mutants with N-glycolylneuraminic acid do not differ in their tumorigenic or metastatic potential from wild-type cells which synthesize N-acetylneuraminic acid (Dennis, 1986).

As well, the level and linkage of sialic acid on cell surface glycoconjugates appear to influence tumor cell invasion and metastasis, possibly by decreasing cellular adhesion to the ECM (Dennis et al., 1989). WGA-resistant MDAY-D2 lymphoma cells demonstrate enhanced adhesion to collagen, laminin and fibronectin, whereas revertants adhere poorly to the ECM (Dennis, 1985). Furthermore, the level of α2-3-linked sialylation of Asn-linked oligosaccharides is greater in metastatic cells than in nonmetastatic subtypes of B16 melanoma cells (Passaniti and Hart, 1988). As well, β1-6 branching of Asn-linked oligosaccharide can inhibit the action of certain α2-6 linked sialyltransferases, ultimately leading to increased α2-3 sialylation. In fact, many of the tri- and tetra-antennary oligosaccharides in malignant cells are terminated by α2-3 linked sialic acid (Santer et al., 1989). Thus it appears that sialylation in general and perhaps sialylation of specific structures is crucial to metastasis, yet the precise effects of these changes are not fully understood.

2.2.4.6.2 Blood group antigens in cancer.

Cancer cells, especially those of epithelial origin, commonly exhibit changes in expression and structure of blood group antigens (Bloom et al., 1990). These alterations include the expression of antigens that should not be expressed, the deletion of antigens
that should be expressed and the synthesis of novel antigens related to the blood group determinants.

### 2.2.4.6.2.1 Lewis antigens.

The Lewis (Le) antigens are fucosylated derivatives of either type 1 or type 2 chains (Figure 2.11). The Le\(^a\) and Le\(^b\) antigens are derived from type 1 chains, whereas Le\(^x\) and Le\(^y\) structures are modifications of type 2 chains (Figure 2.11). Le\(^x\) is often referred to as the SSEA-1 antigen since it is recognized by mAb SSEA-1 (Solter and Knowles, 1978). The Le\(^a\) and Le\(^x\) antigens can be sialylated to form sialyl-Le\(^a\), also called CA 19-9, and sialyl-Le\(^x\), respectively.

<table>
<thead>
<tr>
<th>Type 1</th>
<th>Type 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>H: Galβ1-3GlcNAc-R</td>
<td>H: Galβ1-4GlcNAc-R</td>
</tr>
<tr>
<td>Fuc</td>
<td>Fuc</td>
</tr>
<tr>
<td>A: GalNAcα1-3Galβ1-3GlcNAc-R</td>
<td>A: GalNAcα1-3Galβ1-4GlcNAc-R</td>
</tr>
<tr>
<td>Fuc</td>
<td>Fuc</td>
</tr>
<tr>
<td>B: Galα1-3Galβ1-3GlcNAc-R</td>
<td>B: Galα1-3Galβ1-4GlcNAc-R</td>
</tr>
<tr>
<td>Fuc</td>
<td>Fuc</td>
</tr>
<tr>
<td>Le(^a): Galβ1-3GlcNAc-R</td>
<td>Le(^x): Galβ1-4GlcNAc-R</td>
</tr>
<tr>
<td>α1-4</td>
<td>α1-3</td>
</tr>
<tr>
<td>Fuc</td>
<td>Fuc</td>
</tr>
<tr>
<td>Le(^b): Galβ1-3GlcNAc-R</td>
<td>Le(^y): Galβ1-4GlcNAc-R</td>
</tr>
<tr>
<td>α1-2</td>
<td>α1-3</td>
</tr>
<tr>
<td>Fuc</td>
<td>Fuc</td>
</tr>
<tr>
<td>Sialyl Galβ1-4GlcNAc-R</td>
<td>NeuNAc</td>
</tr>
<tr>
<td>α2-3</td>
<td>Fuc</td>
</tr>
</tbody>
</table>

Figure 2.11: Blood group antigens on type 1 and type 2 chains. Blood group antigens A, B, H and Lewis (Le) structures are depicted.

There is neoexpression or overexpression of type 2 Lewis antigens, including Le\(^x\), Le\(^y\) and sialyl-Le\(^x\), in many human carcinomas including breast, bladder (Cordon-Cardo et al., 1988), lung (Zenita et al., 1988) and colon (Singhal et al., 1990). Specifically, increased levels of both Le\(^x\) and Le\(^y\)-related structures are associated with colon carcinomas (Hakomori, 1984). Since Le\(^x\) and Le\(^y\) antigens are expressed in low
levels in the normal proximal colon and are completely absent in the distal colon (Bloom et al., 1990), the increased expression and/or reexpression of these determinants is cancer-associated. Still, the degree of expression does not appear to relate to tumor stage or degree of differentiation (Itzkowitz et al., 1986b). Furthermore, sialylation and polyfucosylation of Lewis antigens is commonly observed in colon cancers, with sialyl-Le$^X$ being detected in the serum of colon cancer patients. The structure sialyl-difucosylated-Le$^X$ is relatively cancer specific (Itzkowitz et al., 1986b) and first appears during early stages of cancer progression (Yuan et al., 1987). Interestingly, studies by Singhal et al. (1990) have suggested that Le$^X$ and Le$^Y$-related antigens found in the serum of colon cancer patients are present only on glycoproteins and not glycolipids.

Type 1 chain based Lewis antigens also display incompatible expression, reexpression or loss of expression in colon carcinomas (reviewed in Bloom et al., 1990). For example, Le$^a$, which is expressed throughout the colon is often absent in colon tumors and Le$^b$ antigen is often missing in tumors from the proximal colon (Ernst et al., 1984). Sialyl-Le$^a$ is newly synthesized in a variety of cancers and is detected in the serum of patients with colorectal cancers (Atkinson et al., 1982).

The functional significance of aberrant Lewis antigen expression in cancers may be linked to the adhesive properties of these determinants. Sialylated Le$^X$ and Le$^a$ are ligands for cell adhesion lectins, termed selectins, found on endothelial cells and platelets (refer to Section 2.3.2). It is postulated that the increased presence of these sialylated structures on cancer cells allows for interaction between the cancer cells and host cells during spread of cancer cells through the circulation. Indeed, sialyl-Le$^X$ is expressed at high levels on mucins isolated from liver metastases (Hoff et al., 1989), suggesting a role for this determinant in spread of cancer cells to the liver.
2.2.4.6.2.2 Synthesis and expression of ABH blood group antigens in normal tissues.

The ABH blood group determinants are the carbohydrate epitopes that confer ABO blood group status in humans. Patients of blood group A express A determinants on their erythrocytes, individuals of blood type B express B determinants on their erythrocytes and individuals of O blood type express neither A nor B determinants on their erythrocytes. Epithelial and endothelial tissues also express ABH determinants and these are compatible with blood type status (Marcus, 1969). ABH antigens reside on type 2 chains on erythrocytes however, they are mainly present on type 1 chains in epithelial tissue, including the gastrointestinal tissues.

The H antigen, the common precursor of A and B determinants, is formed by the addition of a fucose residue in an α1-2 linkage to either type 1 or type 2 chains (Figure 2.12). Synthesis of the H antigen on type 1 and type 2 backbones is carried out by two fucosyltransferases of differing specificities. The H gene encodes an α1-2 fucosyltransferase (FucT) that is specific for type 2 chains and the secretor (Se) gene encodes a transferase that fucosylates type 1 chains (Piau et al., 1994). The H FucT is typically present in erythrocytes and can be activated in neoplastic tissues, whereas the Se FucT is present in normal epithelial tissues, including the colon.

A and B determinants are synthesized by adding an N-acetylgalactosamine (GalNAc) or galactose (Gal), respectively, in an α1-3 linkage to the H determinant (Figure 2.13). These reactions are catalyzed by α1-3 N-acetylgalactosaminyltransferase (A transferase) and α1-3 galactosyltransferase (B transferase), which are highly specific enzymes that recognize only fucosylated type 1 and 2 chains (Yamamoto et al., 1990). Interestingly, cDNAs encoded A and B transferases are nearly identical, except for 7 nucleotide positions. This slight difference results in a four amino acid difference between the two enzymes which confers specificity for either UDP-GalNAc or UDP-Gal (Yamamoto et al., 1990). Individuals of blood group O (H) possess a gene containing a
Figure 2.12: Biosynthesis of ABH blood group determinant. The oligosaccharide structures and enzymes responsible for synthesis of the ABH blood group antigens on type 1 and 2 extension chains are shown. Please note that the \( \alpha_1-2 \) fucosyltransferases responsible for synthesis of the H antigen on type 1 and type 2 chains are different where as the A and B Transferase act on both type 1 and type 2 H structures.

A frame shift mutation which encodes a non-functional protein and thus neither A nor B determinants are synthesized.

Normal adult colon exhibits a proximal to distal gradient of ABH expression in which the distal colon is completely devoid of ABH determinants (Figure 2.13) (Szulman, 1962). However in fetal colonic mucosa the ABH determinants are expressed throughout the length of the large intestine (Figure 2.13). Thus, adult development is typified by a loss of ABH expression in the distal portion the colon (Szulman, 1964; Yuan et al., 1985).

2.2.4.6.2.3 Alterations to ABH antigens in cancer.

Alterations to ABH antigens have been detected in pancreatic (Itzkowitz et al., 1987), lung (Matsumoto et al., 1993), ovarian (Metoki et al., 1989), stomach and colon carcinomas (Denk et al., 1974). Like the Lewis antigens, ABH antigens display four general types of alterations in cancers, including re-expression or
increased expression, loss of expression, incompatible expression and neoexpression of ABH and AB-like structures. For example, expression of A determinants is detected in pancreatic cancers from Syrian Hamsters (Egami et al., 1990). Since the A antigen is normally only expressed in fetal or developing pancreas and absent in adult pancreas, these data suggest that the A antigen is a pancreatic tumor marker (Egami et al., 1990). As well, the A determinant is expressed on multi-antennary Asn-linked complex glycans in this model (Hirota et al., 1992). In contrast, loss of ABH antigens appears to be associated with metastatic potential in lung carcinomas (Matsumoto et al., 1993).

Some of the most well documented ABH alterations are seen in colorectal cancers. The majority of colon cancers display increased or re-expression of ABH antigens (Itzkowitz et al., 1986a). Specifically, cancers of the distal colon, which is normally devoid of ABH antigens, express high levels of these determinants (Cooper and Haesler, 1978). This alteration appears to be a relatively early event in cancer progression since adenomatous polyps frequently display ABH antigens (Bloom et al., 1990). As well, data by Itzkowitz and associates (1986a) suggested that the expression of ABH antigens is related to the tumor stage, with more advanced tumors showing increased expression. Interestingly, neoplasia is often associated with an increase in synthesis of A and B
determinants on both type 1 chains and type 2 chains (Yuan et al., 1985). Since the ABH determinants normally reside on type 1 chains in epithelial tissues, the presence of ABH determinants on type 2 chains appears to be a cancer-associated marker. Furthermore, colon tumors occasionally display incompatible expression of ABH determinants (Cooper et al., 1980; Itzkowitz et al., 1986a). However, it is possible that these A and B structures are not true A and B antigens but A or B-like structures. For instance, 'A-like' determinants expressed in blood group O patients have been identified as both Forssman glycolipid antigen and Tn antigens (Hakomori et al., 1977; Hirohashi et al., 1985). These A or B-like structures may be immunologically related to, but differ in structure and synthesis from true A or B antigens. Deletion of A and B determinants in cancers of the proximal colon has also been documented (Denk et al., 1974 and Kim et al., 1974). These deletions often result in increased precursor levels, i.e. H antigen, due to loss of A or B transferase activity (Yuan et al., 1985).

2.2.4.6.2.4 Functional significance of altered ABH antigen expression.

The role of ABH antigens in normal and neoplastic states remains largely unknown. Unlike the Lewis structures, i.e. sialyl Le\(^x\) and Le\(^a\), which have been implicated as ligands for the selectins, evidence linking ABH antigen involvement in cell adhesion is lacking. Recent reports suggest that addition of A structures to sialyl-Le\(^b\) decreases selectin recognition and cell adhesion (Larkin et al., 1992). However, it is unclear whether this is the biological role for the ABH antigens. As well, the A antigen has been detected on glycoprotein receptors such as the epithelial growth factor (EGF) receptor (Childs et al., 1984; Feizi, 1985). Yet again, the functional significance of its presence is not clear. Feizi and Childs (1985) suggested that the A antigen may be a receptor for regulators of cell proliferation, but there is no definitive evidence to substantiate this claim. Still, the ABH antigens are developmentally regulated.
carbohydrate epitopes in tissues such as the pancreas and colon, suggesting that there is a role for these structures in tissue development and function.

Analysis of ABH antigen expression in lung carcinomas suggest that a loss of the ABH structures is associated with metastatic potential. Patients who have lung cancers that remain ABH positive have a better prognosis and survival rate (Matsumoto et al., 1993; Lee et al., 1991). Likewise, loss of A determinants in bladder cancer is related to poorer prognosis (Ørntoft et al., 1996). In contrast, in patients with gastric carcinomas, increased helix pomatia staining (a plant lectin which recognizes terminal GalNAc residues (Prokop et al., 1965)), is indicative of lower survival rate (Kakeji, 1991). Interestingly, persons of blood group A status typically have an increased frequency of gastric or stomach carcinomas (Doll et al., 1960).

2.2.4.6.2.5 Involvement of glycosyltransferase activity in the regulation of ABH antigen expression.

Studies examining glycosyltransferase activities in human colon cell lines and colon tumors have suggested that expression of ABH structures is controlled by the activity of α1-2 fucosyltransferase provided that the low activities of A and B transferase are not limiting. Studies by Ørntoft et al., (1991), indicated that the activity of both the H and Se Fuc T was increased significantly in human tumor samples, whereas the activity of A transferase was unchanged. As well, these data indicated that only Fuc T had a higher activity in the caecum as compared to the rectum of normal colon (Ørntoft et al., 1991), implying that the loss of ABH determinants in the distal colon is a result of decreased Fuc T activity. In contrast, examination of glycosyltransferase activities in normal colon, colon tumors and colon carcinoma cell lines have suggested that there are no significant changes in fucosyltransferase, A or B transferase activity, implying the expression of ABH antigens is regulated by something other than glycosyltransferase activity in the colon (Dahiya et al., 1989; Itzkowitz et al., 1990). Since ABH
determinants are often extensions of type 2 chains in neoplastic states, regulation of type 2 chain expression may ultimately regulate ABH determinant synthesis.

2.3 Glycoproteins associated with cancer progression.

There have been a large number of reports describing alterations in the structure and expression of glycoproteins in a variety of cancers. Detailed characterization and analysis of these glycoproteins is beginning to provide some understanding of their function in normal and neoplastic cells. Glycoproteins involved in cellular adhesion and recognition events have been the most extensively studied to date. However, studies of growth factor receptors and ECM components have provided interesting insights into the role of glycan structure in the function of glycoproteins (reviewed in Varki, 1993).

2.3.1 Mucins.

Malignancy in epithelial tissues is often accompanied by alterations in mucin expression and atypical carbohydrate structure (Kim, 1992). Mucins are high molecular weight, extensively glycosylated, oligomeric glycoproteins that are the predominant macromolecules in mucous secretions which produce a protective physical barrier between epithelial cells and the environment (Strous and Dekker, 1992). Mucins contain numerous O-linked glycans terminated by sialic acid and sulphate residues, which give the mucin a high negative charge and an extended structure. As well, these O-linked glycans bear a number of antigenic determinants, including Lewis and ABH blood group structures (Kim, 1992). Asn-linked oligosaccharides are also present on mucins and appear to be critical to efficient oligomerization of these glycoproteins (Dekker and Strous, 1990).

The polypeptide moiety of mucins, called apomucins, are encoded by at least four related MUC genes (MUC1-4) (reviewed in Kim, 1992). These genes encode polypeptides that contain a large conserved domain of tandem repeats with many O-linked
glycosylation sites. Initial studies suggested that apomucin expression is organ specific
(Yonezawa et al., 1991). However, studies by Ogata et al., (1992) indicated that all four
mucin genes are expressed in normal colon. Increased expression of MUC1 has been
documented in malignancy (Zotter et al., 1988). Increased presence of MUC1 mucins on
cancer cells and the resultant increase in sialic acid on the cell surface appears to increase
the resistance of these cells to lysis by natural killer cells (Zotter et al., 1988). Other data
have documented decreased expression of MUC2 and MUC3 mucins in cancers and
perhaps increased MUC4 expression in colon cancers (Ogata et al., 1992).

In addition, mucins from carcinomas typically display a variety of carbohydrate
modifications. Increased levels of modified Le\textsuperscript{X} and Le\textsuperscript{Y} structures have been detected on
mucins isolated from colon cancers (Itzkowitz et al., 1986b). As well, mucins from
colon and hepatocellular carcinomas contain poly-N-acetyllactosamine repeats (Miyake et
al., 1989). Furthermore, cancer-derived mucins display cryptic core structures due to
incomplete glycosylation, including Tn, sialyl Tn and T antigens (refer to Figure 2.6 for
structures) which are typically absent from normal tissues (Kim, 1992).

Recently, studies have documented altered expression of cell membrane-
associated mucin-type glycoproteins in tumor cells (reviewed in Hilkens et al., 1992).
These glycoproteins are similar to secretory mucins in oligosaccharide content and
structure, but they contain a hydrophobic transmembrane domain. Glycoproteins
including leukosialin (CD43), glycophorin, epiglycanin and episialin exhibit these mucin-
like properties (Carlsson and Fukuda, 1986; Kemperman et al., 1994; Wesseling et al.,
1995). Significantly, episialin synthesis is increased in carcinoma cells (Zotter et al.,
1988). It has been suggested that expression of these large mucin-like glycoproteins on
tumor cell surfaces mask adhesion molecules that normally prevent the migration of cells
out of their tissues (Hilkens et al., 1992). Notably, the presence of epiglycanin on the
surface of cancer cells prevents adhesion of these cells to laminin (Kemperman et al.,
1994).
2.3.2 Cellular adhesion molecules and receptors.

Adhesion events are critical to the development and spread of cancer. As described in Section 2.1.2, metastasis of tumor cells requires the concerted effort of a number of cell-cell and cell-ECM adhesion molecules (Heino, 1996). With this in mind, it is not surprising that many adhesion molecules and cellular receptors are altered in malignancy. There are several families of receptors that act as cellular adhesion molecules. In general, these can be divided into receptors involved in cell-cell interactions and receptors involved in cell-matrix interactions. However, the distinction between these two types is not rigid, and in fact is becoming more obscure with increasing research. Notably, many members of the integrin superfamily of receptors function in both capacities.

2.3.2.1 Cell-cell interactions.

Cancer cells not only lose contact with cells in their tissue of origin, but also form novel attachments to cells with which they would not normally interact. For example, tumor cells within the bloodstream adhere to platelets and other blood-borne cells to form emboli, and tumor cells arresting at secondary organs must adhere to endothelial cells and cells of the organ. Cell-cell adhesion involves at least five classes of adhesion receptors: cadherins, lectins, immunoglobulin (Ig) superfamily receptors, integrins and perhaps receptor protein tyrosine kinases and phosphatases. Alterations to molecules in each class have been associated with a variety of cancers (Tang and Honn, 1995).

The cadherins, a family of Ca++-dependent transmembrane glycoproteins, are involved in homophilic cell-cell adhesion (reviewed in Takeichi, 1993). Cadherin-mediated interactions transmit signals through the intracellular modulators α and β-catenin (Gumbiner, 1995). Decreased expression of E-cadherin has been associated with metastatic potential in a variety of tumors (reviewed in Honn and Tang, 1992). Specifically, decreased E-cadherin levels in human prostate cancer is indicative of

The mammalian lectins recognize specific carbohydrate structures. Mammalian lectins implicated in cancer and metastasis include the selectins (C-type lectins), P-type lectins, pentraxins and galectins (reviewed in Zhou and Cummings, 1992). The selectins, which include E-selectin (ELAM-1), P-selectin (GMP-140) and L-selectin (LAM-1) are cell type specific receptors (Kuijpers, 1993). Involvement of selectin in lymphocyte-homing, platelet activation, and binding to endothelial cells is well documented (Honn and Tang, 1992) and similar selectin activities have been postulated to occur in metastasis. The selectins recognize the carbohydrate epitopes, sialyl-Le\textsuperscript{X} and sialyl-Le\textsuperscript{a} (Phillips et al., 1990), which are commonly expressed on cancer cells. Thus, interactions between P- or E-selectin and sialyl-Le\textsuperscript{X} or Le\textsuperscript{a} may initiate interactions of tumor cells with endothelial cells during tumor cell arrest at secondary organs (reviewed in Honn and Tang, 1992).

The galectins bind galactose residues on poly-N-acetylactosamine and ABH blood group structures (Sato and Hughes, 1992). Increased expression of galectin-3 has been correlated with metastatic potential in vivo and cell motility and invasion in vitro (Raz and Lotan, 1981; Lotan, 1994; Raz et al., 1990). Galectin-3, which is differentially expressed in certain highly metastatic tumor cell variants, appears to be involved in tumor cell embolization (Raz et al., 1986). Still, the role of galectin-3 in human colon cancer is inconclusive (Lotz et al., 1993). However, recent studies by Schoepppner et al. (1995) suggested that increased galectin-3 expression is associated with increased Duke's staging and increased metastatic potential of colon tumors.

The immunoglobulin superfamily of receptors contain immunoglobulin (Ig)-like folds in their extracellular domains and are often involved in homophilic interaction (Tang and Honn, 1995). Members of this family include T cell receptors, neuronal receptors
(such as N-CAM), growth factor receptors (such as PDGF), platelet and endothelial cell receptors, and tumor antigens (\textit{e.g.} carcinoembryonic antigen (CEA)) (reviewed in Tang and Honn, 1995). The DCC gene product, which is implicated as a tumor suppressor gene in colon cancer, is a neural cell adhesion molecule (N-CAM)-like receptor (Fearon \textit{et al.}, 1991). As well, increased expression of CEA is commonly observed in colorectal carcinomas and is often used as a marker of malignancy (Benchimol \textit{et al.}, 1991; Johnson, 1991). A third member of the Ig receptor superfamily, ICAM-1, is associated with metastatic potential in melanoma cells (Becker and Brocker, 1995). ICAM-1 mediates cell-cell adhesion through interaction with the integrins LFA-1 and Mac-1.

\subsection*{2.3.2.2 Cell-extracellular matrix interactions.}

To migrate and invade through the ECM, tumor cells must form new interactions and break existing interactions with ECM components. This is achieved by changes not only in the expression of cell-ECM receptors, but also modifications of the activity of these receptors. Tumor cells often display abnormalities to a number of known ECM receptors including non-integrin ECM receptors and members of the integrin superfamily of adhesion molecules.

\subsection*{2.3.2.2.1 Laminin receptor.}

Increased expression of the 67 kDa non-integrin, high affinity laminin receptor is associated with metastatic potential in adenocarcinomas of the breast, lung and colon (Cioce \textit{et al.}, 1991). Notably, the 67 kDa receptor is upregulated in crypt cells as compared to the villus cells in the adult intestine and other dividing cell populations (Rao \textit{et al.}, 1994). Upregulation of the 67 kDa laminin receptor in cancer cells is thought to assist in the invasion and entry of cells into the circulation (Cioce \textit{et al.}, 1991).
2.3.2.2 CD44.

CD44 is a cell surface proteoglycan which is the principle hyaluronate binding receptor in many cells including epithelial cells, fibroblasts and leukocytes. The CD44 receptor has been implicated in regulation of cell motility and morphology through cell-cell (Naujokas et al., 1993) and cell-ECM adhesions (Aruffo et al., 1989). Increased expression of the CD44 receptor has been associated with the metastatic potential of epithelial tumors (Günthert et al., 1991) and lymphomas (Jalkanen et al., 1991). Specifically, transfection of a CD44 isoform specific for metastatic rat carcinoma cells into non-metastatic cells produced a metastatic phenotype (Günthert et al., 1991). Furthermore, expression of CD44 isoforms correlated with tumor progression and tumor staging in colon cancer (Gotley et al., 1996). However, studies have shown that only CD44 isoforms that interact with hyaluronate influence tumor cell growth and local invasiveness (Sy et al., 1991), suggesting that the specific interaction between CD44 and hyaluronate within the ECM is critical in the metastatic process. Interestingly, CD44-mediated adhesion to hyaluronate appears to be controlled by Asn-linked glycosylation of CD44 (Bartolazzi et al., 1996). Treatment of cells with tunicamycin, which inhibits synthesis of Asn-linked oligosaccharides, resulted in loss of CD44-mediated cell adhesion to hyaluronate-coated plates, whereas treatment of cells with deoxymannojirimycin (which routes synthesis to high-mannose type Asn-linked glycans), had no effect. It appears that the Asn-linked glycans are necessary for CD44 function. Whether the CD44 receptors undergo differential glycosylation following neoplastic transformation remains to be determined.

2.3.2.3 Integrin superfamily of receptors.

The integrin superfamily of receptors is a large family of heterodimeric glycoproteins which mediate cell-matrix and cell-cell recognition and adhesion (Hynes, 1992). Because of their central role in many crucial adhesion effects, integrins are
essential to mammalian development and disease (Albelda and Buck, 1990). Indeed, a variety of alterations to the expression and structure of integrins are associated with cancer and metastasis (Ruoslahti and Giancotti, 1989).

The integrin receptors are produced by noncovalent interaction of an alpha (α) integrin subunit and a beta (β) integrin subunit (Figure 2.14). Studies to this date have identified eight (8) β subunits and sixteen (16) α subunits (Hynes, 1992; Heino, 1996). The individual β integrin subunits can combine with a variety of α integrin subunits to form unique receptors with diverse specificities for ECM components and cell surface receptors (Figure 2.15). For example, α4 subunits can combine with either β1 or β7 subunits producing α4β1 integrin which recognizes VCAM-1 (a member of the immunoglobulin superfamily of receptors) and α4β7 integrin which binds addressin (Elies et al., 1990). Integrin receptors containing a β1 subunit belong to the VLA (very late-acting antigen) family of receptors (Figure 2.15, blue lines) (reviewed in Hemler, 1990). The various α and β integrin subunits are highly conserved throughout different species and cell types, suggesting that they developed early in evolution and are central to cell function. For instance, the human α3 integrin subunit (Takada and Hemler, 1989) is nearly identical to the chicken α3 integrin subunit (Hynes et al., 1989). In contrast, the specific α and β integrin subunits show variable degrees of homology. Many of the α integrin subunits have little or no homology to one another, whereas the β subunits are approximately 45% homologous to each other (Hynes, 1992).

The α and β integrin polypeptides consist of a large extracellular domain, a single hydrophobic transmembrane domain and a cytoplasmic domain. The extracellular domains of the α and β integrin chains form noncovalent interactions with each other to produce the ligand binding domain (Figure 2.14) (Loftus et al., 1990; Vogel et al., 1990). As well, within the extracellular domain of the α integrin chain there are conserved divalent cation (metal)-binding sites which are essential for receptor function (Calvete et al., 1989; Kirchhofer et al., 1990, 1991) (Figure 2.14). Moreover, certain α
Figure 2.14: The integrin receptor. Schematic diagram of the integrin receptor heterodimer. The noncovalently associated complex is composed of an α integrin chain and a β integrin chain. Portions of both subunits form the ligand binding domain. Certain α integrin subunits are cleaved in their extracellular domain near the membrane and the cleavage products are linked by disulfide bonds (S-S). Adapted from Hynes, 1992.

Figure 2.15: The integrin family of receptors. Interactions between the known alpha (α) and beta (β) integrin subunits to form receptor heterodimers are depicted. The β1 integrin family of receptors is shown in blue. Receptor substrates include collagen I (Cl), collagen IV (CIV), fibronectin (Fn), fibrinogen (Fb), laminin-1 (Ln-1), laminin-5 (Ln-5), vitronectin (Vn). Known cell adhesion counter receptors are underlined in red. Recognition sequences are depicted in outline print. Adapted from Albelda and Buck, 1990.
integrin subunits, including the α3 and α6 integrin chains, undergo post-translational cleavage near the carboxy-terminal end of the extracellular domain. The cleavage products are joined by disulfide bonds (Figure 2.14). Other α integrin subunits contain a 180-200 amino acid insert (called the I domain) within the extracellular region of the polypeptide. The I domain contains a collagen recognition motif, but the precise function of this domain is not understood (Calderwood et al., 1995; Tuckwell et al., 1995). The β integrin chain contains four conserved cysteine-rich motifs at the C-terminal end of the extracellular domain which contribute to integrin conformation. As well, the ligand binding region of the β integrin subunit is stabilized by disulfide bonding between many conserved cysteine residues (Calvete et al., 1989). Proper conformation and heterodimer assembly are required for cell surface expression and function of the integrin receptor.

The cytoplasmic tails of the integrin subunits do not interact with each other, but they do interact with a number of cytoskeletal and intracellular proteins (Tapley et al., 1989). For example, β1 integrin interacts with α-actinin and talin (Otey et al., 1993). The interactions between integrin chains and intracellular proteins not only allows for transmission of signals from the extracellular surroundings to the intracellular machinery of the cell, but also signaling from within the cell to the cell surface, termed 'outside-in and inside-out signaling' (reviewed in Clark and Brugge, 1995; Dedhar, 1995; Yamada and Miyamoto, 1995).

The integrin receptors recognize specific peptide sequences within their respective ligands (Figure 2.15, outline type). Many integrin receptors recognize an arginine-glycine-asparate (RGD) sequence in the ligand (e.g. α5β1 integrin-fibronectin interaction). However, recent studies have identified the sequences KQAGDV, DGEA and EIVDV as integrin recognition sequences (reviewed in Hynes, 1992). The conformation surrounding the recognition site appears to determine which integrin will bind the ligand (Ruoslahti and Pierschbacher, 1987). For example, α5β1 integrin recognizes an RGD sequence in fibronectin, whereas αvβ5 recognizes an RGD sequence
in vitronectin (Figure 2.15). Furthermore, ligands often possess two or more binding sites that are specific for certain integrin receptors. For instance, the laminin receptors α6β1, α3β1 and α2β1 integrin recognize a site on the long arm of laminin-1 but α1β1 integrin recognizes a site on the cross region of the laminin-1 molecule (Mercurio, 1990).

Integrins are expressed in a cell-specific, developmentally-regulated manner. Indeed certain integrins are restricted to one or a few cell types. For instance, integrins such as αIIbβ3, LFA-1 (αLβ2 integrin) and Mac-1 (αMβ2 integrin) are specific for blood-borne cells, such as platelets and leukocytes, and are involved in cell-cell interactions in these cell types (Springer, 1990). As well, α6β4 integrin resides only on epithelial cells (Kajji et al., 1989). In contrast, the majority of integrins involved in cell-matrix interactions are expressed at various levels on many different cell types. In point of fact, most mammalian cells express on their surface from two to ten different integrin receptors which mediate the binding of these cells to the ECM (Heino, 1993).

Cells also modulate the binding properties and activity of the integrin receptors on the cell surface. Often the affinity of integrin receptors for ligands is dependent on cell type and the surrounding environment. For example, α2β1 integrin expressed on platelets specifically recognizes collagen (Staatz et al., 1989), but α2β1 integrin expressed by other cell types recognizes both collagen and laminin-1 (Elices and Hemler, 1989). The confusion is increased when it is noted that different lipid content (Conforti et al., 1990) and the presence of certain divalent cations (Kirchhofer et al., 1990, 1991) within the cell can alter the ligand specificity of certain integrin receptors. In addition, integrin receptors undergo activation and deactivation depending on cell status. For example, αIIbβ3 integrin on resting circulating platelets does not bind soluble ligands but can bind surface-bound fibrinogen. Only with activation of the platelets does αIIbβ3 integrin recognize soluble fibrinogen. These alterations to integrin specificity are a result of conformational changes to the receptor (Shattil and Brugge, 1991). Furthermore, the integrin receptors undergo additional conformational changes upon ligand binding.
(Frelinger et al., 1990; 1991). As well, phosphorylation/dephosphorylation of the intracellular regions of integrin subunits have been implicated in the regulation of integrin activity. Phosphorylation of the integrin receptor has been implicated in the activation of integrin ligand binding in platelets and carcinoma cells (Shattil and Brugge, 1991). Notably, the β1 integrin subunit is phosphorylated on a tyrosine residue by pp60c-src which decreases β1 integrin binding to talin and fibronectin (Tapley et al., 1989). As well, rapid phosphorylation of α3β1 integrin occurs upon adhesion and spreading of fibroblasts (Guan et al., 1991). These crucial studies emphasize the dynamic complexity of integrin-mediated adhesions -- adhesion events that are meticulously regulated and continually modified by cells.

2.3.2.3.1 Role of oligosaccharides in integrin function.

All integrin receptors are glycoproteins which are extensively glycosylated with Asn-linked and O-linked oligosaccharides (Hynes, 1992). Recent studies have shown that glycosylation is critical to the structure and function of integrins. For instance, the treatment of cells with enzymes which remove Asn-linked glycans from α5β1 integrin abolished adhesion of these cells to fibronectin (Zheng et al., 1994). The lack of adhesion was due to dissociation of the α5 and β1 integrin subunits on the cell surface. Similar studies of α6β1 integrin from B16-F10 melanoma cells indicated that binding of laminin is dependent on the presence of Asn-linked oligosaccharides (Chammas et al., 1991). As well, the presence of α-galactosyl residues on the α integrin chain is important for laminin binding, whereas the β1-6 branch of Asn-linked glycans on the β chain is associated with cell spreading (Chammas et al., 1993). Moreover, glycosylation of the β1 integrin subunit is necessary for melanoma cell adhesion to fibronectin and collagen substrates, since immature high-mannose forms of the β1 integrin chain have reduced binding to these substrates (Veiga et al., 1995). However, binding to laminin appears to be independent of the glycosylation state in these cells. Similar studies of the
β2 integrin suggested that Asn-linked oligosaccharides are necessary for effective interaction of β2 integrins with ICAM-1 in neutrophil-endothelial cell binding (Sriramarao et al., 1993). As well, the level of ICAM-1 glycosylation influences the adhesion between Mac-1 integrin and ICAM-1. Specifically, Mac-1 integrin binds more efficiently to ICAM-1 with smaller Asn-linked oligosaccharides, or deglycosylated ICAM-1 (Diamond et al., 1991). Interestingly, different cell types have differential glycosylation of ICAM-1. These data suggest that altered integrin glycosylation and ligand glycosylation may influence the adhesive properties of cancer cells.

2.3.2.3.2 Integrins and Cancer

Cancer cells display a variety of alterations to the expression and activity of integrin receptors. It has been speculated that cancer cells alter their adhesive and migratory properties by changing integrin function, thus aiding in the dissemination of these cancer cells. Overall, studies of integrins and cancer have not pin-pointed a single specific integrin or change to all cancers, rather it appears that individual integrins or groups of integrins are important in specific types of cancer. The complex role of integrins in various cancers has been reviewed elsewhere (Heino, 1993; Cress et al., 1995; Nip and Brodt, 1995). Therefore, this section will only highlight select findings significant to the studies discussed in this thesis.

Early studies demonstrated that injection of RGD-containing peptides in tumor-bearing animals reduced growth formation of tumors in a number of systems (Humphries et al., 1986). Moreover, RGD-containing peptides can inhibit invasion and metastasis of colon cancer cells (Saiki et al., 1990). Since the RGD containing peptides block the function of many integrin receptors, these data suggested a role for integrins in tumor formation and metastasis.

Cell surface expression of various integrin receptors is frequently modified in transformed and tumor cells. Transformed cells have reduced α5β1 integrin, a
fibronectin receptor, on their cell surface (Plantefaber and Hynes, 1989). Decreased levels of α5β1 integrin accentuated the migration of these transformed cells, due to decreased fibronectin matrix deposition and decreased adhesion to fibronectin (Ruoslahti and Giacobetti, 1989). As well, antibodies that bind α5 integrin subunits and block receptor function, increase the migration of certain cell types (Akyiama et al., 1989).

Inhibiting β1 integrin receptor function with antibodies directed against the β1 integrin subunit decreased the migration of many tumor cell lines on fibronectin (Newton et al., 1995), suggesting that the β1 integrin receptor family (refer to Figure 2.15) may be important to tumor development and metastatic spread. Indeed, laminin and laminin receptor expression appear to promote tumor formation since colon cancer cells which adhere to laminin form larger tumors. (Kinz et al., 1994). This tumor formation can be inhibited by anti-β1 integrin antibodies. Likewise, anti-β1 integrin antibodies can prevent spontaneous metastasis of tumor cells (Kinz et al., 1994; Newton et al., 1995). As well, anti-β1 antibodies can inhibit liver metastasis of colon cancer cells in vivo (Fujita et al., 1992). Indeed, integrin receptors containing a β1 subunit are often overexpressed in colon carcinoma cells compared to normal colon cells, and overexpression of the β1 subunit correlates with lymph node metastases and depth of invasion (Fujita et al., 1995).

Similarly, LFA-1 expression is often upregulated in metastatic cell lines (Johnson et al., 1995). It has been suggested that the increased presence of LFA-1 integrin, the ICAM-1 counter receptor, on the surface of metastatic cells may aid in cancer cell survival in the circulation via tumor emboli formation. Moreover, lymphoma cells employ LFA-1 and fibronectin receptors in adhering to liver cells (Ross and Rossien, 1987).

Other integrins or integrin families appear to play specific roles in only one or a few types of cancer. For example, the collagen-laminin binding integrins, including α2, α3, α6 and β4 integrin subunits, exhibit decreased expression in non-small cell carcinomas of the lung. Notably, decreased α3 integrin subunit expression correlated with poorly differentiated tumors (Smythe et al., 1995). In contrast, αvβ3 integrin and

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α4β1 integrin appear to be important in melanomas. Specifically, increased surface expression of αvβ3 and α4β1 integrin was associated with increased adhesion to vitronectin and fibrinogen and increased metastatic potential (Boukerche et al., 1990). As well, antibodies against αvβ3 integrin prevented melanoma tumor formation in nude mice, however they did not prevent invasion in vitro (Genhlsen et al., 1992). Recent studies have linked αvβ3 integrin-vitronectin binding to urokinase plasminogen-activator (uPA) proteolysis in melanomas (reviewed in Nip and Brodt, 1995), suggesting that integrin function is important in ECM proteolysis and tumor cell invasion. Specifically, αvβ3 integrin-vitronectin binding leads to activation of signaling pathways and increased uPA synthesis (Nip and Brodt, 1995). Furthermore, αvβ3 integrin-vitronectin binding is important for melanoma cell arrest at lymph nodes (Tawil et al., 1996).

Integrins α6β1 and α6β4 appear to be important in the invasiveness of prostate cancer cells (reviewed in Cress et al., 1995). Indeed, cancer cells isolated from invasive prostate carcinomas have low levels of α2, α4, α5 and αv integrin subunits but increased surface expression of α6β1 integrin and α6β4 integrin compared to non-invasive cells. As well, increased α6β4 integrin expression has been detected in metastatic skin tumors (Tennenbaum et al., 1995). It has been postulated that increased surface expression of α6β4 and α6β1 integrin increases tumor cell adhesion to laminin, which assists in invasion and migration through ECM surrounding nerves, blood vessels and muscles (Kemperman et al., 1994). In addition, metastatic mammary carcinoma cells bind liver cells via α6β4 integrin (Kemperman et al., 1994).

**Integrin α3β1**

Integrin α3β1 expression is altered in many cancer cell lines and tumor models, yet the precise effect of these alterations is not clear. Indeed, the function of α3β1 integrin in normal cells is ambiguous since this integrin binds a variety of ECM proteins, including collagens, fibronectin, laminin-1 (Takada et al., 1988) and laminin-5 (Carter et
and mediates homophilic interactions (Sriramarao et al., 1993). As well, expression of the α3 integrin chain is increased in actively proliferating cells (Varner et al., 1992). Transformed cells display increased levels of α3β1 integrin on their surface (Plantefaber and Hynes, 1989). As well, many tumor cell lines have increased expression of α3β1 integrin. Notably, metastatic breast cancer cell lines have higher levels of α3β1 integrin compared to non-metastatic cells. Increased cell surface expression of α3β1 integrin is believed to improve adhesion of these metastatic cells to fibronectin-rich matrices secreted at lymph nodes (Tawil et al., 1996). In contrast, transfection of α3 integrin subunit cDNA into human rhabomyosarcoma cells leads to increased tumor cell adhesion to collagen and decreased spontaneous and experimental metastasis (Tawil et al., 1996). Interestingly, in glioma cells, antibodies to α3β1 integrin increase the expression and activity of MMP-2 and subsequently the invasiveness of these cells (Chintala et al., 1996).

A role for α3β1 integrin in colon cancer is unclear and studies are often contradictory. Studies by Boku et al. (1995) suggested that increased expression of α3 integrin subunit is indicative of more invasive or "contracted" types of colon and gastric cancers compared to less invasive cancers. In contrast, studies by Pignatelli et al. (1990) indicated that colon carcinomas typically display a decrease or loss of collagen receptors, including α2β1 and α3β1 integrins. This loss of α3β1 integrin was associated with loss of tumor differentiation (Pignatelli et al., 1990).

### 2.3.3 The role of glycoproteins bearing β1-6 branched Asn-linked oligosaccharides in cancer.

As described in Section 2.2.4.4.4, increased expression of β1-6 branched Asn-linked oligosaccharides is directly associated with increased metastatic potential. However, it is not known whether it is the branch itself or modifications to the branch which are important in the metastatic process. Studies in transformed and tumor cell lines
have identified a select number of glycoproteins which bear β1-6 branched Asn-linked oligosaccharides. These include the lysosomal membrane associated proteins (lamps), the galectin-3 binding protein family (Ullrich et al., 1994; Koths et al., 1993), CEA (Benchimol et al., 1989), laminin (Jin et al., 1995) and members of the integrin receptor family. Significantly, many of these glycoproteins are implicated in cellular adhesion and recognition events.

2.3.3.1 Lysosomal membrane associated proteins (lamps)

Major carriers of β1-6 branched Asn-linked glycans in metastatic cells are lysosomal membrane associated protein-1 (lamp-1) (Laferté and Dennis, 1989) and lamp-2 (Carlsson et al., 1988). Lamp-1 and lamp-2 are also carriers of poly-N-acetyllactosamine repeats in normal cells (reviewed in Fukuda, 1992; Carlsson et al., 1988; Laferté and Dennis, 1989). Indeed, the β1-6 branch is the primary site for type 2 chain extensions since GnT III (i) preferential acts on this branch (refer to Section 2.2.4.4.4). Lamp proteins are highly glycosylated proteins containing both Asn-linked and O-linked oligosaccharides (Maemura and Fukuda, 1992). Lamp-1 and lamp-2 are primarily localized to the lysosomal membrane where they are believed to protect the membrane from proteolytic digestion. Normally, lamp-1 and lamp-2 are present on the cell surface in small amounts and it appears that the lamps can cycle between the cell surface and the lysosome compartment (Granger et al., 1990). Increased cell surface expression of lamp-1 and lamp-2 is commonly observed in metastatic cells. For instance, highly metastatic human colon carcinoma cells express more lamp-1 and lamp-2 on the cell surface than their low metastatic counterparts (Saitoh et al., 1992). Furthermore, lamp-1 and lamp-2 isolated from highly metastatic colon cancer cells contain more poly-N-acetyllactosamine structures which are terminated with sialyl-LeX structures compared to lamps isolated from non-metastatic cells (Saitoh et al., 1992). Significantly, these highly metastatic cells adhere more strongly to E-selectin-expressing cells, a phenomenon
which can be inhibited by the addition of soluble lamp-1 (Sawada et al., 1993). These studies suggested a role for cell surface lamp-1 and lamp-2 in cell adhesion. It has been postulated that increased poly-N-acetyllactosamine, due to increased β1-6 branching, provides sites for addition of carbohydrate antigens involved in cell adhesion (Fukuda, 1991).

2.3.3.2 Integrins

Integrin chains including α5, α6, αv, β1 (Demetrious et al., 1995; Chammas et al., 1993; Jasiulious et al., 1996) have been shown to display β1-6 branched Asn-linked oligosaccharides. Transfection of lung epithelial cells with GnT V resulted in increased presence of β1-6 branched glycans on lamp-2, α5, αv and β1 integrin subunits (Demetrious et al., 1995). These transfected cells were less adhesive to fibronectin and collagen type IV-coated surfaces compared to untransfected cells, even though the surface levels of the integrin receptors was unchanged. In other studies, α6β1 integrin has been shown to be the major carrier of β1-6 branched Asn-linked glycans in ras-transformed fibroblasts (Jasiulionis et al., 1996). EJ-ras transfection of NIH 3T3 fibroblasts resulted in increased expression of α6β1 integrin and increased migration on laminin-1 substrate. As well, β1 integrin chains isolated from the ras-transformed cells were L-PHA reactive. However, migration was independent of glycosylation state. Nonetheless, β1-6 branched Asn-linked oligosaccharides appear to be important in cell spreading (Chammas et al., 1993). These studies suggested that the presence of the β1-6 branch on receptors may influence the function and/or specificity of receptors. Indeed, the presence of the β1-6 branch on integrin subunits may affect the conformation and/or function of the glycoprotein. Alternatively, the presence of the β1-6 branch may generate new carbohydrate antigens on the glycoprotein which in turn influence cell adhesion. Thus the role of β1-6 branched Asn-linked oligosaccharides in adhesion and migration is complex.
2.4 Use of an animal model of colon carcinogenesis to study the role of
glycoproteins bearing β1-6 branched Asn-linked oligosaccharides in colon
cancer progression.

With the evidence implicating the β1-6 branch of Asn-linked oligosaccharides in
metastasis and recent data suggesting an involvement of this branch in regulating cell
adhesion and migration, studies were needed to address the involvement of glycoproteins
bearing β1-6 branched Asn-linked oligosaccharides in colon cancer. Studies in our
laboratory have employed an animal model of colon carcinogenesis, in addition to human
colon carcinoma cell lines and human colon tumor samples, to address the role of β1-6
branch bearing glycoproteins in colon cancer.

2.4.1 The azoxymethane-induced rodent model of colon carcinogenesis.

Colon carcinogenesis can be induced in rats using the carcinogen azoxymethane.
Azoxy methane, when activated in the liver, is a potent DNA methylating agent that causes
DNA damage and eventually tumor formation (Deschner, 1974). This carcinogen
produces tumors in both the small and large intestine. Azoxy methane-induced cancer in
rats produces a number of tumors in each animal. These colon tumors, which range from
polyps to invasive carcinomas, closely resemble human colon tumors. However,
metastasis to the liver is rarely seen in this rodent model before animal morbidity occurs
(Bird et al., 1985). Since numerous tumors of varying stages occur in a single animal,
direct comparison of biochemical markers related to cancer progression is possible.
Furthermore, the Sprague-Dawley rodent strain used in many studies expresses

2.4.2 Preparation of monoclonal antibody 3A7.

Hybridoma technology was employed to prepare reagents for the study of
glycoproteins bearing β1-6 branched Asn-linked oligosaccharides in colon cancer which
could be detected with the lectin L-PHA. Monoclonal antibodies (mAb) were prepared against the membrane fraction of an azoxymethane-induced rat colon tumor and screened for their ability to detect L-PHA binding glycoproteins differentially expressed in colon carcinoma and normal colon (Laferté et al., 1995). One such antibody, mAb 3A7, recognized an epitope expressed at higher levels in azoxymethane-induced colon tumors compared to normal colon, suggesting that this antibody may be useful in studies of colon cancer. Specifically, rat colon carcinoma detergent lysates contained mAb 3A7-immunoreactive glycoproteins ranging in size from 60-200 kDa, with a major species of 140 kDa (Figure 2.16B, lane 5). The 140 kDa glycoprotein recognized by mAb 3A7 was also shown to be an L-PHA binding glycoprotein (Figure 2.16A, lane 5).

2.4.3 Expression of the 3A7 epitope in normal and neoplastic rat colon.

Immunohistochemical studies were performed in our laboratory to examine the expression of the 3A7 epitope in azoxymethane-induced rat colon tumors and normal colon. In normal rat colon, expression of the epitope was highest in the caecum (Figure 2.17A), followed by ascending (data not shown) and descending colon (Figure 2.17B). In the caecum, the antibody staining was dispersed throughout the entire epithelium with the strongest staining localized within the crypts. In contrast, staining in the ascending and descending colon was confined to the lower half of the crypts which is the proliferative zone of the colonic epithelium (Louvard et al., 1992). Staining was primarily contained within columnar absorptive cells (Laferté, et al., 1995).

Staining of azoxymethane-induced rat colon tumors with mAb 3A7 revealed altered expression and localization of the epitope compared to normal tissues (Figure 2.17, panels C-H). In general, most areas of invasive cancers were strongly positive (Duke's B2, Figure 2.17D). In contrast, earlier lesions often had large areas that were only slightly positive (Duke's A, Figure 2.17C). However, increased expression of the
Figure 2.16: Blotting of normal and neoplastic rat colon with Leucophytoagglutinin (L-PHA). A: Detergent lysates prepared from normal rat colon (lanes 1, 2, 5 and 6) and an azoxymethane-induced rat colon tumor (lanes 3, 4, 7 and 8) were immunoprecipitated with NMS (lanes 1, 3, 5 and 7) or mAb 3A7 (lanes 2, 4, 6 and 8), separated by 7.5% SDS-PAGE and blotted with L-PHA lectin followed by normal rabbit serum (NRS) (lanes 1-4) or anti-L-PHA serum (lane 5-8). Colour development was performed as described in Section 4.4.6. B: Detergent lysates were prepared from normal rat colon (lanes 3 and 6) and azoxymethane-induced rat colon polyp (lanes 1 and 4) and invasive tumor (lanes 2 and 5), separated by 7.5% SDS-PAGE and blotted with L-PHA lectin followed by NRS (lanes 1-3) or anti-L-PHA serum (lanes 4-6).
Figure 2.17: Immunohistochemical staining of normal rat intestine and rat colon tumors with mAb 3A7. Segments of small and large intestine as well as asoxymethane-induced rat colon tumors were examined by immunohistochemical staining using either normal mouse serum or mAb 3A7 (1:500 dilution) as described in Section 4.4.5. Staining patterns with mAb 3A7 are shown. Parallel staining of sections with NMS yielded negative results. A: Normal caecum depicting generalized staining of the epithelium with the strongest staining present in the crypt zones x200. B: Normal mucosa from the descending colon illustrating immunopositivity for the antibody predominantly in the crypt zones (original magnification, x150). C: Duke's stage A colonic adenocarcinoma (tumor specimen Azo 1.6), x200. D: Conventional Duke's B2 adenocarcinoma (tumor specimen Azo 1.7), x40. E: Collision colonic adenocarcinoma with both conventional adenocarcinoma (arrowhead) and a signet-ring carcinoma pattern (arrow), x30. F: Hematoxylin and eosin-stained micrograph from an area of a step section roughly equivalent to Figure 2.17E, x30. G: High power view of the mucosa adjacent to the tumor in Figure 2.17E. This highlights the irregularity in the staining of this transitional mucosa, with some focal areas close to the lumen markedly positive. Note the submucosal vessels depicting vascular invasion (arrow), x200. H: Hematoxylin and eosin-stained micrograph is from an area of a step-section roughly equivalent to Figure 2.17G. In contrast to Figure 2.17G, no overtly abnormal areas are seen in the transitional mucosa, x200.
3A7 epitope appeared to be an early event in colon carcinogenesis, since many of the early-stage carcinomas (Duke's A and B1) stained intensely with the antibody. Moreover, intensity of staining appeared to be indicative of tumor stage, with invasive tumors (Duke's B2) staining the most intensely. However, a lymph node metastases (Duke's C) did not stain with mAb 3A7, suggesting that a decrease in expression of the epitope may be important in tumor spread (Laferté et al., 1995). Still, mAb 3A7 staining was detected at sites of angioinvasion (Figure 2.17G, arrow). Finally, the mucosa adjacent to colonic tumors exhibited increased and more dispersed staining than normal mucosa (Figure 2.17G). Hematoxylin and eosin-stained sections (Figure 2.17 F and H) did not reveal any abnormal areas in the mucosa adjacent to the tumor, suggesting that the antibody may detect subtle early abnormalities in the colonic mucosa and hence be a useful marker for detection of disease.

2.4.4 Expression of 3A7-immunoreactive glycoproteins in normal rat tissues.

Immunohistochemical studies depicted the expression and localization of the 3A7 epitope in normal and neoplastic rat colon, yet they did not provide any information in regard to the macromolecules bearing the 3A7 epitope. Preliminary observations suggested that the epitope was not present on neutral and acidic glycolipids extracted from rat colon. Western blotting analysis of detergent-solubilized rat tissues revealed that the epitope detected by mAb 3A7 is present on glycoproteins expressed by gastrointestinal tissues, including the stomach, small intestine and to a lesser extent, the large intestine (Laferté, et al., 1995). Glycoprotein species of 130-140 kDa, 90 and 60 kDa were detected in the proximal small intestine, whereas in the large intestine mAb 3A7 detected a species of approximately 140 kDa (Laferté, et al., 1995).

Analysis of fetal and neonatal rat intestine demonstrated that expression of the 3A7 epitope is developmentally regulated in rat intestine (Laferté et al., 1995).
Specifically, expression of the epitope coincided with appearance of simple epithelium in the intestine, with the levels of mAb 3A7 staining increasing in the large intestine until the time of weaning. Following weaning, expression of the epitope in the colon decreased until it reached the levels seen in adult colon. These data indicated that the epitope detected by mAb 3A7 is oncodevelopmentally regulated in the rat intestine. As well, these studies suggested a potential use for mAb 3A7 in the study of glycoproteins bearing β1-6 branched Asn-linked oligosaccharides in colon cancer. Further study of the 3A7 epitope and glycoproteins bearing this epitope in colon cancer was required.

3.0 RATIONALE AND OBJECTIVES

Studies revealing that the 3A7 epitope and/or glycoproteins bearing this epitope are oncodevelopmentally regulated in rat colon suggested that examination of the 3A7 epitope and the 140 kDa glycoprotein bearing this epitope were warranted. Moreover, the relevance of the 3A7 epitope to human disease needed to be addressed.

Preliminary examination of detergent lysates from human colon carcinoma cell lines revealed that mAb 3A7 detects a major species of 140 kDa and minor species ranging from 60-200 kDa (Figure 3.1). Four human colon carcinoma cell lines established from different histopathological grades were examined. These included the moderately well differentiated cell line HT29 (blood group A) established from a Duke's B2/grade II primary tumor, the poorly differentiated cell line SW1417 (blood group B) established from a Duke's C1/grade III primary tumor, the poorly differentiated cell lines SW480 and SW620 (blood group A) established from a Duke's C2/grade III/IV primary tumor and lymph node metastases, respectively, isolated from the same patient.

A 140 kDa glycoprotein, denoted gp140, was immunoprecipitated by mAb 3A7 from [35S]-methionine labeled HT29 and SW480 cell lysates (Figure 3.1, lanes 2 and 6, respectively). In contrast, SW620 cell lysates failed to synthesize 3A7-immunoreactive gp140 (Figure 3.1, lane 8). These data suggested that loss of the 3A7 epitope or gp140
Figure 3.1: Expression of glycoproteins detected by mAb 3A7 in human colon cancer cell lines. The human colon cancer cell lines HT29 (Duke’s B2, moderately differentiated), SW480 (Duke’s C2, poorly differentiated), SW620 (lymph node metastasis isolated from SW480 primary tumor) and SW1417 (Duke’s C2) were radiolabeled for 24 hours with [35S]methionine (50 μCi/ml). Radiolabeled proteins from cell lysates (2 x 10⁶ cpm) were immunoprecipitated with normal mouse serum (lanes 1, 3, 5 and 7) or mAb 3A7 (lanes 2, 4, 6 and 8) and separated by 7.5% SDS-PAGE. Bands were visualized following fluorography and autoradiography.
was associated with lymph node metastasis. As well, gp140 was not immunoprecipitated by mAb 3A7 from SW1417 cells, a blood group B cell line, suggesting that the epitope defined by mAb 3A7 may be a blood group A or A-like determinant. Further studies were required to determine the structure of the epitope detected by mAb 3A7 and the identity and role of 3A7-immunoreactive gp140 in human colon cancer. The specific objectives of these studies are as follows:

1) to determine the relevance of the 3A7 epitope and glycoproteins bearing this epitope to human colon cancer.

2) to elucidate the structure of the epitope detected by mAb 3A7.

3) to generate antibodies to the polypeptide portion of gp140 and determine the identity of gp140.

4) to examine whether altered synthesis of 3A7-immunoreactive gp140 in human colon carcinoma cell lines results from alterations in glycosylation or polypeptide expression.

5) to study the mechanism(s) regulating expression of the 3A7 epitope in normal and neoplastic colon

6) to examine the potential function(s) of gp140 in colon cancer and the contribution of glycan structure, in particular the β1-6 branch of Asn-linked oligosaccharides, to this function.

Ultimately these studies should improve our understanding of glycoproteins bearing β1-6 branched Asn-linked oligosaccharides in colon cancer progression.
4.0 MATERIALS AND METHODS

4.1 Cell lines.

The human colon carcinoma cell lines HT29 (HTB 38), SW480 (CCL 228), SW620 (CCL 227), SW1417 (CCL 238), LS123 (CCL 255), CaCo-2 (HTB 37) SW1116 (CCL 233), SW403 (CCL 230), SW48 (CCL 231), LoVo (CCL 229) and T84 (CCL 248) as well as the non-secreting mouse myeloma cell line F0 (CRL 1646) and the fibroblast cell line 3T3 Swiss albino (CCL 92) were obtained from the American Type Culture Collection (A.T.C.C., Rockville, MD, USA) and cultured in Dulbecco's minimum essential media (DMEM, high glucose, Gibco-BRL, Burlington, ON, Canada) containing 10% fetal bovine serum (FBS, Gibco-BRL). The cell lines KM20C and KM12SM were obtained from Dr. I. J. Fidler at MD Anderson Cancer Centre (Houston, TX, USA) and the cell lines COLO320DM (CCL 220), LS174T, KM12C, KM12L4A and WiDr were obtained from Dr. J. Xiang at the Saskatoon Cancer Centre (Saskatoon, SK, Canada) These cells lines were cultured in DMEM containing 10% FBS, 2 mM L-glutamine (Gibco-BRL), 1 mM sodium pyruvate (Gibco-BRL), 1% non-essential amino acid supplement (Gibco-BRL) and 2% MEM vitamine solution (Gibco-BRL).

All cell lines were grown in a 37°C, 5% CO2 incubator and maintained by passage every 3-4 days using standard trypsinization protocols. Briefly, cells were grown to approximately 80-90% confluency on 100 x 15 mm tissue culture plates (Nunc, Gibco-BRL), washed once with 15 ml calcium and magnesium free-phosphate buffered saline (CMF-PBS, pH 7.2), coated with 1.5 ml of 0.025% trypsin-0.265 mM EDTA (TE, Gibco-BRL) in CMF-PBS, aspirated to remove excess TE and incubated at 37°C until
cells were rounded. Cells were removed from the plate by gentle pipetting with DMEM containing 10% FBS, diluted 1:10 with the aforementioned medium and plated.

4.2 Monoclonal antibodies.

The monoclonal antibody specific for blood group H determinant (Fucα1-2Gal-R) was generously provided by Dr. Monica Palcic (University of Alberta, Edmonton, AB, Canada) (Yuan et al., 1985). The rabbit anti-human lysosomal associated membrane protein (lamp)-1 (931-A) and lamp-2 (932-1) antibodies were generous gifts from Dr. M. Fukuda (La Jolla Cancer Centre, CA, USA). Mouse anti-human integrin α3β1/VLA-3 (MAb1992) antibody was purchased from PDI/Joldon (PDI BioScience, Calgary, AB, Canada) and anti-human integrin α3 and anti-human integrin β1 (clone P4C10) monoclonal antibody ascites were obtained from Gibco-BRL. Anti-mouse blood group A determinant monoclonal antibody was purchased from Dako Corporation (Cappinteria, CA, USA). Monoclonal antibodies 3A7, 2F7, 4F3 and 8D5 were prepared by Dr. S. Laferté as described (Laferté et al., 1995). Rabbit anti-L-PHA (SL6.1) antisera was prepared by Dr. S. Laferté.

Monoclonal antibodies described herein were prepared by standard protocols (Harlow and Lane, 1988). Briefly, female balb/c mice were injected intraperitoneally with 50 μg of antigen (see Results Section 5.6) in sterile PBS emulsified with Freund's incomplete adjuvant (Gibco-BRL). Two, four and nine weeks later, the mouse was boosted with intraperitoneal injections of 50 μg of antigen; serum was collected and tested for desired immunoreactivity. One month after the final boost, the mouse was given three injections of 25 μg of antigen divided equally between a single tail vein injection and intraperitoneal injections. Twenty-four hours prior to cell fusion, spleen cells from five non-immunized mice were harvested, washed with DMEM, incubated for 7 minutes with 0.167 M ammonium chloride to lyse the red blood cells, resuspended in 150 ml of hybridoma (Hb) medium (DMEM containing 10% FBS, 100 units/ml.
penicillin/streptomycin (Gibco-BRL), 16 µg/ml gentamycin, 2 mM L-glutamine, 1 mM sodium pyruvate, 0.2 µM 2-mercaptoethanol) and plated in 96-well culture plates (Nunc, Gibco-BRL). Three days after the final tail vein injection, the immunized mouse was sacrificed, spleen cells were harvested, washed with DMEM, incubated for 7 minutes with 0.167 M ammonium chloride, pelleted, washed with DMEM and counted. Spleen cells (1 x 10^8) and F0 myeloma cells (2 x 10^7) were pelleted, the medium was removed and 1 ml of 40% polyethylene glycol 1540 (Boehringer Mannheim, Montreal, PQ, Canada), prewarmed to 37°C, was added over 2 minutes to initiate cell fusion. Pre-warmed DMEM (20 ml) was added over 3-5 minutes with continuous, gentle shaking. The cells were pelleted and resuspended in 15 ml of Hb medium containing 0.1 µM selenium (Gibco-BRL) and incubated in a 100 x 15 mm plate for 6 hours. The cells were resuspended in 75 ml of Hb medium supplemented with 5% FBS, 0.1 µM selenium and 3X HAT (0.3 mM hypoxanthine, 48 µM thymidine and 1.2 µM aminopterin (Sigma, St. Louis, MO, USA)) and plated in the 96-well culture plates containing a spleen cell feeder layer. Hybridoma clones were maintained by replenishing Hb media containing 1X HAT (0.1 mM hypoxanthine, 16 µM thymidine, 0.4 µM aminopterin), as required. Colonies were tested after 2-3 weeks for immunoreactivity and maintained in Hb media containing 1X HT (0.1 mM hypoxanthine and 16 µM thymidine). Clones which tested positive were transferred to 24-well culture plates (Nunc, Gibco-BRL) containing a spleen cell feeder layer and retested for immunoreactivity. Positive hybridoma lines were subcloned twice by limiting dilution. Briefly, 96-well plates containing spleen cells were prepared 24 hours prior to cloning. Hybridoma cells were harvested from 24-well plates, pelleted, resuspended in 0.5 ml of Hb media and counted. Cells were diluted with Hb media to a density of approximately 30 cells per ml and plated in the 96-well culture plates containing the spleen cell feeder layer. The subcloned colonies were tested for desired immunoreactivity, transferred to 24-well plates and stored in liquid nitrogen. Ascites were produced by intraperitoneal injection of 3 x 10^5 hybridoma cells into Balb/c mice
(>6 weeks old) that were primed with 0.3 ml Freund's incomplete adjuvant 24 hours prior to injection. Ascites were collected 7-14 days after injection of hybridoma cells and subsequently used for immunodetection analyses and IgG purification.

Isotyping of hybridomas was carried out using an Isotyping Kit (BioRad Laboratories, Mississauga, ON, Canada). Antigen was adsorbed to Immulon 2 microtiter plates (Dynatech Laboratories, Chantilly, VA, USA) by incubating with 100 µl of antigen solution (0.5 µg per well diluted in PBS) overnight at 4°C. The plates were washed four times with PBS containing 0.05% Tween-20 (BioRad Laboratories) (PBST), blocked for 1 hour at room temperature with 4% bovine serum albumin (BSA, Sigma) in PBST, and after washing, incubated with ascites (1:3000 dilution in PBST) for 1 hour. The wells were washed and incubated for 1 hour with 100 µl aliquots of immunoglobulin subclass specific rabbit anti-mouse sera, washed and then incubated for 1 hour with horseradish peroxidase-conjugated goat anti-rabbit Ig (1:3000 dilution in PBST). Wells were washed four times with PBST, once with PBS and incubated 30 minutes with 100 µl of peroxidase substrate solution. Colour development was stopped by the addition of 2% oxalic acid (Sigma) (100 µl per well) and the absorbance read at 415 nm.

4.2.1 Purification of monoclonal IgG from mouse ascites.

IgG was purified from mouse ascites using a MAbTrap GII column (Pharmacia Biotech, Montreal, PQ, Canada) according to manufacturers instructions. Briefly, 0.5 ml of ascites was diluted with an equal volume of binding buffer (20 mM sodium phosphate, pH 7.0), applied to the column pre-equilibrated in binding buffer, and incubated for 1 hour at room temperature. The resin was washed with 10 volumes of binding buffer to remove unbound material and the IgG was collected by passing 5 volumes of elution buffer (0.1 M glycine-HCl, pH 2.7) through the resin. Eluted fractions were immediately neutralized by the addition of 75 µl of neutralization buffer (1 M Tris) per ml of elution buffer. Eluted protein was detected by monitoring absorbance at 280 nm. Aliquots of eluted fractions were analyzed for the presence of heavy and light IgG chains by 10%
SDS-PAGE and Coomassie Blue staining, as described in Section 4.3. Fractions containing IgG were pooled, dialyzed against PBS and concentrated to 1 ml using a Centricon 10 microconcentrator (Amicon, Beverly, MA, USA).

4.3 SDS-polyacrylamide gel electrophoresis.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed as described by Laemmli (1970). Briefly, proteins were separated on 6.0, 6.5, 7.5, 10 or 15% polyacrylamide gels for 1-1.5 hrs at 100 volts using a Mini-Protean II electrophoresis system (BioRad Laboratories). Proteins were visualized by staining with 0.088% Coomassie Blue R-250 (BioRad Laboratories) in 40% ethanol, 8% acetic acid for 20 minutes and destained in a solution consisting of 10% acetic acid, 20% methanol. [35S]-methionine labeled proteins separated by SDS-PAGE were visualized by fluorography (Bonner and Laskey, 1974), using Enlightning (NEN, DuPont, Mississauga, ON, Canada). Alternatively, gels were immediately subjected to electrophoretic transfer and Western blotting, as described in Section 4.4.6.

4.4 Immunodetection.

4.4.1 Dot blot analysis.

The dot blotting procedure performed was developed from protocols originally described in Sharon et al. (1979). Aliquots of up to 7 µl were spotted directly onto nitrocellulose (Schleicher and Schull, Mandel Scientific, Guelph, ON, Canada) or larger aliquots (up to 100 µl) were dried overnight, resuspended in 3 µl of water and spotted onto nitrocellulose. The nitrocellulose was allowed to dry at room temperature and blocked in 4% BSA in TBST (50 mM Tris-HCl, pH 8.0, 0.15 M sodium chloride (NaCl), 0.1% BSA, 0.025% Tween-20, 0.02% sodium azide (NaN3)) for 1 hour at room temperature or overnight at 4°C. The nitrocellulose was washed three times for 5 minutes with TBST, incubated at room temperature for 2 hours with the primary antibody
diluted in TBST, washed three times with TBST and incubated for 1 hour with alkaline phosphatase-conjugated affinity-purified goat anti-mouse Ig (BioRad Laboratories) (1:3000 dilution in TBST). The nitrocellulose was washed four times with TBST, once with TBS (50 mM Tris-HCl, pH 8.0, 0.15 M NaCl, 0.02% NaN₃) containing 0.05% Tween-20, once with TBS and once with developing buffer (0.1 M sodium bicarbonate (NaHCO₃), pH 9.8, 1 mM magnesium chloride (MgCl₂)). Immunoreactivity was detected colourimetrically by incubating the nitrocellulose in developing buffer containing 3.67 µM p-nitroblue tetrazidium chloride (NBT) and 3.46 µM 5-bromo-4-chloro-3-indolyl-phosphate-toluidine salt (BCIP) for 5 to 30 minutes at room temperature, in the dark (AP Colour Development Reagents, BioRad Laboratories).

**4.4.2 Enzyme-linked immunosorbent assay.**

Enzyme-linked immunosorbent assays (ELA) were executed following methods described in Laferté et al. (1995). Samples were applied, in a final volume of 100 µl, to Immulon 2 microtiter plates (Dynatech Laboratories) and incubated overnight at 4°C. Plates were washed with TBST and blocked at room temperature for 1 hour with TBST containing 4% BSA. After washing with TBST, plates were incubated for 2 hours with 100 µl of monoclonal antibody diluted in TBST, washed in TBST and then incubated for 1 hour with 100 µl of alkaline phosphatase-conjugated goat anti-mouse Ig (1:3000 dilution in TBST)(BioRad Laboratories). Plates were washed three times with TBST, once with TBS supplemented with 0.05% Tween-20, and twice with TBS. Immunoreactive material was detected colourimetrically at 405 nm following the addition of 100 µl of p-nitrophenyl phosphate substrate per well (0.5 mg/ml in 10 mM diethanolamine buffer)(Bio-Rad EIA kit, BioRad Laboratories).

**4.4.3 Immunofluorescence analysis.**

Immunofluorescence analysis was carried out following procedures originally described in Osborn and Weber (1982) with modifications described in Laferté and Loh (1992). Subconfluent cultures were washed twice with CMF-PBS and incubated with 2
ml of PBS containing 5 mM EDTA (PBS/EDTA) in a 37°C incubator until cells were partially detached. The cells were removed from the plate with DMEM containing 10% FBS, washed with CMF-PBS and counted. For analysis of live cells, 2 x 10⁵ cells were pelleted in borosilicate glass tubes (6 x 50 mm) and incubated for 1 hour with a 1:500 dilution of normal mouse serum (NMS) or monoclonal antibody in immunofluorescence (IF) buffer (PBS containing 3% FBS and 5.5 mM glucose). Following four washes with 400 µl IF buffer, the cells were incubated for 30 minutes with fluorescein isothiocyanate (FITC)-labeled goat anti-mouse Ig (Zymed, Dimension Laboratories, Mississauga, ON, Canada), diluted 1:50 in IF buffer. Cells were resuspended in 50 µl PBS:glycerol (1:1), dropped onto glass slides, mounted and examined under a fluorescence microscope (Nikon). For analysis of fixed and permeabilized cells, cells harvested with PBS/EDTA were washed, resuspended in CMF-PBS at a concentration of 2 x 10⁶ cell per ml and 10 µl was spotted per well on 8-well toxoplasmosis slides (Bellco Glass Inc., Vineland, NJ, USA). The slides were air-dried and fixed by incubation at -20°C for 4 minutes with methanol and 2 minutes with acetone. Slides were air-dried and stored at -20°C. Prior to use, slides were washed for 5 minutes in PBS. Slides were incubated with primary antibody and FITC-conjugate as described above. Slides were washed by dipping in three washes of PBS, coverslips were mounted with PBS:glycerol (1:1) and slides were viewed as described above.

4.4.4 Flow Cytometry

Human colon carcinoma cell lines were grown to approximately 70% confluency, washed with CMF-PBS and the cells removed from the culture plates with trypsin-EDTA, as described in Section 4.1. The cells were washed three times with CMF-PBS and 1 x 10⁶ cells were resuspended in 1 ml PBS, 0.1% BSA, 0.2% NaN₃ (FC buffer) and incubated with ascites at a 1:300 final dilution for 1 hour on ice. The cells were subsequently washed three times with FC buffer and incubated for 30 minutes on ice with 100 µl of a 1:50 dilution of FITC-conjugated goat anti-mouse Ig (Zymed, Dimension
Laboratories). The cells were washed three times with FC buffer, resuspended in 1 ml of FC buffer, filtered through 100 μm nylon mesh and kept on ice until use. Flow cytometry was carried out using an EPICS XL flow cytometer (Coulter, Miami Lakes, FLA, USA).

4.4.5 Immunohistochemical staining of normal and neoplastic tissues.

Five micron-thick sections were deparaffinized and prepared for immunohistochemical staining, as previously described (Hsu, et al. 1981). Briefly, sections were incubated in two xylene baths for 10 minutes each, rehydrated by incubating in 100%, 95%, 70% ethanol and tap water and washed in PBS. Sections were either pre-incubated with 0.5% hydrogen peroxide for 30 minutes or directly blocked with 4% heat inactivated normal goat serum (NGS) (4% NGS/PBS) and subsequently incubated for 1 hour at room temperature or overnight at 4°C with either NMS or monoclonal antibody (1:500 dilution in 4% NGS/PBS). Sections were washed with PBS, incubated for 30 minutes with biotinylated goat anti-mouse IgM (1:500 dilution in 4% NGS/PBS)(Jackson Laboratories, Bio/Can Scientific, Mississauga, ON, Canada) followed by incubation for 1 hour with avidin-biotin-complex (ABC reagent), according to manufacturer's instructions (Vector, Dimension Labs, Mississauga, ON, Canada). Immunoreactivity was detected by incubating sections for 5 minutes with 2.8 mM 3,3'-diaminobenzidine peroxidase substrate (DAB, Sigma). Sections were counterstained with Meyer's hematoxylin for 3.5 minutes, dehydrated by dipping 10 times in 70%, 75%, 95%, 100% ethanol and xylene and mounted in Entellan (BDH, Saskatoon, SK, Canada).

4.4.6 Western blotting analysis.

Proteins were transferred to nitrocellulose by wet transfer methods using a Mini-Protean II Trans-Blot Transfer Cell (BioRad Laboratories) (Tobwin et al. 1979). Briefly, proteins separated by SDS-PAGE were transferred onto nitrocellulose by electrophoresis for 1 hour at 100 volts in buffer containing 25 mM Tris, 188 mM glycine and 20%
methanol. The nitrocellulose blots were then blocked for 1 hour at room temperature or overnight at 4°C in TBST/4% BSA, washed with TBST and incubated for 2 or 3 hrs with either NMS, primary antibody (1:3000 dilution in TBST) or biotinylated lectin (0.4 μg/ml dilution in TBST, E-Y Labs). Following washes with TBST, antibody blots were incubated with either alkaline phosphatase-conjugated goat anti-mouse Ig or alkaline phosphatase-conjugated goat anti-rabbit Ig (1:3000 diluted in TBST) for 1 hour. Blots treated with biotinylated lectins were washed with TBST and subsequently incubated for 20 minutes with streptavidin-conjugated goat anti-mouse Ig (1:4000 diluted in TBST, Gibco-BRL). Membranes incubated with the lectin L-PHA (0.4 μg/ml dilution in TBST, Sigma) were washed with TBST, incubated for 1 hour with rabbit anti-L-PHA (1:3000 dilution in TBST), washed with TBST and subsequently incubated for 1 hour with alkaline phosphatase-conjugated goat anti-rabbit Ig (1:3000 diluted in TBST). The substrates NBT and BCIP were used for colour development (BioRad Laboratories), as described in Section 4.4.1.

4.5 Haemagglutination assay.

Haemagglutination assays were carried out as described in Widmann (1985) and performed by the staff at the Royal University Hospital (Saskatoon, SK, Canada). Briefly, appropriate dilutions of monoclonal antibody ascites were mixed with a 4% suspension of human A1, A2, B or O erythrocytes, mixed by gentle shaking and centrifuged. Tubes were inspected for the degree of agglutination and the minimum antibody concentration required to achieve agglutination was determined.

4.6 Preparation of blood group containing neoglycoproteins.

H type 2-BSA and A type 2-BSA conjugates were gifts from Dr. Monica Palcic (University of Alberta, Edmonton, AB, Canada). H type 2 oligosaccharides with an 8-methoxycarbonyl aglycone were chemically synthesized following a procedure described
by Hindsgaul et al. (1982), enzymatically converted to the A type 2 structure (Compston et al., 1993) and coupled to bovine serum albumin (BSA) via their acyl azide, as described by Pinto and Bundle (1983). The carbohydrate content of the neoglycoproteins was determined using the phenol-sulfuric assay (Dubois et al., 1956) with A type 2 as a reference standard. An incorporation of 57 moles of oligosaccharide per mole of BSA was achieved (n=57). The other neoglycoproteins were generous gifts from the Alberta Research Council (Edmonton, AB, Canada). The incorporations achieved for the compounds were as follows: H type 2-BSA (n=19); A disaccharide-BSA (n=17); A type 1-BSA (n=13); A type 4-BSA (n=17); A type 6-BSA (n=13); B disaccharide-BSA (n=19); B type 2-BSA (n=18); B type 4-BSA (n=14); B type 5-BSA (n=15); B-type 6-BSA (n=16). Neoglycoproteins were diluted to a standard concentration of 50 nmol oligosaccharide per ml. Appropriate dilutions were applied to 96-well microtitre plates for EIA (Section 4.4.2) or digested with glycosidases (Section 4.18).

4.7 Induction of rat colon tumors with azoxymethane.

Female Sprague-Dawley rats (6-8 weeks old) were administered 8 weekly intraperitoneal injections of azoxymethane (15 mg/kg, Sigma) (Bird et al., 1985). Four to six months later, animals were sacrificed, the intestine removed and washed in ice-cold PBS. Tumors were detected in the small intestine as well as the colon. Tumors from the colon were excised and divided into two equivalent sections. One half of the sample was fixed in 10% formaldehyde in PBS and embedded in paraffin while the other half was frozen immediately and stored at -70°C. Five micron-thick sections of rat colon tumors were examined histologically following staining with hematoxylin and eosin and classified according to the Astler-Coller modification of Duke's staging system for colorectal cancer (Astler and Coller, 1954).
4.8 *Collection of intestinal tissues from untreated rats.*

The large and small intestines were removed from female Sprague Dawley rats (4-6 months of age), washed in ice cold phosphate buffered saline (PBS) and stored at -70°C. Alternatively, the large intestine was subdivided into the caecum, ascending and descending colon and the mucosal tissue was collected from each segment by scraping the tissue with a glass slide. Representative samples of normal intestine were collected, fixed in 10% formaldehyde in PBS and embedded in paraffin.

4.9 *Lectin affinity chromatography.*

4.9.1 *Concanavalin A-Sepharose affinity chromatography.*

*Concanavalin A* (Con A)-Sepharose lectin chromatography was carried out as described by Merkle and Cummings (1987a). Desalted, lyophilized glycopeptide (25 mg) was resuspended in 1 ml Con A Buffer (0.1 M Tris-HCl, pH 7.4, 0.5 M NaCl, 1 mM manganese chloride (MnCl₂), 1 mM calcium chloride (CaCl₂), 0.02% NaN₃) and applied to a 8 ml Con A-Sepharose column (13 mg lectin/ml gel, Pharmacia Biotech, Montreal, PQ, Canada) equilibrated with Con A buffer. The sample was allowed to enter the resin and bind for 2 hours at room temperature. Unglycosylated peptides and glycopeptides containing complex-type Asn-linked oligosaccharides or O-linked oligosaccharides which do not bind Con A-Sepharose, were removed by washing with 10 volumes of Con A buffer (Ogata et al., 1975). The Con A-Sepharose column was eluted with 5 volumes of Con A buffer containing 10 mM methyl α-D-mannopyranoside (Sigma) followed by 5 volumes of Con A buffer containing 100 mM methyl-α-D mannopyranoside, to remove bound glycopeptides containing biantennary/hybrid Asn-linked oligosaccharides and high-mannose type Asn-linked oligosaccharides, respectively (Ogata et al., 1975). The methyl-mannopyranoside solutions were prewarmed to 60°C to increase the efficiency of elution. Fractions of 2 ml were collected throughout the procedure, assayed for
immunoreactivity, pooled, lyophilized, desalted and subjected to further chromatographic procedures.

4.9.2 *Dolichos biflorus*-agarose affinity chromatography.

Samples (0.5-1 ml) were resuspended in DBA buffer (TBS pH 7.4, 0.02% NaN₃) and allowed to enter the resin of a *dolichos biflorus* (DBA)-agarose column (4 mg lectin/ml gel, E-Y Labs, Intermedico, Markham, ON, Canada) pre-equilibrated in the aforementioned buffer. Unbound material was collected by washing the column with 10 volumes of DBA buffer adjusted to 0.5 M NaCl and bound material was eluted from the column with 100 mM N-acetyl-D-galactosamine (Sigma) in DBA buffer. Fractions of 0.5 or 1 ml were collected and assayed for immunoreactivity and protein content. A 2 ml DBA-agarose column was used in glycopeptide separation whereas columns of 5 and 10 ml of packed gel were used for glycoprotein separation. For the separation of detergent-solubilized samples, 0.1% 3-[(3-Cholamidopropyl)-dimethylammonio]-1-propane-sulfonate (CHAPS) (Sigma) was included in all buffers.

4.9.3 *Helix pomatia*-agarose affinity chromatography.

Samples (1 ml) were applied to a 5 ml column of *helix pomatia* (HPA)-agarose (1.9 mg lectin/ml gel, Sigma) pre-equilibrated in HPA buffer (TBS pH 7.4, 0.1% CHAPS, 0.02% NaN₃). After extensive washing of the column with 10 volumes of HPA buffer adjusted to 0.5 M NaCl, the bound material was eluted with 5 volumes of HPA buffer containing 100 mM N-acetyl-D-galactosamine. Fractions of 1 ml were collected and assayed for immunoreactivity. Immunoreactive fractions were pooled and concentrated to 0.5 ml using a Centricon 10 microconcentrator (Amicon).

4.9.4 Wheat germ agglutinin-Sepharose affinity chromatography.

A 17.5 ml wheat germ agglutinin (WGA)-Sepharose column was prepared by coupling WGA lectin (*triticum vulgaris*, Sigma) to CNBr-activated Sepharose 4B (Pharmacia Biotech) (Bassett, 1975). Briefly, 5 g of Sepharose 4B powder was resuspended in 1 mM HCl and washed for 15 minutes on a sintered glass filter with 1
mM HCl. WGA lectin (25 mg) was resuspended in 5 ml of coupling buffer (0.1 M NaHCO₃, pH 8.0, 0.5 M NaCl), mixed with the washed Sepharose 4B bead suspension and 1% N-acetyl-D-glucosamine (GlcNAc, Sigma) and allowed to incubate for 2 hours at room temperature with gentle mixing. The WGA-Sepharose gel was washed with coupling buffer and incubated for 1 hour in 100 ml of 0.1 M Tris-HCl, pH 8.0 to block remaining amino groups. After blocking, the gel was washed with coupling buffer (50 ml), followed by three alternating cycles of 0.1 M sodium acetate, pH 4.0, 0.5 M NaCl (50 ml) and 0.05 M Tris-HCl, pH 8.0, 0.5 M NaCl (50 ml). After the final wash, the gel was resuspended in 50 mM Tris-HCl pH 8.0, packed in a 1 x 20 cm column and equilibrated in 50 mM Tris-HCl, 0.5 M NaCl, 0.5% CHAPS, 0.02% NaN₃. Cell lysates were applied to the WGA-Sepharose column and allowed to enter the resin. The column was subsequently washed with 20 column volumes of WGA buffer (50 mM Tris HCl pH 7.4, 0.5 M NaCl, 0.1% CHAPS, 0.02% NaN₃) and eluted with 5 column volumes of WGA buffer containing 200 mM N-acetyl-D-glucosamine. Immunoreactive fractions were detected by Western blotting, dot blot and EIA as described in Sections 4.4.6, 4.4.1 and 4.4.2, respectively. Immunoreactive fractions were pooled and concentrated to 1 ml using a Centricon 10 microconcentrator. The unbound material was reapplied to WGA-Sepharose until the unbound fraction no longer contained immunoreactive material.

4.10 Gel filtration chromatography.

Samples of 0.5 ml were loaded onto the gel filtration resins, Biogel P6 (1.5 x 120 cm, BioRad Laboratories) or Biogel A5m (1.5 x 120 cm, BioRad Laboratories), equilibrated in sterile distilled water or TBS pH 7.4, 0.1% CHAPS, 0.02% NaN₃, respectively. Columns were maintained at 4°C. Proteins were eluted at a flow rate of 0.05 ml per minute in the aforementioned buffers and collected in 1.6 ml fractions. Fractions were assayed for immunoreactivity and immunoreactive fractions were pooled and concentrated. Biogel P6 column fractions were also assayed for sugar content using
the phenol-sulfuric assay for hexose detection (Dubois et al., 1956). Briefly, 100 µl aliquots of column fractions were incubated for 30 min at room temperature in new borosilicate glass tubes (15 x 100 mm) containing 0.3 ml of 5% phenol solution and 2 ml of concentrated sulfuric acid. The absorbance was read at 484 nm using a Spectronic 700 spectrophotometer (Bausch and Lomb).

4.11 Preparation of total glycopeptide fraction.

Glycopeptides were isolated from rat intestine as described by Finne and Krusius (1982). The tissue (57.9 g) was delipidated by extraction with four volumes of chloroform:methanol (2:1) for 30 minutes at room temperature and then filtered through Whatman #42 filter paper. The extraction procedure was repeated a second time and the residue was allowed to dry. The dried, delipidated tissue was then resuspended at a protein concentration of 50 mg/ml in digestion buffer (0.1 M Tris-HCl, pH 8.0, 1 mM CaCl₂, 0.02% NaN₃) and digested for 72 hours at 60°C with 3% (w/w) Protease E (Type XIV from Steptomyces griseus, Sigma) that had been pre-incubated at 60°C for 15 minutes. The protease was replenished every 24 hours. The digest was diluted 1:1 with water and adjusted to 0.4 M NaCl in preparation for the removal of polyanionic material by dropwise addition of 0.1 volume of 0.1 M cetylpyridinium chloride (C₂₁H₃₈NCl) solution containing 0.1 M sodium sulfate (Na₂SO₄). Following a 20 minute incubation at 37°C, the digest was clarified by centrifugation at 15 000 g for 15 min and filtered through Whatman #1 filter paper. The filtrant was cooled to 4 °C, precipitated with 0.1 volume of cold 0.1 M sodium thiocyanate (NaSCN) overnight at 4 °C and centrifuged at 20 000 g for 30 min to separate the excess cetylpyridinium salt from the glycopeptides. Samples were lyophilized and desalted by gel filtration chromatography using a Biogel P2 column (2.5 x 50 cm, BioRad Laboratories) equilibrated in 25 mM pyridine acetate, pH 5.0. Lyophilized glycopeptides were resuspended in 2 ml of 25 mM pyridine acetate, applied to the column and eluted with 5 volumes of 25 mM pyridine acetate, pH 5.0.
Fractions of 2 ml were collected and assayed for immunoreactivity by dot blot using mAb 3A7 (refer to Section 4.4.1). Immunoreactive fractions were pooled, lyophilized and subjected to lectin affinity chromatography first on Con A Sepharose and subsequently on DBA-agarose, as described in Section 4.9.

4.12 Preparation of detergent-solubilized cell and tissue lysates.

Detergent-solubilized cell and tissue lysates were prepared either from whole cell and tissue pellets or from microsomes of cells and tissues. Microsomes were isolated using methods described in Simonds et al. (1980) as follows. Cells and tissues were homogenized in four volumes of 50 mM Tris-HCl, 0.25 M sucrose, 1 mM ethylenediaminetetraacetic acid (EDTA), 1 mM phenylmethylsulfonyl fluoride (PMSF, Sigma), 1 mM benzamidine (Sigma), 0.02% NaN₃ at 4°C using a Polytron homogenizer (Brinkmann Instruments, Edmonton, AB, Canada) and centrifuged at 8000 g for 6 minutes to remove cellular debris. Microsomes were collected by centrifugation of the supernatant at 100 000 g for 1 hour at 4°C. The microsomal pellets were either resuspended in 0.5-1 ml of 10 mM HEPES (Gibco-BRL), pH 7.6, 0.25 M sucrose and stored at -70°C until use or solubilized immediately, as described below.

Cell pellets, tissues and microsomes, with the exception of those used for glycosyltransferase assays, were extracted in ten volumes of extraction buffer containing TBS pH 7.4, 1 mM PMSF, 1 mM benzamidine, 0.5 μM leupeptin (Sigma), 0.7 μM pepstatin (Sigma), 10 μg/ml aprotinin (Sigma) supplemented with 1% Triton X-100 (BioRad Laboratories) or 0.5% CHAPS (Sigma) for 1 hour on ice. Cell and tissue microsomes used in glycosyltransferase assays were resuspended in 10 mM HEPES, 0.25 M sucrose and solubilized with two volumes extraction buffer consisting of 50 mM HEPES, pH 7.0, 1 mM EDTA, 0.25% glycerol supplemented with 0.2% Triton X-100 for 1.5 hours in a Wheaton homogenizer (Stroup et al., 1990). Detergent lysates were clarified by centrifugation at 20 000 g for 30 minutes.
4.13 Determination of protein concentration.

The concentration of protein in samples was determined using the BioRad DC Protein Assay Kit (BioRad Laboratories) based on original methods described by Lowry et al. (1951). Briefly, appropriate dilutions of sample in a final volume of 50 μl, were incubated at room temperature for 30 minutes with 200 μl of alkaline copper tartrate solution (reagent A: supplemented with 20 μl of reagent S per ml of reagent A when detergent was present in the samples) and 2 ml of Folin reagent (reagent B). The absorbance was measured at 750 nm. Gamma-globulin was used to generate a standard curve ranging from 0.2 to 1 mg/ml.

4.14 Isolation of the 140 kDa glycoprotein from HT29 cells.

HT29 cells (60 ml packed cells) were lysed by repeated freeze/thawing of the cells in four volumes of 50 mM Tris-HCl, pH 7.4, 0.25 M sucrose, 1 mM EDTA, 1 mM PMSF, 1 mM benzamidine, 0.02% NaN3. Cellular debris was removed by centrifugation at 8,000 g for 6 minutes and the microsomal fraction was collected after centrifugation at 100,000 g for 1 hour. The microsome pellet was solubilized on ice for 1.5 hours in 10 volumes of extraction buffer containing 0.5% CHAPS. The HT29 detergent lysate was clarified by centrifugation at 20,000 g for 45 minutes and applied directly to a WGA-Sepharose column (17.5 ml gel bead) equilibrated in 50 mM Tris-HCl, pH 7.4, 0.5 M NaCl, 0.5% CHAPS, 0.02% NaN3. WGA-Sepharose affinity column chromatography was performed, as described in Section 4.9.4. Immunoreactive fractions were detected by Western blotting, dot blot and EIA, as described in Sections 4.4.6, 4.4.1 and 4.4.2, respectively. Immunoreactive material, which was present exclusively in the eluted fractions of WGA-Sepharose, was pooled, dialyzed exhaustively in buffer containing TBS, pH 7.4, 0.1% CHAPS, 0.02% NaN3 and concentrated to 1 ml using a Centricon 10 microconcentrator. WGA-Sepharose eluted material was applied to a 10 ml column of DBA-agarose equilibrated in TBS pH 7.4, 0.1% CHAPS, 0.02%
NaN₃ and allowed to bind to the resin overnight at 4°C. The column was subsequently washed and bound material was eluted with 100 mM GalNAc, as described in Section 4.9.2. Column fractions were assayed by Western blotting, dot blot and EIA with mAb 3A7 (1:3000 dilution). Immunoreactive material which failed to bind DBA-agarose, following repeated application, was concentrated to 1.0 ml using a Centricon 10 microconcentrator and applied to a 5 ml HPA-agarose column pre-equilibrated in TBS pH 7.4, 0.1% CHAPS, 0.02% NaN₃. The HPA-agarose column was washed and bound material was eluted with 100 mM GalNAc, as described in Section 4.9.3. Column fractions were assayed by Western blotting and EIA with mAb 3A7 (1:3000 dilution) and immunoreactive material, present exclusively in the eluted fractions, was pooled and concentrated to 0.5 ml with a Centricon 10 microconcentrator. HPA-agarose eluted material was further purified by gel filtration chromatography on a Biogel A5m column (1.5 x 120 cm) equilibrated in TBS pH 7.4, 0.1% CHAPS, 0.02% NaN₃, as described in Section 4.10. Fractions of 1.6 ml were collected and monitored for protein content at absorbance of 280 nm and immunoreactive material by Western blotting and EIA. Immunoreactive fractions were pooled and concentrated to 0.5 ml.

4.15. Metabolic labeling of cells.

Cell monolayers grown to 70-80% confluency were washed with CMF-PBS and incubated for 1 hour in methionine-free RPMI media (Gibco-BRL) containing 10 mM HEPES prior to addition of [³⁵S]-methionine. Cells were incubated for 24 hours in methionine-free RPMI media containing 10% FBS, 100 μM unlabeled methionine (Gibco-BRL) and 50 μCi/ml [³⁵S]-methionine (NEN, DuPont). The plates were washed three times with cold PBS and the cells were harvested with a rubber policeman and pelleted. Cell pellets were solubilized with 1% Triton X-100 as described in Section 4.12.
4.16 Treatment of cells with glycosylation inhibitors.

HT29 cells were incubated in the absence or presence of the glycosylation inhibitors 1-deoxymannojirimycin (DMJ) (50 μM, Calbiochem, La Jolla, CA, USA), swainsonine (1.73 μM, Boehringer Mannheim, Montreal, PQ, Canada), tunicamycin (6.13 μM, Boehringer Mannheim) (Elbein, 1987), benzyl-α-N-acetyl-D-galactosamine (BzαGalNAc) (2 mM, Sigma) or benzyl-α-N-acetyl-glucosamine (BzαGlcNAc) (2 mM, Sigma) (Kuan et al., 1989). In experiments using radiolabeled cells, the drug was added twenty-four hours prior to the addition of [35S]-methionine and maintained in the culture medium throughout the radiolabeling period. For unlabeled cells, the drug was maintained in the culture medium for 72 hours, with replenishment every 24 hours. Since the stock solution of tunicamycin included dimethylsulfoxide (DMSO, Sigma) as diluent, a plate containing an equivalent amount of DMSO was included as a control.

4.17 Immunoprecipitation analysis.

Unlabeled samples (30-100 μg of protein) or radiolabeled cell lysates (5 x 10^6 cpm) were incubated overnight at 4°C with 3 μl of normal mouse serum, 3 μl of ascites or 0.5 ml of hybridoma supernatant. Following incubation, 100 μl of a 1:1 suspension of protein A Sepharose-4B beads (Pharmacia Biotech) in immunoprecipitation (IP) buffer (50 mM Tris-HCl pH 8.0, 1 mM PMSF, 1 mM benzamidine, 1% deoxycholate (Sigma), 1% Triton X-100, 0.02% NaN₃) was added and the suspension was gently rocked at 4°C for 1 hour. The beads were washed twice with IP buffer containing 0.5 M NaCl, twice with IP buffer containing 0.1% SDS and once with IP buffer. The immunoprecipitated proteins were eluted by either boiling for 5 min in SDS-PAGE sample buffer (0.125 M Tris-HCl pH 6.8, 2% SDS, 2% 2-mercaptoethanol, 10% glycerol) or boiling for 1 min in enzyme buffer prior to glycosidase digestion (Section 4.18) (Laferté and Loh, 1992).
4.18 Glycosidase digestion.

Immunoprecipitated proteins or BSA glycans (5-10 pmole) were resuspended in 30 μl of the appropriate glycosidase digestion buffer, incubated for 1-2 minutes at 100°C and allowed to cool. Samples were digested for indicated lengths of time at 37°C. Alternatively, samples to be digested with glycosidases were excised from the dried, unfixed gel and rehydrated in glycosidase buffer. Samples were boiled for 5 minutes, cooled and incubated with enzyme. Digested samples were either adjusted to 1X SDS sample buffer, boiled for 5 minutes and separated by SDS-PAGE (Section 4.3) or diluted to 100 μl with TBS and applied to Immulon 2 microtiter plates for EIA (Section 4.4.2). The glycosidases, their respective buffers and incubation times are as follows: (a) neuraminidase type X from Clostridium perfringens, 0.1 unit (Sigma) incubated in 25 mM Tris-malate, pH 5.0, 1 mM calcium acetate, 0.1% SDS, 1% Triton X-100 for 2 or 16 hours (Saito et al., 1979), (b) N-glycanase, 0.3 unit (Genzyme, Cambridge, MA, USA) incubated in 50 mM sodium phosphate, pH 7.4, 0.1% SDS, 1% 2-mercaptoethanol, 1% Triton X-100, 50 mM EDTA, pH 7.4, 4 mM benzamidine for 0.25, 0.5, 1, 2, 4, 16 or 24 hours (Chu, 1986), (c) O-glycanase, 0.3 unit (Genzyme) incubated in 25 mM Tris-malate, pH 5.8, 0.1% SDS, 1 mM calcium acetate, 1% Triton X-100 for 16 hours (Adolf et al., 1991), (d) endo-β-galactosidase, 0.01 unit (ICN Biomedicals Ltd., Montreal, PQ, Canada) incubated in 50 mM sodium acetate, pH 5.8, 0.1% SDS, 1% 2-mercaptoethanol, 1% Triton X-100 for 16 hours (Scudder et al., 1984), (e) endoglycosidase H (endo H), 1 unit (Boehringer-Mannheim) incubated in 50 mM sodium acetate, pH 5.5, 0.1% SDS, 1% 2-mercaptoethanol, 1% Triton X-100 for 16 hours (Schwarz and Elbein, 1986), (f) α-N-acetyl-D-galactosaminidase from Acremonium sp., 2 units (Seikagaku, PDI BioScience, Calgary, AB, Canada) incubated in 0.1 M sodium citrate buffer, pH 4.5 for 24 hours (Salimath et al., 1995) and (g) α-D-galactosidase from green coffee beans, 5 units (Oxford GlycoSystems, Rosedale, NY, USA) incubated in 0.1 M sodium phosphate citrate, pH 6.5 for 24 hours (Salimath et al., 1995).
4.19 Limited proteolytic peptide mapping.

Limited proteolytic peptide mapping was carried out as described by Cleveland et al., (1977) and modified as previously described (Loh, 1991). [35S]-methionine labeled detergent solubilized cell lysates were immunoprecipitated with NMS or ascites (Section 4.17) and separated on 6% SDS-polyacrylamide gels (Section 4.3). Unstained, unfixed gels were dried and exposed to X-ray film (Kodak X-omact ARS). Gel slices corresponding to the desired bands on the autoradiograph were excised, rehydrated in buffer consisting of 0.125 M Tris-HCl, pH 6.8, 1 mM EDTA, 0.1% SDS, 30% glycerol and placed in wells of a 1.5 mm thick SDS-polyacrylamide gel. The gel slice was covered with staphylococcal V8 protease (0.1 µg/well, ICN Biomedicals Canada, Ltd.) in enzyme buffer (0.125 M Tris-HCl, pH 6.8, 1 mM EDTA, 0.1% SDS, 10% glycerol). The proteins were electrophoresed through the stacking gel for 4 hours at 12 mA to allow proteolysis of the proteins, followed by separation of the peptides in a 15% SDS-polyacrylamide gel. The gel was stained with Coomassie Blue R-250, destained, dried and bands were visualized by fluorography as described in Section 4.3.

4.20 Isolation of gp140 for partial protein sequencing.

A 5 ml pellet of HT29 cells was extracted in 4 volumes of IP buffer containing 1% Triton for 1 hour on ice and then clarified by centrifugation at 20 000 g for 45 minutes at 4°C. The detergent lysate was immunoprecipitated overnight at 4°C with 3 µl mAb 7A8 per ml of extract. One hundred microliters of a 1:1 suspension of protein A Sepharose per ml of sample was added to the immunoprecipitates and incubated for 1 hour at 4°C. The protein A Sepharose beads were washed as described in Section 4.17, the immunoprecipitated proteins eluted with SDS-PAGE sample buffer and separated on 6% SDS-polyacrylamide gels. The proteins were electrophoretically transferred at 100 volts for 1 hour to an Immobilon-P membrane (Millipore, Missassauga, ON, Canada) pre-washed in methanol. The membrane containing immobilized proteins was washed
with distilled water and stained with 0.1% Ponceau S (Sigma) in 0.1% acetic acid for 2 minutes. The membrane was washed with 0.1% acetic acid until protein bands were visible. The two protein bands at approximately 135 and 145 kDa were excised and the membrane was washed repeatedly with distilled water. The excised bands were frozen at -20°C and later sent to Dr. Bill Lane at Harvard BioChem (Harvard University, Boston, MA, USA) for protein sequence analysis. Each sample was digested with trypsin and the resultant peptides separated by HPLC using a reversed-phase column. Three peptides from the 135 kDa species and two peptides from the 145 kDa species were sequenced.

4.21 Assay of glycosyltransferase activity.

Glycosyltransferase assays were performed using methods described by Palcic et al. (1988). Microsomes isolated from rat intestine, rat colon tumors or human colon carcinoma cell lines were prepared and solubilized in HEPES buffer containing 0.2% Triton X-100 as described in Section 4.12. Detergent lysates were incubated with appropriate radiolabeled sugar donor and appropriate hydrophobic acceptor at 37°C for 30 minutes to 3 hours. The reaction was terminated by addition of 0.5 ml of distilled water. Reaction mixtures were loaded onto C18 Sep-Pak Cartridges (Waters, Mississauga, ON, Canada) pre-equilibrated in distilled water. Unbound material was removed by washing the Sep-pak columns with 50 ml of distilled water. Bound products were eluted with 3.5 ml of methanol, collected in scintillation vials and counted to determine the number of disintegrations per minute (dpm). The specific activity (nmoles of product/minute/mg of protein at 37°C) of each glycosyltransferase was determined in each sample. All carbohydrate acceptors and GDP-fucose donor were generous gifts from Dr. O. Hindsgaul (University of Alberta, Edmonton, AB, Canada). All other nucleotide sugar donors were purchased from Sigma and the radiolabeled nucleotide sugars were obtained from American Radiolabeled Chemicals (St. Louis, MO, USA). The specific reaction conditions for the glycosyltransferases assayed are as follows:
4.21.1 α1-3 N-acetylgalactosaminyltransferase (A transferase) assay.

Ten microlitres of detergent lysate was incubated for 1 hour (16 hours for human colon cancer cell extracts) in a reaction mixture (final volume 33 μl) containing 50 mM sodium cacodylate, pH 6.9, 20 mM MnCl₂, UDP-GalNAc donor (96 μM) plus [³H]UDP-GalNAc (approximately 70 000 dpm) and αFuc(1→2)βGal-O-(CH₂)₈COOCH₃ (Fucose-Galactose-R) (80 μM) or αFuc(1→2)βGal(1→4)βGlcNAc-O-(CH₂)₈COOCH₃ (H type II-R) (800 μM) acceptor (Compston et al., 1993).

4.21.2 α1-2 fucosyltransferase (FucT) assay.

Twenty microlitres of detergent lysate was incubated for 90 minutes in a reaction mixture (final volume 40 μl) consisting of 20 mM HEPES, pH 7.0, 20 mM MnCl₂, 0.2% BSA, 50 μM GDP-fucose donor containing approximately 60 000 dpm of [³H]GDP-fucose and 3 mM phenyl-β-D-galactopyranoside (Sigma), 2.5 mM βGal(1→3)β[4 deoxy]GlcNAc-O-(CH₂)₈COOCH₃ (4-deoxy LeC) or 2.5 mM βGal(1→4)β[3 deoxy]GlcNAc-O-(CH₂)₈COOCH₃ (3-deoxy LacNAc) acceptor, for assaying total α1-2 Fuc T activity, α1-2 FucT activity specific for type 1 chains and α1-2 FucT activity specific for type 2 chains, respectively (Palcic et al., 1988).

4.21.3 N-acetylglucosaminyltransferase I (GnTI) assay.

Fifteen microlitres of detergent lysate was incubated for 1 hour in reaction mixture (final volume 20 μl) consisted of 20 mM HEPES, pH 7.0, 20 mM MnCl₂, 0.2% BSA containing 700 μM 3,6-di-O-(α-D-mannopyranosyl)-β-D-mannopyranoside-O-(CH₂)₈COOCH₃ (Trimannoside-R) acceptor and 400 μM UDP-N-acetylglucosamine (GlcNAc) donor containing approximately 200 000 dpm of [³H]UDP-GlcNAc (Palcic et al., 1988).
4.21.4 β1–6 N-acetylglicosaminytransferase (Core 2 transferase) assay.

Reaction mixtures containing 2 μM UDP-GlcNAc donor with approximately 2 x 10^5 dpm [³H]UDP-GlcNAc, 12.5 μM βGal(1→3)αGalNAc-O-(CH₂)₃COOCH₃ (Tn-R) acceptor and 40 μM GlcNAc were lyophilized to dryness and resuspended in 30 μl of buffer containing 100 mM HEPES, pH 6.5, 2% Triton X-100. The reaction was initiated by addition of 50 μl of detergent lysate and extended for 90 minutes.

4.21.5 β1–3 galactosyltransferase (Core 1 transferase) assay.

Twenty microlitres of detergent lysate was incubated for 3 hours in a reaction mixture (final volume of 50 μl) consisting of 343 mM sodium cacodylate, pH 7.0, 0.0675% Triton X-100, 68 mM MnCl₂, 0.135% BSA, 500 μM UDP galactose with 1 x 10^5 dpm [³H]UDP-galactose and 1 mM β-N-acetyl-galactopyranoside-O-(CH₂)₈COOCH₃ (GalNAc-R) acceptor.

4.21.6 Galactosyltransferase assay.

Twenty microlitres of detergent lysate was incubated for 1 hour in a reaction mixture (final volume of 55 μl) consisting of 250 mM sodium cacodylate, pH 7.4, 25 mM MnCl₂, 743 nM NaCl, 182 μM UDP-galactose with 105 000 dpm [³H]UDP-Galactose and 372 μM β-N-acetyl-glucopyranoside-O-(CH₂)₈COOCH₃ (GlcNAc-R) acceptor (Palcic et al., 1988).

4.22 Cell adhesion assays.

Adhesion assays were performed as described by Takada et al. (1988) with the following modifications. Extracellular matrix (ECM) proteins, laminin-1 (Ln-1) (Sigma), fibronectin (Fn) (Sigma), collagen Type I (C-I) (Sigma) and collagen Type IV (C-IV) (Sigma) were coated onto wells of Immulon 2 microtitre plates by incubating 0.01 - 5 μg of protein per well in 50 μl PBS overnight at 4°C. The wells were washed three times with PBS and blocked with 1% BSA in PBS for 60 minutes at 37°C. Human colon carcinoma cells were harvested by standard trypsinization protocols as described in
Section 4.1, washed twice with DMEM and counted. Aliquots of $1 \times 10^5$ cells in DMEM were added to each well of the coated Immunolon 2 plates and incubated for 6 hours in a 37°C incubator. Non-adherent cells were removed by washing several times with 200 μl of PBS. The plates were washed until the BSA control wells were free of cells. The adherent cells were stained with 1% crystal violet in methanol for three minutes, washed with distilled water and allowed to air dry overnight. The absorbance was read at 565 nm using a BioRad EIA plate reader. In some experiments, cells were incubated with IgG purified from NMS or monoclonals 7A8, 7B11, 8H7 (refer to Section 4.2.1). In these assays, the cells were incubated with 0.001, 0.1, 10 or 1000 ng purified IgG per $1 \times 10^5$ cells, for 30 minutes on ice prior to addition to adhesion plates. Purified IgG was maintained in the media throughout the duration of the assay.

4.23 Cell migration and invasion assays.

Migration and invasion assays were performed using 6.5 mm Transwell cell culture chambers with pore size of 8 μm (#3422 Costar, Corning, Fisher Scientific, Edmonton, AB, Canada) as described (Jasiulionis et al., 1996) with the following changes. For migration assays, the underside of the filter was coated with ECM protein by incubation for 2 hours at room temperature in a solution of ECM proteins diluted to 50 μg/ml with DMEM. Filters were removed from the substrate solution, washed three times with CMF-PBS and blocked with 0.5% BSA in PBS for 1 hour in a 37°C incubator. Following three washes in PBS, the filters were placed in 24-well cluster dishes (Costar, Corning) containing 600 μl of serum-free F12/DMEM media (Gibco-BRL) supplemented with 2 μg/ml insulin, 2 μg/ml transferrin, 0.2 x10^{-9} M T3 and 20 ng/ml EGF (SF media). One hundred microliters of SF media containing $2 \times 10^5$ cells was added to the top of the filter and cells were allowed to migrate to the underside of the filter for 24 hours. The extent of cell migration was assayed colourimetrically using the MTT assay as described by Imamura et al. (1994). Briefly, 20 μl of a 5 mg/ml MTT (3-
[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; thiazolyl blue, Sigma) solution was added to the bottom chamber of the transwell and incubated for 4 hours in a 37°C incubator. Non-migrating cells were removed from the top of the filter using a cotton swab. The blue MTT-formazan crystals produced were dissolved by transferring the filter to a second 24-well plate containing 200 µl of DMSO per filter. Alternately, cells on the top and the bottom of the filter were assayed colourimetrically to determine total cells. The absorbance of the dissolved formazan solution was read at 565 nm and the ratio of migrating cells to total cells was calculated.

For cell invasion assays, the wall of the upper well of the transwell chamber was coated with melted paraffin wax, dried and incubated with 70 µl Matrigel (Collaborative Research Inc., Bedford, MA, USA) (0.2 mg/ml dilution in distilled water) overnight in a laminar flow hood. Fifty microliters of SF medium containing 2 x 10^5 cells was added to the upper well. Conditioned medium (600 µl) derived from 3T3 Swiss albino fibroblasts was placed in the lower well as a chemoattractant (Muir et al., 1993). Following incubation of the chambers in a 37°C CO₂ incubator for 72 hours, the non-invasive cells were removed with a cotton swab and the extent of cell invasion was determined using the MTT assay, as described above for the migration assay.

In some migration and invasion assay experiments, cells were incubated with IgG purified from NMS or monoclonal antibodies 7A8, 7B 11 or 8H7 (refer to Section 4.2.1). In these assays, the cells were incubated with purified IgG (0.1, 10 or 1000 ng protein per 2 x 10^5 cells) for 30 minutes prior to addition to chambers. Purified IgG was maintained during the assays and replenished every 6 hours.
5.0 RESULTS

5.1 Expression of the 3A7 epitope in normal and neoplastic colon.

5.1.1 Immunohistochemical staining of normal and neoplastic human colon.

Immunohistochemical staining of a panel of human colon tumors with mAb 3A7 was conducted to determine the relevance of the 3A7 epitope to human disease. Human colon tumors and normal colon samples were obtained from Dr. Dan Sadowski at the University of Alberta (Edmonton, AB, Canada) and were staged according to the Astler-Coller classification system (Table 5.1, refer to Figure 2.3 for description of staging). Representative sections of human colonic tumors stained with mAb 3A7 are displayed in Figure 5.1 (brown colouration of sections represents reactivity with antibody). Most tumor samples stained moderately to strongly with mAb 3A7 (Table 5.1). Yet, staining was not as intense as that observed in the azoxymethane-induced rat colon tumors (Figure 2.17). Antibody staining was distributed throughout the neoplastic tissue. Some tumors contained only a few areas of positive cells (Table 5.1, e.g. Specimens 1 and 4), while other tumors contained mostly immunoreactive cells (Table 5.1, e.g. Specimens 6 and 16). In general, more invasive tumors (Duke's C1 and C2) displayed strong staining in more cells within the tumor (Table 5.1, Figure 5.1B). In contrast, no detectable staining was observed in the epithelial cells of histologically normal colon adjacent to the tumors. Variable results were obtained with antibody staining of lymph node metastasis. Of the five lymph nodes examined, three stained weakly and two stained moderately to strongly with mAb 3A7 (Figure 5.1C, Table 5.1), suggesting that the 3A7 epitope may be important in the formation of lymph node metastases. In contrast, cancerous tissue
Figure 5.1: Immunohistochemical staining of human colon tumors with mAb 3A7. Five micron paraffin-embedded sections of human colon tumors were examined by immunohistochemical staining using either NMS or mAb 3A7 (1:500 dilution) as described in Section 4.4.5. Staining patterns with mAb 3A7 are shown, parallel staining of sections with NMS yielded negative results. A: Duke's A colon tumor showing weak staining with the antibody. B: Duke's C2 primary tumor showing intense staining with mAb 3A7. C: Duke's C2 lymph node metastasis showing intense staining of tumor cells. D: Duke's D liver metastasis showing no staining of neoplastic cells.
Table 5.1: Expression of the 3A7 epitope in paraffin-embedded sections of human colon tumors, lymph nodes and liver.

<table>
<thead>
<tr>
<th>Specimen</th>
<th>Duke’s Staging</th>
<th>Grade</th>
<th>Primary tumor</th>
<th>Lymph node</th>
<th>Liver metastasis</th>
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<tbody>
<tr>
<td>1</td>
<td>A</td>
<td>II</td>
<td>++ (5)</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>A</td>
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<td>-</td>
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<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>B2</td>
<td>n.a.</td>
<td>+ (1)</td>
<td>-</td>
<td>n.a.</td>
</tr>
<tr>
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<td>+ (100)</td>
<td>+ (100)</td>
<td></td>
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<tr>
<td>6</td>
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<td>+++ (100)</td>
<td>-</td>
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</tr>
<tr>
<td>7</td>
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<td>II</td>
<td>-</td>
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</tr>
<tr>
<td>8</td>
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<td>II</td>
<td>+++ (20)</td>
<td>n.a.</td>
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<tr>
<td>9</td>
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<td>+ (100)</td>
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<tr>
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<td>II</td>
<td>+ (100)</td>
<td>+++ (100)</td>
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<tr>
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<td>C2</td>
<td>II</td>
<td>+ (100)</td>
<td>+ (100)</td>
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</tr>
<tr>
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</tr>
<tr>
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<td>C2</td>
<td>II</td>
<td>++ (100)</td>
<td>-</td>
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<tr>
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<td>++ (50)</td>
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</tr>
<tr>
<td>17</td>
<td>D</td>
<td>II/III</td>
<td>++ (10)</td>
<td>n.a.</td>
<td>-</td>
</tr>
</tbody>
</table>

n.a. not available
Positivity is scored as negative (-), weak (+), moderate (++) and strong (+++). The number in parenthesis indicates the percentage of the tumor which is positive.

within the one liver metastasis examined did not stain with mAb 3A7 (Figure 5.1D). However, the primary tumor isolated from the same patient (Table 5.1, Specimen 17) contained only a small percentage of tumor cells that stained moderately with mAb 3A7. Thus, the role of 3A7 epitope expression in cancerous spread to the liver remains unclear.

A striking difference was observed when human normal and tumor sections were compared to rat samples. MAb 3A7 staining in the rat samples was confined to epithelial cells (Figure 2.17). Specifically, mAb 3A7 staining was confined to epithelial cells in normal rat colon and tumor cells in the azoxymethane-induced rat colon tumors. In contrast, in human normal colon and tumor sections the antibody recognized epitopes on endothelial cells and smooth muscle cells. Notably, intense staining of blood vessels was present in the human samples (Figure 5.1B, arrow), indicating that expression of the 3A7
epitope is more widespread in human colon compared to rat colon. Nonetheless, expression of the 3A7 epitope on epithelial cells is clearly a cancer-related phenomenon.

These data confirmed the relevance of the 3A7 epitope in human colon cancer and suggested that expression of the 3A7 epitope may be significant in colon cancer progression and spread. Thus, in-depth study of the 3A7 epitope and glycoproteins bearing this epitope in a rodent model and human colon carcinoma cell lines should contribute to our understanding of this disease.

5.1.2 Localization of the 3A7 epitope in human colon carcinoma cell lines.

Initial studies indicated that mAb 3A7 immunoprecipitated a major glycoprotein species of 140 kDa (gp140) from the human colon carcinoma cell lines, HT29 and SW480 (Figure 3.1). Indirect immunofluorescence analyses were conducted to establish the location of 3A7-immunoreactive glycoconjugates within these cells. Analysis of live HT29 cells with mAb 3A7 produced intense membrane fluorescence (Figure 5.2E) indicative of cell surface expression of the epitope. Analysis of permeabilized HT29 cells indicated that the 3A7 epitope is also present intracellularly (Figure 5.2B) which is likely due to the presence of the 3A7 epitope on glycoconjugates transiting through the Golgi apparatus. Interestingly, although the HT29 cell line appears morphologically homogeneous, only 50-60% of the cells within the HT29 cell line stained with mAb 3A7. Analysis of the SW480 cell line with mAb 3A7 resulted in intense membrane fluorescence of approximately 60% of the cells within the population (data not shown). SW620 cells stained only weakly with mAb 3A7 (data not shown), which is consistent with previous findings indicating an absence of 3A7-immunoreactive gp140 in SW620 cell extracts (Figure 3.1).
Figure 5.2: Indirect immunofluorescence staining of HT29 cells with mAbs 3A7 and 8D5. Methanol/acetone-fixed HT29 cells (panels A to C) and unfixed cells (panels D to F) were incubated with NMS (A and D), mAb 3A7 (B and E) or mAb 8D5 (C and F) followed by FITC-labeled goat anti-mouse Ig, as described in Section 4.4.3.
5.1.3 Western blotting analysis of detergent-solubilized normal rat colon and rat colon tumors.

Immunohistochemical staining provided information regarding the localization of the 3A7 epitope in normal and neoplastic tissues; still, it did not provide any information regarding the macromolecules possessing the epitope. The presence of 3A7-immunoreactive glycoproteins was assessed in a panel of azoxymethane-induced rat colon tumors of differing histopathological grades. Western blotting analysis of detergent-solubilized azoxymethane-induced rat colon tumors and corresponding segments of normal large intestine with mAb 3A7, revealed a quantitative increase in 3A7-immunoreactive glycoproteins in tumor samples (Figure 5.3 displays representative normal and neoplastic samples examined). MAb 3A7 detected glycoproteins ranging in size from 60-200 kDa with a major species of 140 kDa. In the majority of tumor samples (Figure 5.3, lanes 3-6 and 8-21), 3A7-immunoreactive glycoproteins were present at higher levels than in normal caecum, ascending colon and descending colon (Figure 5.3, lanes 1, 2 and 7, respectively). To control for differences in 3A7 epitope expression throughout the length of the colon, tumors of the ascending colon (Figure 5.3, lanes 3-6) were compared to normal ascending colon (Figure 5.3, lane 2) and tumors of the descending colon (Figure 5.3, lanes 8-21) were compared to normal descending colon (Figure 5.3, lane 7). As seen in Table 5.2, results obtained from immunohistochemical analysis (see Figure 2.17 for examples) and Western blotting analysis (Figure 5.3) of colonic tumors were in general agreement. Differences in results obtained with the two techniques could be explained by the observation that some tumors possessed only a small percentage of cells that stain intensely with mAb 3A7 or that residual normal mucosa was present within the solubilized samples. Both cases would result in decreased intensity of the signal in Western blotting.

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Figure 5.3: Western blotting analysis of normal colon and azoxymethane-induced rat colon tumors with mAb 3A7.

Microsomes were prepared and solubilized with 0.2% Triton X-100, as described in Section 4.12. Detergent lysates (30 μg protein) were separated by 7.5% SDS-PAGE and Western blotted with mAb 3A7 (1:3000) for 2 hours, as described in Section 4.4.6. Lane 1, caecum; lane 2, ascending colon; lane 3, Azo26-6; lane 4, Azo24-3; lane 5, Azo29-4; lane 6, Azo22-17; lane 7, descending colon; lane 8, Azo25-10; lane 9, Azo25-17; lane 10, Azo26-16; lane 11, Azo26-17; lane 12, Azo27-12; lane 13, Azo28-13; lane 14, Azo28-14; lane 15, Azo28-15; lane 16, Azo28-9; lane 17, Azo26-11; lane 18, Azo25-15; lane 19, Azo27-11; lane 20, Azo28-10; lane 21, Azo26-10. Lanes 3-6 and lanes 8-21 respresent tumors derived from the ascending colon and descending colon, respectively. Numbers in the left hand margin represent molecular weight markers. Lettering above blots denotes Duke's stage classification of tumor (see Table 5.2).
Table 5.2: Immunohistochemical and Western blotting analysis of azoxymethane-induced rat colon tumors.

<table>
<thead>
<tr>
<th>Tumor</th>
<th>Tumor Classification (Staging)</th>
<th>Western blotting</th>
<th>Immunohistochemistry</th>
</tr>
</thead>
<tbody>
<tr>
<td>25.10</td>
<td>Adenoma, villous</td>
<td>+/-</td>
<td>++</td>
</tr>
<tr>
<td>1.6</td>
<td>A</td>
<td>+++</td>
<td>+++</td>
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<td>3.11</td>
<td>A</td>
<td>+</td>
<td>+++</td>
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<td>6.9</td>
<td>A</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>7.8</td>
<td>A</td>
<td>+++</td>
<td>++</td>
</tr>
<tr>
<td>26.6</td>
<td>A</td>
<td>+/-</td>
<td>++</td>
</tr>
<tr>
<td>25.17</td>
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<td>29.4</td>
<td>B1</td>
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<td>+++</td>
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<tr>
<td>2.8</td>
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<td>C2</td>
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</tr>
<tr>
<td>4.15</td>
<td>C2</td>
<td>+/-</td>
<td>+</td>
</tr>
</tbody>
</table>

5.1.4 Western blotting analysis of detergent solubilized human colon tumors.

Detergent lysates were prepared from a few human colon tumors and subjected to Western blotting to determine the molecular size of 3A7-immunoreactive glycoproteins in...
human samples. MAb 3A7 detected species of 40, 60 and 140 kDa in the human colon tumors lysates (Figure 5.4, lane T), demonstrating that the relative molecular weights of the 3A7-immunoreactive glycoproteins in human tumors is similar to those observed in the rat colon tumors and human colon carcinoma cell lines. Notably, 3A7-immunoreactive glycoproteins were detected in tumors resected from blood group A and AB patients (Figure 5.4), but not from tumor samples resected from blood group O patients (data not shown), suggesting that the presence of the 3A7 epitope is related to blood group status. Due to the small number of samples available, no definitive statement regarding expression of 3A7-immunoreactive glycoproteins and human colon cancer progression could be formulated. As well, mucosa (M) isolated from areas adjacent to the tumors also stained in some cases (Figure 5.4, lane M). MAb 3A7 detected species of approximately 75, 90 and 120 kDa in adjacent normal mucosa. Detailed examination of the epitope detected by mAb 3A7 and isolation of the major 3A7-immunoreactive glycoprotein were required.

5.2 Characterization of monoclonal antibodies prepared against an azoxymethane-induced rat colon tumor.

The results obtained from immunohistochemistry and Western blotting analyses warranted further study of the 3A7 epitope and glycoproteins bearing this structure. In addition to mAb 3A7, three other mAbs 2F7, 4F3 and 8D5 were generated against azoxymethane-induced rat colon tumors (refer to Section 2.4.2). These antibodies immunoprecipitate a glycoprotein species that comigrates with the 140 kDa glycoprotein immunoprecipitated by mAb 3A7 from human colon carcinoma cell lines. As well, staining of HT29 with mAb 8D5 (Figure 5.2C and F) is analogous to mAb 3A7 staining of these cells (Figure 5.2 B and E) in indirect immunofluorescence analyses, suggesting that both mAbs 8D5 and 3A7 are detecting cell surface glycoproteins of similar size.
**Figure 5.4:** Western blotting analysis of human tumors and adjacent normal mucosa with mAb 3A7. Detergent lysates (30 µg protein) were separated by 7.5% SDS-PAGE and blotted with mAb 3A7 (1:3000). The primary tumor (lane T) and adjacent normal mucosa (lane M) from an adenomatous polyp (Adn) and Duke's C2 tumor from the ascending colon and Duke's B2 and C2 tumors from recto-sigmoid colon are represented.
5.2.1 Specificity of mAbs 2F7, 3A7, 4F3 and 8D5.

Preliminary investigations suggested that the epitopes detected by mAbs 2F7, 3A7, 4F3 and 8D5 were carbohydrate in nature. These antibodies recognized multiple species ranging in size from 60 to 200 kDa by Western blotting. As well, antibody reactivity was lost following treatment of formalin-fixed tissue sections with sodium periodate (S. Laferté, unpublished observations), which is known to alter oligosaccharide structure (O'Shannessy et al., 1984). In addition, reactivity of tissue samples or cell lines with mAb 3A7 was dependent on blood group status, suggesting that the epitope recognized by mAb 3A7 may be blood group related. The specificity of mAbs 2F7, 3A7, 4F3 and 8D5 was investigated by examining their ability to agglutinate erythrocytes and recognize neoglycoproteins expressing blood group determinants of defined structure.

MAb 3A7 was found to agglutinate both A₁ and A₂ erythrocytes (which differ by their ability to bind to the blood group A specific lectin *dolichos biflorus* (Bird, 1952)) at titres of 1:51,000 and 1:12,800, respectively (Table 5.3). There was also a significant amount of reactivity of this antibody to blood group B erythrocytes (titre of 1:12,800). (Refer to Figure 2.11 for structures of blood group determinants.) In contrast, mAbs 2F7, 4F3 and 8D5 were able to agglutinate both A₁ and A₂ erythrocytes with reasonable titres but these antibodies were unable to agglutinate blood group B erythrocytes at any

<table>
<thead>
<tr>
<th>Blood Type</th>
<th>3A7</th>
<th>2F7</th>
<th>4F3</th>
<th>8D5</th>
</tr>
</thead>
<tbody>
<tr>
<td>A₁</td>
<td>51000</td>
<td>3200</td>
<td>6400</td>
<td>12800</td>
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<td>12800</td>
<td>1600</td>
<td>3200</td>
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<td>O</td>
<td>&lt;100</td>
<td>&lt;100</td>
<td>&lt;100</td>
<td>&lt;100</td>
</tr>
</tbody>
</table>

*The numbers represent the reciprocal of the maximum dilution of antibody which results in definite hemagglutination of erythrocytes following a 30 minute incubation at room temperature.
titre (Table 5.3). None of the antibodies agglutinated blood group O erythrocytes even at a 1:100 dilution of the antibody (Table 5.3). These studies suggested that mAbs 2F7, 4F3 and 8D5 recognize epitopes on the blood group A determinant while mAb 3A7 detects an epitope on blood A and B determinants.

In order to elucidate the structure of determinants recognized by mAbs 2F7, 3A7, 4F3 and 8D5, we tested the ability of these antibodies to detect in Western blotting and EIA, neoglycoproteins consisting of bovine serum albumin (BSA) chemically modified with oligosaccharides of defined structure (refer to Table 5.4 for structures). Individual neoglycoproteins were analyzed by Western blotting with either normal mouse serum or mAbs 2F7, 3A7, 4F3, 8D5 (Figure 5.5). Results obtained from analysis of the reactivities of the antibodies with the panel of neoglycoproteins concurred with the data obtained in haemagglutination assays. Specifically, mAb 3A7 recognized A type 2-BSA (Figure 5.5D, lane 4), A type 6-BSA (Figure 5.5D, lane 6) and B type 2-BSA (Figure 5.5D, lane 8). No binding was detected with H type 2-BSA (Figure 5.5D, lane 1), A and B disaccharides (i.e. no α1-2 linked fucose) (Figure 5.5D, lanes 2 and 7, respectively) or with any of the neoglycoproteins containing A and B determinants on a type 1

Table 5.4: Structure of Oligosaccharide Moiety of Neoglycoproteins.

<table>
<thead>
<tr>
<th>Name</th>
<th>Structure</th>
</tr>
</thead>
<tbody>
<tr>
<td>H type 2</td>
<td>Fucα1-2Galβ1-4GlcNAc-BSA</td>
</tr>
<tr>
<td>A disaccharide</td>
<td>GalNAcα1-3Gal-BSA</td>
</tr>
<tr>
<td>A type 1</td>
<td>GalNAcα1-3[Fucα1-2]Galβ1-3GlcNAc-BSA</td>
</tr>
<tr>
<td>A type 2</td>
<td>GalNAcα1-3[Fucα1-2]Galβ1-4GlcNAc-BSA</td>
</tr>
<tr>
<td>A type 4</td>
<td>GalNAcα1-3[Fucα1-2]Galβ1-3GalNAc-BSA</td>
</tr>
<tr>
<td>A type 6</td>
<td>GalNAcα1-3[Fucα1-2]Galβ1-4Glc-BSA</td>
</tr>
<tr>
<td>B disaccharide</td>
<td>Galα1-3Gal-BSA</td>
</tr>
<tr>
<td>B type 2</td>
<td>Galα1-3[Fucα1-2]Galβ1-4GlcNAc-BSA</td>
</tr>
<tr>
<td>B type 4</td>
<td>Galα1-3[Fucα1-2]Galβ1-3GalNAc-BSA</td>
</tr>
<tr>
<td>B type 5</td>
<td>Galα1-3[Fucα1-2]Galβ1-3Gal-BSA</td>
</tr>
<tr>
<td>B type 6</td>
<td>Galα1-3[Fucα1-2]Galβ1-4Glc-BSA</td>
</tr>
</tbody>
</table>

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Figure 5.5: Western blotting analysis of neoglycoproteins. The neoglycoproteins (1 nmol oligosaccharide), depicted in Table 5.4, were separated by 7.5% SDS-PAGE and blotted with mAb 2F7 (1:3000 dilution, panel A), mAb 4F3 (1:2000 dilution, panel B), mAb 8D5 (1:3000 dilution, panel C), mAb 3A7 (1:3000 dilution, panel D) or NMS (1:3000 dilution, panel E) for 2 hours followed by secondary antibody, as described in Section 4.4.6. Lane 1, H-type 2-BSA; lane 2, A disaccharide-BSA; lane 3, A type 1-BSA; lane 4, A type 2-BSA; lane 5, A type 4-BSA; lane 6, A type 6-BSA; lane 7, B disaccharide-BSA; lane 8, B type 2-BSA; lane 9, B type 4-BSA; lane 10, B type 5-BSA; lane 11, B type 6-BSA. The numbers in the left margin represent sizes of prestained molecular weight standards.
chain(Galβ1-3GlcNAc) (Figure 5.5D, lanes 3, 5, 9, 10). In contrast, mAbs 2F7 (Figure 5.5A), 4F3 (Figure 5.5B) and 8D5 (Figure 5.5C) recognized A type 1 (lane 3), A type 2 (lane 4), A type 4 (lane 5) and A type 6 (lane 6) containing neoglycoproteins. No binding was detected with H type 2-BSA (lane 1), A disaccharide (lane 2) or any neoglycoproteins containing B determinants (lanes 7-11). These data suggested mAb 3A7 recognizes an epitope on type 2 chains bearing either blood group A or B determinants, whereas mAbs 2F7, 4F3 and 8D5 detect epitopes exclusively on a blood group A determinant.

The relative binding affinity of mAbs 2F7, 3A7, 4F3 and 8D5 for the neoglycoproteins was examined by EIA. As illustrated in Figure 5.6B, the relative binding of mAb 3A7 was greatest for A type 2-BSA (GalNAcα1-3[Fucα1-2]Galβ1-4GlcNAc-BSA, black squares) and B type 2-BSA (Galα1-3[Fucα1-2]Galβ1-4GlcNAc-BSA, green triangles). The relative binding of mAb 3A7 for A type 6-BSA (GalNAcα1-3[Fucα1-2]Galβ1-4Glc-BSA, blue circles) was much lower than with A- and B type 2-BSA. Very little binding of the antibody to other neoglycoproteins was detected. In contrast, mAbs 2F7 (Figure 5.6A), 4F3 (Figure 5.6C) and 8D5 (Figure 5.6D) showed the strongest reactivities toward A type 1-BSA (GalNAcα1-3[Fucα1-2]Galβ1-3GlcNAc-BSA, red open circles), and A type 6-BSA (GalNAcα1-3[Fucα1-2]Galβ1-4Glc-BSA, blue circles), followed by A type 4-BSA (GalNAcα1-3[Fucα1-2]Galβ1-3GalNAc-BSA, hatched yellow squares) and A type 2-BSA (GalNAcα1-3[Fucα1-2]Galβ1-4GlcNAc-BSA, black squares). The higher level of binding of these antibodies to A type 1-BSA compared to A type 2-BSA suggested a preference for type 1 carrier chains (Galβ1-3GlcNAc-R) over type 2 carrier chains (Galβ1-4GlcNAc-R). The inability of any of these antibodies to bind to A disaccharide-BSA (GalNAcα1-3Gal-BSA, cross) and H type 2-BSA (Fucα1-2Galβ1-4GlcNAc-BSA, closed diamond) suggested that an α1-2 linked fucose and terminal α1-3-linked N-acetyl-D-galactosamine are absolute requirements for antibody binding.
Figure 5.6: Solid phase immunosorbent assay (EIA) of neoglycoproteins with mAbs 2F7, 3A7, 4F3 and 8D5. Serial dilutions of neoglycoproteins (10–0.01 pmol oligosaccharide/well), depicted in Table 5.4, were incubated on 96-well plates overnight at 4°C. EIAs were performed as described in Section 4.4.2 using mAbs 2F7 (A) at 1:200 dilution, 3A7 (B), 4F3 (C) and 8D5 (D) at a 1:3000 dilution. Colour development was monitored at 405 nm following addition of p-nitrophenyl phosphate substrate. Each point represents the mean ± standard deviation (standard deviation falls below graphic resolution on some data points and therefore does not appear). H type 2-BSA (●); A disaccharide-BSA (★); A type 1-BSA (●); A type 2-BSA (★); A type 4-BSA (●); A type 6-BSA (●); B disaccharide-BSA (★); B type 2-BSA (★); B type 4-BSA (★); B type 5-BSA (★); B type 6-BSA (★).
To investigate the requirement of α1-3 linked sugars for antibody recognition, neoglycoproteins bearing A type 1, A type 2 and B type 2 determinants were digested with the enzymes α-N-acetyl-D-galactosaminidase from *Acremonium* sp. or α-D-galactosidase from green coffee beans to remove the terminal α1-3 linked GalNAc and α1-3 linked galactose, respectively. The reactivity of mAb 3A7 to both A type 2-BSA and B type 2-BSA was greatly reduced following enzyme treatment (Table 5.5). As well, the reactivity of mAb 8D5 to A type 1-BSA was almost completely abolished following enzyme treatment (Table 5.5). To ensure that loss of reactivity was not a result of cleavage or breakdown of sugar products, antibodies toward the H type 2 determinant (the expected product after glycosidase digestion) were used as a control. Untreated A or

Table 5.5: Effects of partial deglycosylation of A and B-BSA glycans on mAb 3A7, mAb 8D5, mAb 11D4 and anti-H binding.

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<thead>
<tr>
<th></th>
<th>mAb 3A7</th>
<th>anti-H</th>
<th>NMS</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>A type 2-BSA</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Untreated</td>
<td>0.959±0.030</td>
<td>0.049±0.004</td>
<td>0.041±0.004</td>
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<tr>
<td>20 mU α-N-acetylgalactosaminidase</td>
<td>0.069±0.047</td>
<td>0.343±0.055</td>
<td>0.028±0.002</td>
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<tr>
<td><strong>B type 2-BSA</strong></td>
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<tr>
<td>Untreated</td>
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<td>20 mU α-galactosidase</td>
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<td>0.814±0.005</td>
<td>0.057±0.001</td>
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<tr>
<td><strong>A type 1-BSA</strong></td>
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<td></td>
<td></td>
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<tr>
<td>Untreated</td>
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<td>nd</td>
<td>.028±0.003</td>
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<tr>
<td>20 mU α-N-acetylgalactosaminidase</td>
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<td>nd</td>
<td>.046±0.003</td>
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<tr>
<td><strong>A type 2-BSA</strong></td>
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<tr>
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<td>0.031±0.009</td>
<td>0.027±0.003</td>
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<tr>
<td>20 mU α-N-acetylgalactosaminidase</td>
<td>0.061±0.027</td>
<td>0.357±0.013</td>
<td>0.031±0.005</td>
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</table>

*A type 1-BSA, A type 2-BSA or B type 2-BSA conjugates (5 pmol oligosaccharide) were digested with α-N-acetyl-D-galactosaminidase from *Acremonium* sp. or α-D-galactosidase from green coffee beans and assayed by EIA with normal mouse serum (NMS) or mAb 3A7, 8D5 and 11D4 at a 1:3000 dilution or affinity-purified anti-H immunoglobulin (300 µg/ml). Absorbance at 405 nm was recorded 3 hours after addition of substrate, as described in Section 4.4.2. Numbers indicate the mean absorbance of triplicate samples and the standard deviation minus control wells containing TBS pH 8.0. The presence of H type 1 structures following enzyme treatment of A type 1-BSA could not be determined since the anti-H immunoglobulin is specific for type 2 chains (nd).
B type 2-BSA exhibited little reactivity to anti-H type 2 antibody. However, the enzyme-treated A or B-type 2-BSA showed a dramatic increase in reactivity with anti-H type 2 antiserum. These findings indicated that the terminal α1-3 linked sugar of the A or B-type 2-BSA is required for mAb 3A7 binding. Likewise, the terminal α1-3 linked sugar of the A type 1-BSA was necessary for mAb 8D5 binding. Still, examination of 3A7 and 8D5-immunoreactive glycoconjugates from native sources was required to determine the epitopes detected by these antibodies under biological conditions.

5.2.2 Analysis of the structural relatedness of gp140 recognized by mAbs 2F7, 3A7, 4F3 and 8D5.

MAbs 2F7, 3A7, 4F3 and 8D5 recognize carbohydrate determinants on a major glycoprotein species of 140 kDa. To determine the structural relatedness of the glycoproteins immunoprecipitated from [3S]-methionine labeled HT29 detergent cell lysates by mAbs 3A7 (Figure 5.7A, lane 2), 8D5 (Figure 5.7A, lane 3), 4F3 (Figure 5.7A, lane 4) and 2F7 (Figure 5.7A, lane 5), the immunoprecipitated bands corresponding to gp140 were excised from unstained, unfixed SDS-polyacrylamide gels and subjected to either limited proteolytic peptide mapping with V8 protease, which cleaves at glutamate residues, or N-glycanase digestion, which removes Asn-linked oligosaccharides.

Limited proteolytic peptide mapping with V8 protease provided evidence that gp140 immunoprecipitated by the four antibodies is structurally identical. The V8 protease peptides generated from the 140 kDa species immunoprecipitated by mAbs 3A7 (Figure 5.7B, lane 1), 2F7 (Figure 5.7B, lane 2), 4F3 (Figure 5.7B, lane 3) and 8D5 (Figure 5.7B, lane 4) were identical, suggesting that the antibodies recognize structurally identical glycoproteins or a structurally identical group of comigrating glycoproteins.

As seen in Figure 5.7C, N-glycanase digestion (lanes 5-8) of gp140 immunoprecipitated by each of the antibodies produced the same deglycosylated
Figure 5.7: Analysis of glycoproteins detected by mAbs 3A7, 2F7, 4F3 and 8D5 in HT29 cells. A: The human colon cancer cell line HT29 was radiolabeled for 24 hours with [35S]methionine, harvested and solubilized with 1% Triton X-100, as described in Sections 4.15 and 4.12. Radiolabeled cell lysates (2 x 10⁶ cpm) were immunoprecipitated with NMS (lane 1), mAb 3A7 (lane 2), mAb 2F7 (lane 3), mAb 4F3 (lane 4) or mAb 8D5 (lane 5) and separated by 7.5% SDS-PAGE. B: Radiolabeled cell lysates (5 x 10⁶ cpm) were immunoprecipitated with mAbs 3A7 (lane 1), 2F7 (lane 2), 4F3 (lane 3) or 8D5 (lane 4). Samples were separated by 7.5% SDS-PAGE, as shown in panel A. The major 140 kDa glycoprotein species was excised from the dried, unfixed gel and subjected to limited V8 protease peptide mapping, as described in Section 4.19. C: [35S]methionine labeled HT29 cell lysates (2 x 10⁶ cpm) were immunoprecipitated with mAbs 3A7 (lanes 1 and 5), 2F7 (lanes 2 and 6), 4F3 (lanes 3 and 7) or 8D5 (lanes 4 and 8). Samples were separated by 7.5% SDS-PAGE. The 140 kDa glycoprotein immunoprecipitated with each antibody was excised from the dried, unfixed gel and digested with 3 mU N-glycanase, as described in Section 4.18. Control (lanes 1 to 4) and N-glycanase-treated samples (lanes 5 to 8) were analyzed by 7.5% SDS-PAGE. D: The 75 kDa (lane 1), 110 kDa (lane 2) and 105 kDa (lane 3) bands derived from the 140 kDa following N-glycanase treatment depicted in panel A (lanes 5-8) were excised from the dried, unfixed gel and subjected to V8 protease peptide mapping, as described in Section 4.19.
products, including major species of 110 and 105 kDa and minor species ranging from 75-105 kDa. The presence of multiple deglycosylated species could be the result of microheterogeneity in the glycan moiety or the presence of different comigrating glycoproteins. V8 protease digestion of the two major deglycosylated species of 110 (Figure 5.7D, lane 2) and 105 kDa (Figure 5.7D, lane 3) resulted in nearly identical peptide maps suggesting that these species are derived from a single polypeptide or closely related polypeptides with heterogeneous glycosylation. The peptide map obtained from the 75 kDa deglycosylated species (Figure 5.7D, lane 1) differed from the maps generated by digestion of the 110 and 105 kDa species, suggesting that the 75 kDa species may be derived from a distinct glycoprotein.

5.2.3 Localization of epitopes defined by mAbs 3A7 and 8D5 on gp140.

Glycosylation inhibitors were employed to investigate the location of the carbohydrate epitopes recognized by mAbs 3A7 and 8D5 on gp140 (Figure 5.8). Pretreatment of HT29 cells with the glycosylation inhibitor tunicamycin, which prevents synthesis of Asn-linked oligosaccharides (Elbein, 1987), abolished immunoprecipitation of gp140 with either mAb 3A7 (Figure 5.8B, lane 3) or mAb 8D5 (Figure 5.8C, lane 3). This finding indicated that the epitope detected by each of these antibodies is present on Asn-linked oligosaccharides. Significantly, treatment of HT29 cells with the glycosylation inhibitor swainsonine, which blocks synthesis of Asn-linked oligosaccharides prior to initiation of β1-6 branch synthesis (Elbein, 1987), only prevented immunoprecipitation of gp140 by mAb 3A7 (Figure 5.8B, lane 4). In contrast, mAb 8D5 was still able to immunoprecipitate gp140 from swainsonine treated HT29 cells (Figure 5.8C, lane 4), although the immunoprecipitated species migrated at an apparent molecular weight of 116 kDa, which is consistent with smaller oligosaccharides. These findings imply that the 3A7 epitope is present primarily on the β1-6 branch of Asn-linked
Figure 5.8: Effects of glycosylation inhibitors on biosynthesis of epitopes detected by mAbs 3A7 and 8D5. HT29 cells were radiolabeled with [35S]methionine in the absence (lanes 1 and 2) or presence of the glycosylation inhibitors tunicamycin (lane 3) or swainsonine (lane 4), as described in Section 4.16. To control for the presence of DMSO in the tunicamycin stock solution, cells were radiolabeled in the presence of DMSO alone (lane 2). Cell lysates were immunoprecipitated with NMS (panel A), mAb 3A7 (panel B) or mAb 8D5 (panel C). Samples were analyzed by 7.5% SDS-PAGE and bands were visualized, as described in Section 4.3.
oligosaccharides whereas the 8D5 epitope is present on an alternate branch of Asn-linked oligosaccharides.

The presence of β1-6 branched Asn-linked oligosaccharides on gp140 was confirmed by lectin blotting. MAb 3A7 (Figure 5.9, lane 2), 8D5 (Figure 5.9, lane 3) and 4F3 (Figure 5.9, lane 4) immunoprecipitated gp140 from HT29 or SW480 cell lysates that was L-PHA sensitive. However, L-PHA lectin was unable to detect glycoprotein species immunoprecipitated by mAbs 3A7, 8D5 or 4F3 from SW620 and SW1417 cell lysates (Figure 5.9) either due to absence of the β1-6 branch and/or absence of immunoreactive glycoproteins bearing this branch.

5.3 Characterization of the major 3A7-immunoreactive glycoproteins from normal and neoplastic colon.

Studies described in Section 5.1 revealed quantitative changes to the 3A7 epitope and/or glycoproteins bearing the 3A7 epitope in neoplastic colon. Further examination of the carbohydrate structure associated with mAb 3A7-immunoreactive glycoproteins in normal and neoplastic colon was conducted by subjecting detergent lysates of normal rat colon and azoxymethane-induced rat colon tumors of different histopathological types to affinity chromatography on the blood group A specific lectins *dolichos biflorus* (DBA) and *helix pomatia* (HPA). *Dolichos biflorus* lectin exhibits a restricted specificity whereas *helix pomatia* lectin recognizes blood group A determinants irrespective of the core oligosaccharide (Goldstein and Hayes, 1978). DBA has been shown to bind blood group A1 erythrocytes with a higher affinity than blood group A2 erythrocytes (Bird, 1952) and although the structural basis of this difference is unclear, studies have shown that the best inhibitor of DBA binding is a type 2 chain blood group A oligosaccharide having the structure GalNAcα1-3[Fucα1-2]Galβ1-4GlcNAcβ1-6-R (Ettler and Kabat, 1970). Most of the mAb 3A7-immunoreactive glycoproteins from normal colon (Figure 5.10A, lanes 1 and 2) were adsorbed to DBA-agarose (note that 100-fold more normal
Figure 5.9: Detection of gp140 immunoprecipitated from human colon carcinoma cell lines by mAbs 3A7, 8D5, and 4F3 with leucophytohemagglutinin (L-PHA). HT29, SW480, SW620 and SW1417 detergent cell lysates were immunoprecipitated with NMS or mAbs 3A7, 8D5 or 4F3, separated by 7.5% SDS-PAGE and blotted with lectin L-PHA (0.4 μg/ml), followed by anti-L-PHA antisera (1:3000), secondary antibody and colour development, as described in Section 4.4.6.
colon protein was applied to the column compared to tumor extracts because of the low level of 3A7-immunoreactive glycoproteins in normal rat colon. Glycoproteins ranging in size between 60 and 200 kDa were specifically eluted from the column, including a major species of 140 kDa (Figure 5.10A, lane 2). Similar analyses were carried out on a panel of azoxymethane-induced rat colon tumors which included a Duke's A intramucosal carcinoma (Figure 5.10A, lanes 3 and 4), a Duke's B1 carcinoma (Figure 5.10A, lanes 5 and 6), a B2 carcinoma (Figure 5.10A, lanes 11 and 12), a B2 collision tumor (conventional adenocarcinoma and signet-ring cell carcinoma; Figure 5.10A, lanes 7 and 8), the original rat tumor used for the production of mAb 3A7 (Figure 5.10A), lanes 9 and 10) and a lymph node metastasis (Duke's C2) (Figure 5.10A, lanes 13 and 14). Western blotting analysis of DBA-agarose unbound (U) and eluted (E) fractions from each sample revealed a cancer-associated decrease in the ability of 3A7-immunoreactive glycoproteins to bind to DBA-agarose. In contrast to normal colon, glycoproteins from Duke's A, B1, B2 and C2 tumors were distributed in both DBA-unbound and eluted fractions. Indeed, the Duke's A tumor (Figure 5.10A, lane 4), one of the B2 tumors (Figure 5.10A, lane 12) and the C2 tumor (Figure 5.10A, lane 14) contained very little material in the DBA-eluted fraction. When mAb 3A7-immunoreactive glycoproteins were subsequently applied to HPA-agarose, nearly all of the immunoreactive material was recovered in the eluted fractions, confirming the presence of blood group A determinants on these glycoproteins (Figure 5.10B). Furthermore, comparison of the glycoprotein profiles of HPA-eluted fractions from various tumors revealed an apparent reduction in the number of glycoprotein species detected by mAb 3A7 in the more aggressive tumors, concomitant with an increased expression of a major glycoprotein species of 140 kDa.

The differential binding of mAb 3A7 immunoreactive glycoproteins to DBA lectin was not unique to rat colon tumors. Affinity chromatography of a detergent lysate of the human colon carcinoma cell line HT29 on DBA-agarose revealed that the 140 kDa glycoprotein, which is the major mAb 3A7-immunoreactive species, was present in the
Figure 5.10: Serial lectin-affinity chromatography of detergent solubilized normal rat colon, rat colon tumors and HT29 cells on *Dolichos biflorus* and *Helix pomatia* agarose. A: Detergent lysates of normal colon (2 g protein), azoxymethane-induced tumor extracts of different histopathological types (2 mg protein) or HT29 cells (2 mg protein) were applied to *Dolichos biflorus* (DBA)-agarose and the column was washed extensively prior to elution with 100 mM N-acetylglactosamine. Unbound (U) and eluted (E) fractions were collected, pooled separately and concentrated to 0.5 ml; 20 μl aliquots were analyzed by Western blotting with mAb 3A7 (1:3000 dilution). Lanes 1, 2, normal colon; lanes 3, 4, Duke's A (Azo 1.6); lanes 5, 6, Duke's B1 (Azo 4.1); lanes 7, 8, Duke's B2 collision tumor (Azo 1.9); lane 9, 10, original rat tumor used in preparation of monoclonal antibodies; lane 11, 12, Duke's B2 (Azo 1.10); lanes 13, 14, Duke's C2 (Azo 4.15); lane 15, 16, HT29 cells. B: DBA-unbound fractions depicted in A (with the exception of normal colon) were subsequently applied to *Helix pomatia* (HPA)-agarose and the column was eluted with GalNAc. HPA-unbound (U), and eluted (E) fractions were concentrated to 0.5 ml; 20 μl aliquots were analyzed by Western blotting with mAb 3A7 (1:3000 dilution). Lanes 1, 2, Duke's A (Azo 1.6); lanes 3, 4, Duke's B1 (Azo 4.1); lanes 5, 6, Duke's B2 collision tumor (Azo 1.9); lane 7, 8, original rat tumor used in preparation of monoclonal antibodies; lane 9, 10, Duke's B2 (Azo 1.10); lanes 11, 12, Duke's C2 (Azo 4.15); lane 13, 14, HT29 cells.
unbound fraction (Figure 5.10A, lane 15). A less abundant 120 kDa species was specifically eluted from DBA-agarose (Figure 5.10A, lane 16). Subsequent chromatography of the DBA-unbound fraction on HPA-agarose resulted in quantitative recovery of the 140 kDa glycoprotein in the eluted fraction (Figure 5.10B, lane 14).

The lectin binding profiles produced by 3A7-immunoreactive glycoproteins from normal and neoplastic colon indicated that in addition to the previously noted quantitative changes in the 3A7 epitope, there are also changes to the structure of the glycans possessing the 3A7 epitope.

5.4 Analysis of 3A7-immunoreactive glycopeptides isolated from normal rat intestine.

Although the EIA assays of blood group bearing neoglycoproteins provided information about the minimum carbohydrate structure detected by mAbs 3A7 and 8D5, these approaches did not define the structures recognized by these antibodies in normal and neoplastic colon. 3A7-immunoreactive glycopeptides were isolated and partially purified from normal rat small intestine and colon to identify the carbohydrate structures recognized by mAb 3A7. Identical studies were performed on glycopeptides isolated from both small intestine and colon. The chromatographic profiles obtained from 3A7-immunoreactive glycopeptides isolated from both tissues were virtually identical. Therefore, only results from analysis of rat colon glycopeptides are shown. However, since normal rat colon contains very low level of 3A7-immunoreactive material, 10 fold more material was used in preparation and analysis of glycopeptides from normal colon compared to small intestine.

The total glycopeptide fraction of rat colon was isolated and subjected to lectin affinity chromatography as described in Section 4.11 and outlined in Figure 5.11. As seen in Figure 5.12, most of the 3A7-immunoreactive glycopeptides were found in the run-through fractions of Con A-Sepharose, as detected by EIA with mAb 3A7,
Figure 5.11: Purification of glycopeptides from normal rat small intestine and colon. Flow chart depicting chromatographic steps in the isolation of mAb 3A7-immunoreactive glycopeptides from normal rat small intestine and colon.

suggesting that the 3A7 epitope is present on either O-linked oligosaccharides or tri- and tetra-antennary complex-type Asn-linked oligosaccharides (Ogata et al., 1975). A small fraction of 3A7-immunoreactive glycopeptides was retained by Con A-Sepharose and eluted with 10 mM $\alpha$-methylmannopyranoside (Figure 5.12), suggesting that the epitope is also present on hybrid or bi-antennary Asn-linked oligosaccharides. The absence of immunoreactive material in the fractions eluted with 100 mM $\alpha$-methylmannopyranoside suggested that the 3A7 epitope is absent from high-mannose Asn-linked structures, as expected for blood group antigens.

The structure of 3A7-immunoreactive glycopeptides in the run-through fraction of Con A-Sepharose was analyzed further by lectin-affinity chromatography on DBA-agarose. Since DBA is a blood group A-specific lectin, it was predicted that 3A7 immunoreactive glycopeptides would be retained by this column and eluted with the competing sugar, N-acetyl-D-galactosamine (GalNAc) (Etzler, 1974). As expected, nearly all of the 3A7-immunoreactive glycopeptides in the Con A-Sepharose run-through fraction were retained on DBA-agarose and subsequently eluted with 100 mM GalNAc (Figure 5.13, closed squares). Analysis of column fractions by EIA using mAb 3A7 indicated that a small fraction of 3A7-immunoreactive glycopeptides was not retained by
Figure 5.12: Lectin affinity chromatography of total rat colon glycopeptides on Concanavalin A-Sepharose. Desalted glycopeptides (25 mg) were applied to Con A-Sepharose; the column was washed and bound material was eluted with 10 mM α-methylmannopyranoside (α-mm) or 100 mM α-mm, as described in Section 4.9.1. Column fractions (100 µl aliquots) were assayed for immunoreactivity by EIA assay with mAb 3A7 (1:1000 dilution) with colour detection at an absorbance of 405 nm, as described in Section 4.4.3.

Figure 5.13: Affinity chromatography of Con A-Sepharose unbound glycopeptides on Dolichos biflorus-agarose. Con A-Sepharose unbound fractions were applied to a 2 ml DBA-agarose column, the column was washed and bound glycopeptides were eluted with 100 mM GalNAc, as described in Section 4.9.2. Column fractions were assayed for immunoreactivity using an EIA assay with mAb 3A7 (1:1000 dilution), with colour detection at an absorbance of 405 nm, as described in Section 4.4.3. The profile of initial application and elution of glycopeptides (●) and the subsequent reloading of unbound immunoreactive material (○) are represented.
DBA-agarose (Figure 5.13, closed squares). However, subsequent reapplication of these fractions to the column resulted in quantitative binding of the 3A7-immunoreactive material to DBA-agarose (Figure 5.13, open circles). These data confirmed the presence of blood group A or A-like structures on 3A7-immunoreactive glycopeptides.

In order to examine the size of the 3A7-immunoreactive glycopeptides, the pooled glycopeptide fraction eluted from DBA-agarose was subjected to gel filtration chromatography on Biogel P6, as described in Section 4.10. Aliquots of column fractions were assayed for the presence of hexose using the phenol-sulfuric assay and immunoreactivity by EIA with mAb 3A7. Initial separation of glycopeptides on Biogel P6 revealed that they eluted in the void volume, suggesting that the glycopeptides had a molecular weight of at least 6 kDa (data not shown). Biogel P6 fractions containing glycopeptides were pooled and redigested with pronase for 72 hours and reapplied to the column. Column fractions were assayed as before by phenol sulfuric assay (Figure 5.14, red diamonds) and EIA with mAb 3A7 (Figure 5.14, black squares). The majority of the 3A7 immunoreactive glycopeptides still eluted in the void volume of the column (Figure 5.14, black squares) indicating a size greater than 6 kDa. This was confirmed by Western blotting with mAb 3A7 which revealed glycopeptides of approximately 12, 20, 32 and 40 kDa and minor species of 8-12 kDa (Figure 5.15, lane 2). These data suggested that either the oligosaccharide component of each glycopeptide is very large or the residual glycopeptides are resistant to pronase possibly due to the predominance of O-linked oligosaccharides (Merkle and Cummings, 1987a).

3A7-immunoreactive glycopeptides isolated by Biogel P6 gel filtration were subjected to structural analysis by mass spectroscopy and nuclear magnetic resonance (NMR) to obtain information regarding the sugar content and linkage of sugars in the carbohydrate component of the glycopeptides. Unfortunately, the presence of large amounts of acetate in the sample interfered with the analyses and as a result, very little useful data was generated. The residual sample was subjected to glycosidase digestion
Figure 5.14: Biogel P6 gel filtration chromatography of DBA-agarose eluted rat intestine glycopeptides. Glycopeptides eluted from DBA-agarose with GalNAc were pooled, lyophilized, desalted and separated on a Biogel P6 column equilibrated in distilled water. Aliquots of 100 µl were coated on Immulon 2 microtiter and assayed for immunoreactivity with mAb 3A7 (1:1000 dilution) in an ELISA assay. Immunoreactivity was detected colorimetrically at an absorbance of 405 nm (■). The presence of glycopeptides within the fractions was determined by subjecting 100 µl aliquots to phenol sulfuric hexose assay and measuring the absorbance at 484 nm (●). The column void volume (V₀) was determined by application of BSA followed by detection of protein at an absorbance of 280 nm and the total column volume (Vₜ) was determined by phenol sulfuric assay after application of mannose.

and lectin blotting which yielded some information regarding the structure and composition of the glycopeptide fraction.

For example, treatment of the partially purified glycopeptide fraction with N-acetyl-D-galactosaminidase from Acremonium sp., which removes the α1-3 linked N-acetylglactosamine, followed by Western blotting with mAb 3A7 abolished immunoreactivity (Figure 5.15, lane 3). This confirmed the requirement of terminal N-acetylglactosamine residues for recognition by mAb 3A7. Lectin blotting of the glycopeptide fraction with biotinylated Datura Stramonium (DSA) (Figure 5.15, lane 4) suggested the presence of short poly-N-acetylactosamine ([Galβ1-4GlcNAc]ₙ-R) repeating structures (n≤3) on the 3A7-immunoreactive glycopeptides (Merkle and
Figure 5.15: Western and lectin blotting analyses of 3A7-immunoreactive rat colon glycopeptides partially purified by lectin chromatography and gel filtration. Glycopeptides (100 μg) isolated from Biogel P6 were left untreated (lanes 1, 2, 4, 5 and 6) or digested with 20 μM α-N-acetylgalactosaminase (lane 3) and separated by 15% SDS-PAGE and blotted with NMS (lane 1), mAb 3A7 (1:3000, 3 hours, lanes 2 and 3), followed by appropriate secondary antibody, DSA-HRP (0.4 μg/ml, 2 hours, lane 4), L-PHA-HRP (0.4 μg/ml, 2 hours, lane 5) or LEA-HRP (0.4 μg/ml, 2 hours, lane 6). Colour development was carried out as described in Section 4.4.6.
Cummings, 1987b). The inability of biotinylated tomato lectin (LEA) (Figure 5.15, lane 6) to bind to these glycopeptides suggested that they lack poly-N-acetyllactosamine structures having more than 4 repeating units. In addition, the isolated glycopeptides stained weakly with the lectin L-PHA (Figure 5.15, lane 5) suggesting that β1-6 branched Asn-linked oligosaccharides are present on the 3A7-immunoreactive glycopeptides. It should be noted that samples containing 100 μg of peptide were used in Western and Lectin blotting studies due to the low levels of 3A7-immunoreactive glycopeptide present in normal rat colon. Therefore, although these structures exist in normal colon they are present at very low levels.

5.5 Isolation of gp140 from HT29 cells.

As depicted in Figure 3.1, 3A7-immunoreactive gp140 is expressed in HT29 and SW480 cell lines established from primary tumors, but undetectable in the SW620 cell line established from a lymph node metastasis. Since all of the antibodies recognizing gp140 detected carbohydrate determinants and recognized multiple glycoproteins, it was unclear whether changes to 3A7-immunoreactive glycoconjugates, including gp140, resulted from altered glycosylation and/or altered polypeptide synthesis. Therefore, gp140 was isolated, using the purification scheme outlined in Figure 5.16. and described in Section 4.14., to obtain information on the structure of gp140 and to generate reagents to be used in the production of antibodies which specifically recognize the polypeptide moiety of gp140.

A detergent cell lysate prepared from the membrane fraction of 60 ml of packed HT29 cells was applied to a WGA-Sepharose column. The majority of the proteins in the cell lysate, as depicted by the absorbance profile at 280 nm (Figure 5.17A, black squares) did not adsorb to WGA-Sepharose. In contrast, all of the mAb 3A7 immunoreactive glycoproteins were recovered in the eluted fractions of WGA-Sepharose, as detected by EIA with mAb 3A7 (Figure 5.17A, red circles).
Figure 5.16: Flow chart depicting chromatographic steps performed for the isolation of 3A7-immunoreactive gp140 from the human colon carcinoma cell line HT29.

Glycoproteins recovered in the WGA-Sepharose eluted fractions were subsequently applied to the blood group A-specific lectin Dolichos biflorus (DBA)-agarose. Confirming earlier observations, nearly all the 3A7-immunoreactive material was recovered in the DBA-agarose unbound fractions (Figure 5.17B, red circles). Analysis of the DBA-unbound and eluted fractions by Western blotting with mAb 3A7 demonstrated that the DBA-unbound fraction contained the majority of the 3A7-immunoreactive glycoproteins, including gp140 (Figure 5.17E, lane 2), whereas the DBA-eluted fraction (Figure 5.17E, lane 1) contained a 3A7-immunoreactive species having an apparent size of 120 kDa and only small amounts of a 140 kDa species. The identity of the 120 kDa species present in the DBA-eluted fraction is not known.

Further purification of glycoproteins in the DBA-unbound fraction was performed by subjecting the material to affinity chromatography on Helix pomatia (HPA)-agarose. As expected, all the 3A7-immunoreactive material bound to HPA-agarose and was
Figure 5.17: Purification of 3A7-immunoreactive gp140 from HT29 cells. Microsomes were prepared from 60 ml of HT29 cells and lysed with 0.5% CHAPS as described in Section 4.14 and subjected to serial lectin affinity chromatography on WGA-Sepharose, DBA- and HPA-agarose and gel filtration on Biogel A5m. Run-through and eluted fractions were monitored for protein content at 280 nm (panels A-D, ■ ) and immunoreactivity by EIA (panels A-D) with mAbs 3A7 (●), 7A8 (▲) or 8H7 (○) and Western blotting with mAb 3A7 (panels E-G). A: The detergent lysate was applied to WGA-Sepharose and bound glycoproteins were eluted with 200 mM GlcNAc. B: mAb 3A7-immunoreactive glycoproteins eluted from WGA-Sepharose were pooled, concentrated and applied to DBA-agarose as described in Section 4.14. Following washing, the column was eluted with 100 mM GalNAc. DBA-unbound (lane 2) and eluted (lane 1) fractions were subsequently pooled, concentrated by ultrafiltration and analyzed by Western blotting using mAb 3A7 (1:3000 dilution) (panel E). C: DBA-agarose unbound material was applied to HPA-agarose, as described in Section 4.14. Following extensive washing, the column was eluted with 100 mM GalNAc. Fractions were assayed for immunoreactivity by EIA and Western blotting with mAb 3A7 (panel F) D: 3A7-immunoreactive glycoproteins eluted from HPA-agarose were pooled, concentrated to 0.5 ml and applied to a Biogel A5m column (size exclusion of 5 x 10⁵ daltons) as described in Section 4.10. Fractions of 1.6 ml were collected and analyzed by EIA and Western blotting with mAb 3A7 (panel G).
subsequently eluted with the competing sugar, as indicated by EIA (Figure 5.17C, red circles) and Western blotting (Figure 5.17F, lanes 51 and 59).

Gel filtration chromatography of the HPA-eluted fraction on Biogel A5m yielded a major immunoreactive species eluting near the void volume of the column, as detected by absorbance at 280 nm (Figure 5.17D, black squares) and EIA with mAb 3A7 (Figure 5.17D, red circles). Analysis of column fractions by Western blotting with mAb 3A7 (Figure 5.17G) indicated that the immunoreactive species migrates as a doublet of 145 and 135 kDa on SDS-polyacrylamide gels. The isolation procedure yielded approximately 300 μg of protein. Analysis of Biogel A5m eluted material by SDS-PAGE followed by silver staining revealed that the major component was gp140 (data not shown).

Lectin blotting of partially purified gp140 revealed that this glycoprotein is the major target for the addition of a number of cancer-associated carbohydrate determinants. Blotting with L-PHA revealed that gp140 bears β1-6 branched Asn-linked oligosaccharides (Figure 5.18A, lane 3). Furthermore, gp140 reacts strongly with biotinylated-DSA lectin (Figure 5.18A, lane 4), but showed no reactivity to biotinylated-LEA (Figure 5.18A, lane 5), suggesting that partially purified gp140 is a carrier of oligosaccharides containing short poly-N-acetyllactosamine repeats.

5.6 Generation and characterization of antibodies recognizing gp140.

Partially purified gp140 obtained from HT29 cells was used for the immunization of a mouse and the generation of monoclonal antibodies. A female balb/c mouse was immunized with purified gp140 and partially purified 3A7-immunoreactive gp140 obtained by single step lectin affinity chromatography on HPA-agarose (50 μg of protein per injection). Following the desired immune response, the spleen was used to prepare hybridomas, as described in Section 4.2, and the hybridomas were
Figure 5.18: Lectin binding profile of gp140 from HT29 cells. A: 3A7-immunoreactive glycoproteins isolated by gel filtration on Biogel A5m (10 µg) were separated by 7.5% SDS-PAGE and blotted with NMS or mAb 3A7 (1:3000) or lectins L-PHA, biotinylated-DSA or biotinylated-LEA, followed by appropriate secondary antibody and colour development, as described in Section 4.4.6. B: HPA-agarose eluted material (30 µg) was immunoprecipitated by mAb 7A8 (lanes 1, 3, 5, 8 and 11) or mAb 8H7 (lanes 2, 4, 6, 9 and 12), separated by 6.5% SDS-PAGE and subjected to Western blotting with NMS (lanes 1 and 2) or mAb 3A7 (lanes 3 and 4) (1:3000 dilution) or lectins L-PHA (lanes 5 and 6), biotinylated-DSA (lanes 7 and 9) and biotinylated-LEA (lanes 10-12).
screened by indirect immunofluorescence analyses, Western blotting of HPA-agarose eluted material and immunoprecipitation analyses.

5.6.1 Screening of hybridomas.

Of the one thousand hybridomas screened, over seven hundred recognized epitopes on permeabilized HT29 cells, as detected by indirect immunofluorescence. The staining patterns observed included cell surface staining, punctate staining or both. Since the staining pattern obtained with mAb 3A7 is primarily cell surface (Figure 5.2B), hybridomas exhibiting this pattern of immunofluorescence staining were selected for further testing (Table 5.6).

Table 5.6: Characterization of monoclonal antibodies and the glycoprotein species recognized.

<table>
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<th>Monoclonal Antibody</th>
<th>Isotype</th>
<th>Species Detected in Western Blotting</th>
<th>Immunofluorescence Pattern</th>
<th>Immunoprecipitation</th>
<th>Presence of 3A7 Epitope</th>
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<td>140 kDa doublet</td>
<td>+</td>
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<td>120 kDa 80 kDa</td>
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Eight clones exhibiting cell surface fluorescence were characterized extensively. The basic characteristics of the monoclonal antibodies and the glycoproteins they detect are summarized in Table 5.6. These monoclonal antibodies were all of the IgG class, with mAbs 6F10, 7B11, 8A4, 9G4 and 11D4 of the IgG₁ subclass, mAbs 7A8 and 8H7 of the IgG₂a subclass and mAb 8C7 of the IgG₂b subclass. All the antibodies were capable of immunoprecipitating protein species of approximately 140 kDa from radiolabeled HT29 detergent cell lysates (Table 5.6). However, the signal obtained in mAbs 6F10 and 8A4 immunoprecipitations was very weak, suggesting these antibodies recognize denatured antigen better. Only mAbs 6F10, 8A4 and 11D4 were capable of recognizing glycoproteins in Western blotting procedures. MAbs 6F10 and 8A4 recognized two species of approximately 80 kDa and 120 kDa, whereas mAb 11D4 recognized a single species at approximately 135 kDa (data not shown). The inability of mAbs 7A8, 7B11, 8C7, 8H7 and 9G4 to detect proteins in Western blotting suggested that these antibodies recognize conformational determinants.

5.6.2 Characterization of glycoproteins recognized by anti-gp140 monoclonal antibodies.

Western blotting of HPA-agarose eluted material immunoprecipitated by mAb 7A8 (lane 3), 7B11 (data not shown), 8C7 (lane 4), 8H7 (lane 5) and 11D4 (lane 6), with mAb 3A7 (Figure 5.19) revealed that gp140 immunoprecipitated by these antibodies contain the 3A7 epitope. MAbs 6F10 and 8A4 immunoprecipitated a glycoprotein that exhibited poor immunoreactivity with mAb 3A7 (Table 5.6). However, the species immunoprecipitated by mAb 9G4 was not 3A7-immunoreactive (Table 5.6). The ability of mAbs 7A8, 7B11, 8C7, 8H7 and 11D4 to recognize a 140 kDa glycoprotein possessing the 3A7 epitope demonstrated that these antibodies recognize the glycoprotein(s) of interest and subsequent studies were carried out using these antibodies.
Figure 5.19: Western blotting analysis of glycoproteins immunoprecipitated by mAb 3A7, 7A8, 8C7, 8H7 and 11D4, with mAb 3A7. HPA-agarose eluted material (30 mg) was immunoprecipitated with NMS (lane 1) or mAbs 3A7 (lane 2), 7A8 (lane 3), 8C7 (lane 4), 8H7 (lane 5) and 11D4 (lane 6), separated by 6.5% SDS-PAGE, and analyzed by Western blotting with mAb 3A7 (1:3000 dilution) followed by secondary antibody and colour detection, as described in Section 4.4.6.
Immunoprecipitation of [35S]-methionine labeled HT29 detergent cell extracts with the panel of monoclonal antibodies and separation on 7.5% polyacrylamide gels produces a single species of approximately 140 kDa, hence the denotation gp140. Analyses of mAbs 7A8 (Figure 5.20A, lane 3) and 7B11 (Figure 5.20A, lane 4) immunoprecipitations on 6.5% gels revealed two species migrating with apparent molecular weights of 145 kDa and 135 kDa under reducing conditions, named gp145 and gp135, respectively. MAbs 8C7 (Figure 5.20A, lane 5) and 8H7 (Figure 5.20A, lane 6) immunoprecipitated gp145, gp135, a species of 160 kDa (gp160) and a faint species of 130 kDa (gp130). MAb 11D4 (Figure 5.20A, lane 7) immunoprecipitated a major band of 135 kDa which appeared to migrate as a single species. Similar studies carried out under non-reducing conditions (Figure 5.20B) revealed that each antibody detected species of approximately 155 kDa and 125 kDa, indicating that the complexes recognized by these antibodies are not associated by interchain disulfide bonding.

The 145 and 135 kDa glycoproteins immunoprecipitated by mAbs 7A8 (Figure 5.21 lane 2) and 7B11 (Figure 5.21, lane 4) produced species of 105 kDa and 95 kDa, respectively (Figure 5.21, lanes 2 and 4) following N-glycanase digestion. Similar treatment of mAbs 8C7 (Figure 5.21, lane 6) and 8H7 (Figure 5.21, lane 8) immunoprecipitates produced species migrating at 130 kDa, 105 kDa and 95 kDa, indicating that all three species recognized by these antibodies are glycoproteins.

Limited proteolytic peptide mapping of the 145 and 135 kDa glycoproteins immunoprecipitated by each of the antibodies confirmed that the antibodies recognize the same 135 (Figure 5.22A) and 145 kDa (Figure 5.22B) glycoprotein species. The 135 kDa species immunoprecipitated by mAbs 7A8 (Figure 5.22A, lane 2), 7B11 (data not shown), 8C7 (Figure 5.22A, lane 3) and 8H7 (Figure 5.22A, lane 4) generated identical peptides. Similarly, gp145 immunoprecipitated by mAbs 7A8, 8C7 and 8H7 (Figure 5.22B, lanes 1, 2 and 3, respectively) generated identical peptide maps. However, the peptide maps produced by immunoprecipitated gp135 (Figure 5.22A) and gp145 (Figure
Figure 5.20: Analysis of species immunoprecipitated by anti-gp140 antibodies. $[^{35}]$-methionine labeled HT29 cell detergent lysates ($2 \times 10^6$ cpm) were immunoprecipitated with NMS (lane 1) or mAbs 7A8 (lane 2), 7B11 (lane 3), 8C7 (lane 4) and 8H7 (lane 5) and separated by 6.5% SDS-PAGE under reducing (panel A) or non-reducing (panel B) conditions. Bands were visualized by fluorography and autoradiography, as described in Section 4.3.
Figure 5.21: Effects of N-glycanase digestion on glycoprotein species immunoprecipitated by mAbs 7A8, 7B11, 8C7 and 8H7. 
[35S]-methionine labeled HT29 cell lysates (2 x 10⁶ cpm) were immunoprecipitated with mAbs 7A8, 7B11, 8C7 and 8H7 and left untreated (lane C) or digested with 0.3 units of N-glycanase (lane F), as described in Section 4.18. Samples were separated by 6.5 % SDS-PAGE and visualized, as described in Section 4.3.
Figure 5.22: Limited V8 protease peptide mapping of species immunoprecipitated by anti-gp140 antibodies. HT29 cells were radiolabeled with [35S]-methionine, harvested and solubilized, as described in Section 4.15. Cell lysates (5 x 10^6 cpm) were immunoprecipitated with mAbs 7A8 (lane 1), 7B11 (lane 5), 8C7 (lane 2), 8H7 (lane 3), 11D4 (lane 4). Samples were separated by 6.0% SDS-PAGE, the 135 kDa (panel A) and 145 kDa (panel B) species were excised from the dried, unfixed gels and subjected to limited V8 protease peptide mapping, as described in Section 4.19.
5.22B) were distinct, suggesting that gp145 and gp135 are different glycoproteins. Still, it is possible that gp145 and gp135 are related glycoproteins. Thus, these antibodies appeared to detect the same complex of glycoproteins. The 160 kDa species immunoprecipitated by mAbs 8C7 and 8H7 generated a peptide map distinct from those of gp145 and gp135 (data not shown). The peptide map obtained from V8 protease digestion of the major species immunoprecipitated by mAb 11D4 (Figure 5.22A, lane 5), varied by only one or two peptides compared to maps generated from gp135 immunoprecipitated by mAbs 7A8, 8C7 and 8H7 (Figure 5.22A, lanes 2–4), suggesting that the species immunoprecipitated by mAb 11D4 is similar but not identical to gp135.

5.7 Sequence analysis of gp140.

Partial protein sequence analysis was performed on tryptic peptides generated from both gp145 and gp135 immunoprecipitated from HT29 cells with mAb 7A8, as described in Section 4.20. The sequences of three tryptic peptides generated from gp145 and two tryptic peptides generated from gp135 were obtained (Table 5.7). Search of these sequences in the Genebank protein sequence data base using the BLAST search system (Altschul et al., 1990) revealed that the partial sequences generated from gp145 and gp135 were contained within the sequences of human β1 integrin and human α3 integrin, respectively.

<table>
<thead>
<tr>
<th>Glycoprotein Species</th>
<th>Partial Amino Acid Sequence</th>
<th>Known Glycoprotein with Matching Sequence</th>
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<tbody>
<tr>
<td>gp145</td>
<td>LKPEDITIQIQPQ</td>
<td>human β1 integrin subunit</td>
</tr>
<tr>
<td>gp135</td>
<td>AGNPGLFGYSVALHR</td>
<td>human α3 integrin subunit</td>
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Table 5.7: Partial amino acid sequence analysis of gp145 and gp135.\textsuperscript{a}

\textsuperscript{a} Glycoprotein species were immunoprecipitated by mAb 7A8 from detergent solubilized HT29 cells, separated by 6.0% SDS-PAGE and transferred to Immobilon-P membrane and visualized with Ponceau S, as described in Section 4.20. Protein bands corresponding to gp145 and gp135 were excised and sent to Dr. Bill Lane (Harvard BioChem) for protein sequence analysis. Samples were digested with trypsin and resultant peptides were sequenced.
5.8 Mapping of epitopes detected by anti-integrin monoclonal antibodies.

5.8.1 Determination of integrin chain specificity of mAbs 7A8, 7B11, 8C7, 8H7, 9G4 and 11D4.

The specificity of mAbs 7A8, 7B11, 8C7, 8H7 and 9G4 was explored initially by depleting $[^{35}S]$-methionine labeled HT29 cell lysates of antigen detected by one antibody and subjecting the resultant supernatant to immunoprecipitation with each of the anti-integrin antibodies. Since $\beta_1$ integrin can associate with a number of different $\alpha$ chains, one would expect that removal of $\beta_1$ integrin using $\beta_1$-chain specific antibodies would deplete cell extracts of all $\alpha$ integrin chains known to associate with $\beta_1$ integrin, including $\alpha_3$ integrin. In contrast, depletion of cell lysates with antibodies specific for the $\alpha_3$ integrin chain would not be expected to affect the amount of $\beta_1$ integrin complexes containing other $\alpha$ integrin chains. Depletion of glycoproteins detected by mAb 7A8 from HT29 cell lysates (Figure 5.23A, lanes 2-6), removed all the glycoproteins immunoprecipitable by both mAbs 7A8 (lane 2) and 7B11 (lane 3), confirming that both antibodies recognize the $\alpha_3\beta_1$ integrin complex. In contrast, depletion of cell lysates with mAb 7A8 did not completely remove glycoproteins detectable by mAbs 8C7, 8H7 and 9G4. MAb 8C7 (Figure 5.23A, lane 4) and 8H7 (Figure 5.23A, lane 5) were still able to immunoprecipitate gp160 and the $\beta_1$ chain, whereas mAb 9G4 (Figure 5.23A, lane 6) was able to immunoprecipitate gp160 from the depleted cell lysate. These results implied that mAbs 7A8 and 7B11 (Figure 5.23B) detect the $\alpha_3$ integrin chain. Conversely, depletion of cell lysates with either mAb 8C7 (data not shown) or 8H7 (Figure 5.23C) completely removed glycoproteins immunoprecipitable by mAbs 7A8 (lane 2), 7B11 (lane 3), 8C7 (lane 4), 8H7 (lane 5) or 9G4 (lane 6). These findings suggested that mAbs 8H7 and 8C7 are specific for the $\beta_1$ integrin chain. The pattern of immunoprecipitation obtained with commercially available antibodies against the $\alpha_3$ chain and the $\beta_1$ chain was consistent with these observations (data not shown). Cell lysates depleted with mAb 9G4 (Figure 5.23D) still contained glycoproteins immunoprecipitable
Figure 5.23: Determination of the chain specificity of mAbs 7A8, 7B11, 8C7, 8H7 and 9G4. [35S]-methionine labeled HT29 cell lysates (2 x 10⁶ cpm) were immunoprecipitated with mAbs 7A8 (panel A), 7B11 (panel B), 8H7 (panel C) or 9G4 (panel D) (lane c). Supernatants depleted with the antibodies were subsequently immunoprecipitated with mAbs 7A8, 7B11, 8C7, 8H7 or 9G4. Samples were separated by 6.5% SDS-PAGE and resultant bands were visualized as described in Section 4.3.
by mAbs 7A8 (lane 2), 7B11 (lane 3), 8C7 (lane 4) and 8H7 (lane 5), suggesting that this antibody does not recognize the α3 or β1 integrin subunit. Still this does not exclude the possibility that mAb 9G4 recognizes the alpha subunit of a different β1 integrin heterodimer.

In collaboration with Dr. Y. Takada (Scripps Institute, La Jolla, CA), we were able to verify the chain specificity of mAbs 7A8, 7B11, 8C7, 8H7 and 9G4 and map the approximate binding sites of these antibodies. The ability of these antibodies to recognize various full-length or truncated human α or β integrin chains expressed in Chinese Hamster Ovary (CHO) cells was assessed using flow cytometry. A schematic diagram of the integrin heterodimer and the approximate binding sites of mAbs 7A8, 7B11, 8C7 and 8H7 is shown in Figure 5.24. MAAb 7A8 recognizes an epitope on the extracellular region of the α3 integrin subunit. MAAb 7B11 also recognized an epitope on the α3 integrin chain. The epitope recognized by mAb 7B11 is at or near the ligand binding domain (Figure 5.24). In contrast, mAbs 8C7 and 8H7 recognized epitopes on the β1 integrin chain at or near the ligand binding domain. As expected, mAb 9G4 recognized a different integrin receptor comprised of the α2 integrin chain. MAAb 11D4 did not recognize any of the CHO transfectants examined, suggesting that it recognizes different glycoproteins or a structure not synthesized by the transfectants.

Figure 5.24: Schematic representation of α3β1 integrin heterodimer and the approximate binding sites of mAbs 7A8, 7B11, 8C7 and 8H7.
5.8.2 Identification of the epitope detected by mAb 11D4.

Based on Western blotting and peptide mapping analyses, MAb 11D4 recognizes a glycoprotein species bearing the 3A7 epitope which is structurally related to the α3 integrin chain. Yet, flow cytometric analysis of CHO transfectants expressing human alpha and beta integrin chains was unable to reveal the specificity of mAb 11D4. Based on our previous experience identifying the specificity of carbohydrate-specific antibodies, we tested the possibility that mAb 11D4 recognizes a blood group determinant. This was executed using neoglycoproteins bearing defined blood group determinants, as described for the epitope mapping of mAbs 3A7, 2F7, 4F3 and 8D5 (Section 5.2). As shown in Figure 5.25, mAb 11D4 was specific for A type 2-BSA alone (Figure 5.25, black squares) indicating that its specificity is distinct from mAb 3A7 specificity. The reactivity of mAb 11D4 for A type 2-BSA was greatly reduced with α-N-acetyl-D-galactosaminidase digestion, indicating that α1-3 linked N-acetylglactosamine is required for antibody recognition (Table 5.5). Thus the determinant recognized by mAb 11D4 is primarily on a single glycoprotein species that is structurally related to α3β1 integrin.

![Absorbance (405 nm) vs. Oligosaccharide (pmole/well)](image)

**Figure 5.25: EIA of neoglycoproteins with mAb 11D4.** Serial dilutions of neoglycoproteins (10-0.01 pmol oligosaccharide/well), depicted in Table 5.4, were added in duplicate to 96-well plates and EIA was performed as described in Section 4.4.2 using mAb 11D4 (1:3000 dilution). Colour development was monitored at 405 nm following addition of p-nitrophenyl phosphate substrate. Each point on the graph represents the mean ± standard deviation (note that for some of the data points, the standard deviation falls below graphic resolution and therefore does not appear. H type 2-BSA (●); A disaccharide-BSA (×); A type 1-BSA (○); A type 2-BSA (●); A type 4-BSA (●); A type 6-BSA (●); B disaccharide-BSA (●); B type 2-BSA (●); B type 4-BSA (●); B type 5-BSA (●); B type 6-BSA (○).
5.9 Characterization of the carbohydrate moiety of $\alpha 3\beta 1$ integrin.

The structure of the glycan moiety of $\alpha 3\beta 1$ integrin was examined using lectin blotting, lectin affinity chromatography, glycosylation inhibitor studies and glycosidase susceptibility analyses. These studies confirmed that $\alpha 3\beta 1$ integrin is a major target for numerous cancer-associated glycosylation changes in human cancer cell lines.

Integrin $\alpha 3\beta 1$ was partially purified from HT29 cells by sequential lectin affinity and gel filtration chromatography (Figure 5.16) as described for the isolation of 3A7-immunoreactive gp140 (Section 5.5.). As expected, mAb 7A8 (Figure 5.17A, green triangles) and 8H7 (Figure 5.17A, blue diamonds) immunoreactive- $\alpha 3\beta 1$ integrin, detected by EIA assay, bound and was subsequently eluted from WGA-Sepharose. This profile parallels the elution of 3A7-immunoreactive material (Figure 5.17A, red circles). As well, 7A8 (Figure 5.17B, green triangles) and 8H7 (Figure 5.17B, blue diamonds) -immunoreactive $\alpha 3\beta 1$ integrin did not bind DBA-agarose. But 7A8 (Figure 5.17C, green triangles) and 8H7 (Figure 5.17C, blue diamonds) -immunoreactive $\alpha 3\beta 1$ integrin co-eluted from HPA-agarose with 3A7-immunoreactive material. Finally, 7A8, 7B11 and 8H7-immunoreactive $\alpha 3\beta 1$ integrin (Figure 5.17D, green triangles, yellow inverted triangles, and blue diamonds, respectively) co-eluted with the 3A7-immunoreactive material (Figure 5.17D, red circles) on a Biogel A5m gel filtration column.

5.9.1 Analysis of Asn-linked oligosaccharides on $\alpha 3\beta 1$ integrin.

The sensitivity of $\alpha 3\beta 1$ integrin immunoprecipitated by mAb 7A8 (Figure 5.26A) and 8H7 (Figure 5.26B) to N-glycanase digestion (lane c) indicated that $\alpha 3\beta 1$ integrin contains Asn-linked oligosaccharides. In fact, Asn-linked oligosaccharides make up approximately 30% of the apparent molecular weight of each species of the glycoprotein complex. The approximate number of Asn-linked chains on the individual integrin subunits was determined by partial digestion with N-glycanase (Figure 5.27). The individual chains immunoprecipitated from $[35S]$-methionine labeled HT29 cells by mAb
Figure 5.26: Susceptibility of α3β1 integrin to glycosidases. "[35S]-methionine labeled HT29 cell lysates (2 x 10^6 cpm) were immunoprecipitated with NMS, mAbs 7A8 or 8H7. Samples were left untreated (lane a) or digested with neuraminidase type X from Clostridium perfringens (lane b), N-glycanase (lane c), O-glycanase (lane d), O-glycanase plus neuraminidase type X (lane e) or endo-β-galactosidase (lane f), as described in Section 4.18. Samples were analyzed by 6.5% SDS-PAGE and bands were visualized as described in Section 4.3.
8H7, namely gp160, gp145 (β1 integrin chain), gp135 (α3 integrin chain) and gp130 were excised from SDS-gels and digested with N-glycanase for varying periods of time.

As shown in Figure 5.27, gp145 (panel B) appears to be glycosylated to a greater extent than gp135 (panel C) since the apparent molecular weight of the deglycosylated species generated from these glycoproteins following 24 hour treatment are 95 kDa (Figure 5.27B, lane f) and 105 kDa (Figure 5.27C, lane f), respectively. The presence of partially deglycosylated intermediates produced during the time course of N-glycanase digestion was consistent with the presence of a minimum of five (5) Asn-linked chains on gp145 (Figure 5.27B, lanes b-f) and a minimum of four (4) chains on gp135 (Figure 5.27C, lanes b-f). Due to the close proximity of bands within the polyacrylamide gels we were unable to determine precisely the maximum number of Asn-linked oligosaccharide chains on the mature glycoprotein chains. Based on protein sequence information, the α3 and β1 integrin chains contain 12 and 13 potential Asn-linked glycosylation sites, respectively (Takada et al., 1988). Similar analysis of gp160 indicated that Asn-linked oligosaccharides account for only about 20% of its mass with the deglycosylated form of gp160 migrating with an apparent molecular weight of 130 kDa (Figure 5.27, lane f). The 130 kDa glycoprotein in the mAb 8H7 immunoprecipitate, which is the least abundant species (Figure 5.27D, lane a), possesses only a few Asn-linked oligosaccharide chains (Figure 5.27D, lanes b-f), accounting for about 13% of its mass.

Further analysis of the Asn-linked oligosaccharide chains present on α3β1 integrin was carried out using inhibitors of Asn-linked oligosaccharide biosynthesis (refer to Figure 2.17 for the Asn-linked glycosylation pathway and sites of inhibitor action). Treatment of HT29 cells with tunicamycin, which inhibits the biosynthesis of the lipid-linked precursor Glc3Man9GlcNAc2-P-P-Dol, prevented immunoprecipitation of α3β1 integrin and gp160 by all of the α3β1 integrin-specific antibodies examined (Figure 5.28, lane c). The inability of these antibodies to recognize α3β1 integrin in tunicamycin treated cells suggested that Asn-linked oligosaccharides are essential for maintaining the
Figure 5.27: Analysis of Asn-linked oligosaccharide content on the α3 and β1 integrin chains. [35S]-methionine labeled HT29 cell lysates (5 x 10⁶ cpm) were immunoprecipitated by mAb 8H7 and the glycoprotein species were separated by 6.0% SDS-PAGE. The four immunoprecipitated bands, gp160 (panel A), α3 integrin chain (gp145) (panel B), β1 integrin chain (gp135) (panel C) and a 130 kDa species (panel D), were excised and left untreated (lane a) or digested with 0.3 units N-glycanase for 15 minutes (lane b), 30 minutes (lane c), 1 hour (lane d), 2 hours (lane e) or 24 hours (lane f). Samples were analyzed by 6.5% SDS-PAGE and visualized as described in Section 4.3.
Figure 5.28: Effect of glycosylation inhibitors on the ability of α3β1 integrin to be immunoprecipitated by mAb 7A8, 8H7 and 11D4. HT29 cells were radiolabeled with [35S]methionine in the absence (lanes a and b) or presence of the glycosylation inhibitors tunicamycin (lane c) or swainsonine (lane d), as described in Section 4.16. Cells were labeled in the presence of DMSO alone (lane b) to control for DMSO in the tunicamycin stock solution. Detergent cell lysates were prepared as described in Section 4.12. Cell lysates were immunoprecipitated (2 x 10⁶ cpm) with NMS or mAbs 7A8, 8H7 or 11D4. Samples were analyzed by 6.5% SDS-PAGE and bands were visualized as described in Section 4.3.
native conformation recognized by each of these antibodies. Alternatively, newly synthesized unglycosylated α3β1 integrin is rapidly degraded. That treatment of radiolabeled HT29 cell lysates with N-glycanase prior to immunoprecipitation also failed to yield immunoprecipitable α3β1 integrin (data not shown) provided additional evidence that glycosylation of α3β1 integrin plays an important role in maintaining its native conformation.

Treatment of HT29 cells with deoxymannojirimycin (DMJ) (Figure 5.29), which inhibits the processing of high mannose to complex type Asn-linked oligosaccharides, and swainsonine (Figure 5.28, lane d), which blocks initiation of β1-6 branch synthesis, had no effect on the ability of anti-integrin antibodies to immunoprecipitate α3β1 integrin. Both mAbs 7A8 and 8H7 were capable of immunoprecipitating α3β1 integrin from cells treated with DMJ (Figure 5.29, lane b). The reduction in apparent molecular weight under non-reducing conditions to 100 and 130 kDa is consistent with the presence of high mannose-type and hybrid-type Asn-linked oligosaccharides, respectively, which are usually smaller in size than complex-type oligosaccharides. These data suggested that although Asn-linked carbohydrates are necessary for maintenance of the native conformation of α3β1 integrin there does not appear to be a requirement for specific types of Asn-linked oligosaccharides.

Treatment of cells with swainsonine did not prevent immunoprecipitation of α3β1 integrin by mAbs 7A8, 7B11, 8C7, 8H7 and 11D4 (Figure 5.28, lane d). The reduction in apparent molecular weight of the 145 and 135 kDa species to 120 kDa and 116 kDa, respectively, confirmed that each species contains β1-6 branched complex type Asn-linked oligosaccharides. In addition, the β1 integrin subunit (gp145) appears to contain more and/or larger β1-6 branched Asn-linked oligosaccharides compared to the α3 integrin subunit (gp135). The 160 kDa species immunoprecipitated by mAb 8C7 (data not shown) and 8H7 (Figure 5.28, lane d) appears to contain β1-6 branched complex type Asn-linked oligosaccharides since there is a slight decrease in apparent molecular
Figure 5.29: Effect of glycosylation inhibitor deoxymannojirimycin on the ability of α3 and β1 integrin chains to associate as a heterodimer. HT29 cells were radiolabeled with [35S]-methionine in the absence (lane a) or presence (lane b) of the glycosylation inhibitor deoxymannojirimycin (DMJ), as described in Section 4.16. Cells lysates were immunoprecipitated with NMS, mAbs 7A8 or 8H7. Samples were separated by 6.5% SDS-PAGE under non-reducing conditions and bands were visualized as described in Section 4.3.
weight of the species immunoprecipitated from swainsonine treated HT29 cell lysates. These findings confirmed the presence of β1-6 branched Asn-linked oligosaccharides on α3 and β1 integrin chains and possibly on gp160. However, the presence of β1-6 branched Asn-linked oligosaccharides does not appear to be essential for antibody binding.

The A type 2 epitope detected by mAb 11D4 is not on the β1-6 branch of complex-type Asn-linked oligosaccharides, since mAb 11D4 immunoprecipitates a species of 120 kDa from swainsonine treated HT29 cell lysates (Figure 5.28, lane d). However, mAb 11D4 was unable to immunoprecipitate a species from DMJ treated HT29 cells, suggesting that the epitope detected by mAb 11D4 is on complex type Asn-linked oligosaccharides (data not shown). As demonstrated in Section 5.2.3., the 3A7 epitope, which appears to be on type 2 chains bearing blood group A or B determinants, is present primarily on the β1-6 branch of Asn-linked oligosaccharides (Figure 5.8B). These data emphasize the uniqueness of the 3A7 epitope and the preferential addition of this epitope to the β1-6 branch.

5.9.2 Analysis of O-linked oligosaccharides on α3β1 integrin.

Treatment of immunoprecipitated α3β1 integrin with O-glycanase, which is specific for Galβ1-3GalNAc-O-R structures, without (Figure 5.27, lane d) or with (Figure 5.27, lane e) prior neuraminidase digestion had little effect on the size of α3β1 integrin detected by SDS-PAGE, suggesting that it contains very little or no O-linked oligosaccharide, or that it contains more complex-type O-linked oligosaccharides which are not cleaved by O-glycanase (Adolf et al., 1991).

A second approach was used to determine whether α3β1 integrin contains O-linked oligosaccharides. Incubation of cells with the compound benzyl-α-N-acetylglactosamine (Bz-αGalNAc), which is incorporated into newly synthesized O-linked chains and prevents further elongation of O-linked structures, resulted in
immunoprecipitation of lower molecular weight species by mAbs 7A8 (Figure 5.30, lane c), 7B11 (data not shown), 8C7 (data not shown) and 8H7 (Figure 5.30, lane c). These data suggested that α3β1 integrin contains O-linked oligosaccharides but that these oligosaccharides are not necessary for antibody recognition. MAbs 3A7 (Figure 5.30, lane c) and 11D4 (Figure 5.30, lane c) were unable to immunoprecipitate α3β1 integrin from cells treated with Bz-αGalNAc. This finding was unexpected given the results obtained with swainsonine treated cells (Figures 5.8 and 5.28) which indicated that at least some of the 3A7 binding structures are present on the β1-6 branch of Asn-linked oligosaccharides. One possible explanation for these conflicting results is that treatment of cells with Bz-αGalNAc inhibits the synthesis of O-linked oligosaccharides on glycosyltransferases. If O-linked oligosaccharides are required for activity of glycosyltransferases involved in the synthesis of the 3A7 epitope, treatment of cells with Bz-αGalNAc would indirectly result in loss of the 3A7 epitope. Further analysis will be required to determine if the 3A7 epitope is present exclusively on the β1-6 branch of Asn-linked oligosaccharides or whether it is also found on complex type O-linked oligosaccharides.

5.9.3 Analysis of terminal oligosaccharides on α3β1 integrin.

Lectin blotting of partially purified α3β1 integrin confirmed that this complex is a major target for the addition of oncodevelopmentally regulated carbohydrate determinants. Blotting with L-PHA verified that Asn-linked oligosaccharides on α3β1 integrin (Figure 5.18B, lanes 5 and 6) bear the β1-6 branch. This result is consistent with earlier studies which showed that gp140 recognized by mAb 3A7 binds L-PHA (Figure 5.18A). As well, α3β1 integrin immunoprecipitated by mAb 7A8 and 8H7 reacted strongly with biotinylated-DSA lectin (Figure 5.18B, lanes 8 and 9), but showed no reactivity to biotinylated-LEA (Figure 5.18B, lanes 11 and 12), indicating that α3β1 integrin is a carrier of oligosaccharides containing short poly-N-acetyllactosamine repeats. These data
Figure 5.30: Effect of O-linked glycosylation inhibitors on \(\alpha3\beta1\) integrin structure. HT29 cells were radiolabeled with \([35S]\)-methionine in the absence (lane a) or presence (lane c) of benzyl-\(\alpha\)-galactosaminyl (Bz-\(\alpha\)-GalNAc), as described in Section 4.16. Cells were also grown in the presence of benzyl-\(\alpha\)-glucosaminyl (Bz-\(\alpha\)-GlcNAc, lane b) to control for treatment with a benzyl compound. Cell lysates were immunoprecipitated with NMS or mAbs 3A7, 7A8, 8H7 or 11D4. Samples were separated by 6.5% SDS-PAGE under reducing conditions and bands were visualized as described in Section 4.3.
agree with lectin reactivity profiles obtained with 3A7-immunoreactive glycopeptides isolated from normal rat colon, suggesting that 3A7-immunoreactive glycoconjugates in human and rodent systems have similar carbohydrate structures. Moreover, the sensitivity of both gp145 and gp135 immunoprecipitated by mAb 7A8 (Figure 5.26A) or mAb 8H7 (Figure 5.26B) to neuraminidase (lane b) indicated that both the β1 and α3 integrin chains are sialoglycoproteins. Furthermore, gp160 detected by mAb 8H7 displayed a slight sensitivity to neuraminidase (Figure 5.26B, lane b), suggesting that it contains some sialic acid residues. Treatment of immunoprecipitated α3β1 integrin with the enzyme endo-β-galactosidase (Figure 5.26A and B, lane f) resulted in little or no change in apparent molecular weight of either the 145, 135 or 160 kDa glycoproteins, suggesting that there is very little poly-N-acetyllactosamine structure on these glycoprotein chains. It should be pointed out, however, that oligosaccharides containing branched poly-N-acetyllactosamine structures are not cleaved by this enzyme very efficiently (Merkle and Cummings, 1987b). In light of this fact and the DSA-lectin staining pattern of α3β1 integrin (Figure 5.18A and B, lanes 8 and 9), it is likely that this receptor contains branched poly-N-acetyllactosamine structures.

5.10 Expression of α3β1 integrin on human colon carcinoma cell lines.

Availability of monoclonal antibodies specific for the polypeptide portion of gp140, which defines α3β1 integrin, made it possible to examine the expression of α3β1 integrin in human colon carcinoma cell lines and compare that expression to the expression of the 3A7 epitope in these cell lines.

Immunoprecipitation studies with [35S]-methionine labeled HT29, SW480 and SW620 cells demonstrated that α3β1 integrin expression is significantly decreased in SW620 cells compared to HT29 and SW480 cells. As shown in Figure 5.31, α3β1 integrin is immunoprecipitated from HT29 and SW480 cell lysates by mAb 3A7 (lanes 2 and 6, respectively), mAb 7A8 (lanes 3 and 7, respectively) and mAb 8H7 (lanes 4 and
Figure 5.31: Expression of glycoproteins detected by mAb 3A7, 7A8 and 8H7 in human colon cancer cell lines. The human colon cancer cell lines HT29, SW480, SW620 were radiolabeled for 24 hours with $[^35]S$methionine. Radiolabeled cell lysates (2 x $10^6$ cpm) were immunoprecipitated with NMS (lanes 1, 5 and 9), mAbs 3A7 (lanes 2, 6 and 10), 7A8 (lanes 3, 7 and 11) or 8H7 (lanes 4, 8 and 12) and separated by 6.5% SDS-PAGE. Bands were visualized as described in Section 4.3.
8, respectively). In contrast, neither mAb 3A7 (Figure 5.31, lane 10) nor mAb 7A8 (Figure 5.31, lane 11) immunoprecipitated α3β1 integrin from radiolabeled SW620 cell lysates, indicating that synthesis of the α3 integrin chain is greatly reduced or abolished in SW620 cells. In addition, the level of integrin complexes immunoprecipitated by mAb 8H7 (Figure 5.31, lane 12) was greatly reduced in SW620 cells compared to SW480 (Figure 5.31, lane 8). Indirect immunofluorescence of fixed and permeabilized HT29, SW480 and SW620 cells confirmed that cell surface expression of α3β1 integrin is lost in SW620 cells. These staining patterns mirrored results obtained earlier with mAb 3A7 (Figure 5.2) and provide evidence that the absence of 3A7-immunoreactive α3β1 integrin (formerly known as gp140) in SW620 cells is a result of loss of the α3β1 integrin polypeptide. Since the SW480 and SW620 cell lines were established from a primary tumor and a lymph node metastasis from the same patient, respectively, the absence of α3β1 integrin in SW620 cells appears to be metastasis-associated. Thus, it appears that loss of α3β1 integrin and/or the 3A7 epitope may be significant to colon cancer progression and metastasis.

The expression of α3β1 in a panel of human colon carcinoma cell lines differing in histopathological grade was examined by flow cytometry to address the significance of α3β1 integrin in colon cancer. In total, twenty cell lines were assessed for integrin expression (Table 5.8) including, seven cell lines isolated from blood group A, AB or B patients. The cell lines used in these studies were obtained from ATCC, Dr. I.J. Fidler and Dr. J. Xiang and cultured as described in Section 4.1. The cell lines tested were originally established from primary tumors of varying histopathological grade, lymph node metastases or liver metastases (Table 5.8). For example, the LS123 cell line was established from a Duke's A tumor while HT29 and SW480 cell lines were established from Duke's B2 and C1 tumors, respectively. SW620 and KM20C cell lines originated from a lymph node metastasis and liver metastasis, respectively (Table 5.8). In addition,
Table 5.8: Analysis of 3A7 epitope and α3β1 integrin expression in a panel of human colon carcinoma cell lines.

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Tumor Classification</th>
<th>Blood Group Status</th>
<th>Mean Fluorescence Intensity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>3A7</td>
</tr>
<tr>
<td>LS123</td>
<td>abnormal epithelium</td>
<td>n.a.</td>
<td>1.16±0.12</td>
</tr>
<tr>
<td>SW1116</td>
<td>Duke's A</td>
<td>O</td>
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</tr>
<tr>
<td>CaCo-2</td>
<td>Duke's B1</td>
<td>O</td>
<td>2.22±0.06</td>
</tr>
<tr>
<td>LS174T</td>
<td>Duke's B1</td>
<td>O</td>
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<tr>
<td>C0LO320DM</td>
<td>Duke's B2</td>
<td>n.a.</td>
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<td>WiDr</td>
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<td>A</td>
<td>29.6±2.8</td>
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<td>Duke's B2</td>
<td>A</td>
<td>34.2±0.26</td>
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<tr>
<td>KM12C</td>
<td>Duke's B2</td>
<td>O</td>
<td>2.83±0.33</td>
</tr>
<tr>
<td>SW403</td>
<td>Duke's C1</td>
<td>O</td>
<td>1.80±0.25</td>
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<td>SW1417</td>
<td>Duke's C2</td>
<td>B</td>
<td>10.8±2.3</td>
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<td>SW480</td>
<td>Duke's C2</td>
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<td>8.44±3.2</td>
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<td>A</td>
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<tr>
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<td></td>
<td>A</td>
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<tr>
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<td>2.44±1.12</td>
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<tr>
<td>SW48</td>
<td>Duke's D</td>
<td>AB</td>
<td>19.8±3.0</td>
</tr>
<tr>
<td>KM20C</td>
<td>Duke's D</td>
<td>A</td>
<td>29.9±3.1</td>
</tr>
<tr>
<td>SW620</td>
<td>lymph node metastases</td>
<td>A</td>
<td>1.91±0.37</td>
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<tr>
<td>LoVo</td>
<td>supraclavicular</td>
<td>B</td>
<td>12.9±2.1</td>
</tr>
<tr>
<td>KM12SM</td>
<td>nude mouse metastases</td>
<td>O</td>
<td>2.34±0.23</td>
</tr>
<tr>
<td>KM12L4A</td>
<td>nude mouse metastases</td>
<td>O</td>
<td>3.40±0.52</td>
</tr>
</tbody>
</table>

n.a. not available; n.d. not determined

*a* cell line established from a tissue in the shoulder region, *b* cell line established from metastases generated in a nude mouse after intracerebral injection of KM12C cells, *c* cell line established from a liver metastasis produced after *in vivo* selection of KM12C cells with enhanced metastatic ability (Morikawa *et al.*, 1988).

cell lines established from metastases generated in a nude mouse model (KM12SM and KM12L4A cell lines) were included in the panel of cell lines tested (Table 5.8). Moreover, the SW480 cell line consists of two morphologically distinct cell types: a population with epithelial-like morphology (SW480-E) and a population with a rounded morphology (SW480-R). These two populations were isolated by limited dilution cloning and analyzed for α3β1 integrin expression. The level of α3β1 integrin expressed by these colon cancer cell lines was analyzed by flow cytometry and immunoprecipitation of [35S]-methionine labeled cell lysates to determine cell surface expression and total expression of α3β1 integrin, respectively.
Firstly, only the cell lines originating from blood group A, AB or B patients expressed the 3A7 epitope as detected by both immunofluorescence (Table 5.8) and immunoprecipitation analyses (see Figure 5.31 for representative samples). The level of expression of this carbohydrate epitope was variable, with the HT29 cell line exhibiting the highest level of 3A7 staining with a mean fluorescence intensity 34.2 and the SW620 cell line exhibiting the lowest level of 3A7 staining of all the blood group A or B derived cell lines (Table 5.8). Secondly, the level of α3β1 integrin expression was highly variable among the cell lines tested (Table 5.8) and expression levels did not appear to correlate with tumor staging. Thirdly, the majority of the cell lines had moderate to high cell surface expression of the β1 integrin subunit, as detected by mAb 8H7 staining (Table 5.8). Flow cytometry data was similar to data obtained by immunoprecipitation of radiolabeled cells with the antibodies, indicating cell surface expression of α3β1 integrin is an accurate assessment of total α3β1 integrin expression in these cell lines, thus only flow cytometric data is shown.

The majority of the cell lines consisted of populations with relatively homogeneous patterns of α3β1 integrin and 3A7 epitope expression (Figure 5.32). As well, individual cells in all the cell lines exhibited similar cell size and cell density. For example, HT29 cells stained intensely with mAbs 3A7 (Figure 5.32A), 7A8 (panel B), 7B11 (panel C) or 8H7 (panel D), indicating a high level of expression of the α3β1 integrin complex and the 3A7 epitope on the cell surface. However, flow cytometric analysis of SW480 cells indicated that there are two subpopulations with respect to α3β1 integrin expression (Figure 5.32F and G) and 3A7 epitope expression (panel E) within this cell line. As stated earlier, the SW480 cell line consists of two morphologically distinct cell type which were isolated by limited dilution cloning. Flow cytometric analysis of the SW480 subpopulations revealed that SW480-R cells expressed both high levels of α3β1 integrin and the 3A7 epitope, whereas SW480-E cells stained weakly with anti-α3 antibodies and mAb 3A7 (Table 5.8). Studies by Tomita, et al
Figure 5.32: Flow cytometric analysis of HT29, SW480 and SW620 cell lines with mAbs 3A7, 7A8, 7B11 and 8H7. Human colon carcinoma cell lines, HT29 (panels A-D), SW480 (panels E-H) and SW620 (panels I-L) were trypsinized by standard protocols and incubated for 1 hour with mAbs 3A7 (panel A, E and I), 7A8 (panel B, F and J), 7B11 (panel C, G and K) or 8H7 (panel D, H and L) followed by incubation with FITC-labeled goat anti-mouse Ig and analyzed with an EPICS flow cytometer (Coulter), as described in Section 4.4.4.
(1992) indicated that the SW480-R cells produced large, more aggressive tumors than SW480-E. The SW620 cell line expressed significantly lower levels of both $\alpha 3\beta 1$ integrin and the 3A7 epitope (Figure 5.32K, L and M). These data suggested a correlation between the presence of $\alpha 3\beta 1$ integrin and expression of the 3A7 epitope on the cell surface.

5.11 Co-expression of $\alpha 3\beta 1$ integrin and the 3A7 epitope on human colon carcinoma cell lines.

Comparison of $\alpha 3\beta 1$ integrin and 3A7 epitope expression in the blood group A, B and AB derived cell lines (Table 5.8) established a direct correlation (Figure 5.33). As seen in Figure 5.33, graphical representation of cell surface expression of the $\alpha 3$ integrin subunit versus the expression of the 3A7 epitope of these human colon carcinoma cell lines produced a straight line. Linear regression analysis on this plot established a regression value ($r$) of 0.970. The proximity of this value to 1.00 provided evidence that expression of the 3A7 epitope on the tumor cell surface is dependent on cell surface expression of the $\alpha 3$ integrin subunit. Similar comparative analysis of cell surface expression of the $\beta 1$ integrin subunit (detected by mAb 8H7) and 3A7 epitope staining yielded a correlation coefficient of 0.852 (data not shown), suggesting that the correlation between $\beta 1$ integrin subunit levels and 3A7 epitope levels is not as strong as the correlation between the levels of the $\alpha 3$ integrin subunit and 3A7 epitope. Therefore, it appears that the $\alpha 3$ integrin chain is the major carrier of the 3A7 epitope in human colon carcinoma cell lines originating from blood group A, AB and B individuals.

5.12 Other possible mechanisms regulating 3A7 epitope expression in normal and neoplastic colon.

The above data suggested that biosynthesis and assembly of $\alpha 3\beta 1$ integrin synthesis plays an important role in regulating the expression of the carbohydrate
Figure 5.33: Correlation between expression of the 3A7 epitope and \( \alpha_3 \) integrin subunit in blood group A, AB and B positive human colon carcinoma cell lines. The fluorescence intensity obtained by staining with mAb 3A7 was plotted against the fluorescence intensity obtained with mAb 7B11 (anti-\( \alpha_3 \) integrin subunit) (Refer to Table 5.8 for mean fluorescence intensity \( \pm \) standard deviation and description of human colon cancer cell lines). Four separate experiments for each cell line are represented on the graph. Curve fit and regression value were calculated using linear regression analysis.
determinant recognized by mAb 3A7. Since the 3A7 epitope is carbohydrate in nature and is therefore synthesized by specific glycosyltransferases, it was important to determine whether specific glycosyltransferases also control 3A7 epitope expression. Based on the minimum structure of the 3A7 epitope, synthesis of this determinant in colon tumors could result from increased activity of α1-3 N-acetylgalactosaminyl transferase (A transferase) which adds GalNAc in an α1-3 linkage to [Fucα1-2]Galβ1-4GlcNAc (H type 2 determinant) (Yamamoto et al., 1990), α1-2 fucosyltransferase which is responsible for synthesis of the H determinant from Galβ1-3/4GlcNAc (Marcus, 1969), or β1-4 galactosyltransferase involved in the synthesis of type 2 carrier chains (Holmes, 1988). Likewise, the increased synthesis of the epitope could be a result of increased synthesis of branches in which the 3A7 epitope is preferentially added. Specifically, synthesis of the β1-6 branch of Asn-linked oligosaccharides, which is initiated by the enzyme, β1-6 N-acetylgalactosaminyltransferase (GnT V) could influence 3A7 epitope synthesis. N-acetylgalactosaminyl transferase I (GnT I), the enzyme responsible for initiating complex type Asn-linked oligosaccharide synthesis was used as a control since activity of this enzyme does not appear to alter in response to neoplasia. Blood group determinants are frequently added to complex-type O-linked oligosaccharides. As well, it was not known if the 3A7 epitope was present on O-linked oligosaccharides. Therefore, enzymes involved in the synthesis of complex type O-glycan synthesis were assayed. The levels of β6 N-acetylgalactosaminyltransferase (Core 2 transferase), initiator of the β1-6 branch on O-linked glycans and β3-galactosyltransferase (Core 1 transferase), initiator of O-linked oligosaccharide synthesis were determined.

5.12.1 Glycosyltransferase activity in human colon carcinoma cell lines.

Microsomes isolated from human colon carcinoma cell lines were solubilized with 0.2% Triton X-100 and the detergent lysates were assayed for glycosyltransferase
activity, as described in Section 4.21. The level of 3A7-immunoreactivity in these cell lines was assessed as described in Section 5.10, Table 5.8. The activities of α1-3-N-acetylgalactosaminyl transferase (A transferase), α1-2 fucosyltransferase, β1-4 galactosyltransferase, GnTI, Core 1 transferase and Core 2 transferase, were determined in detergent lysates prepared from WiDr, HT29, SW1417, SW480, SW620, LoVo and KM20C cells (Figure 5.34A). Attempts were made to assess the activity of GnTV by the 'Sep-pak' method and by an EIA method but the activities were too low to produce reliable and reproducible results.

The activities of the glycosyltransferases examined were quite variable in the different cell lines. Interestingly, of the transferases measured, the specific activity of A transferase was the lowest (Figure 5.34A, closed bar). Of the enzymes tested, Core 1 enzyme (Figure 5.34A, pink bar) and Core 2 enzyme (Figure 5.34A, orange bar), enzymes involved in the synthesis of O-linked glycans, were the most active glycosyltransferases in these human colon carcinoma cell lines. There was no relationship between the activity of any of the transferases and the level of cell surface mAb 3A7 staining. For example, HT29 cells expressed high levels of the 3A7 epitope, whereas SW620 cells expressed undetectable levels of the epitope (Table 5.8). And yet, SW620 cells contained higher activity of A transferase (Figure 5.34A, black bar) and α1-2 fucosyltransferase (Figure 5.34A, blue flecked bar) than HT29 cells. There was a decrease in the level of β1-4 galactosyltransferase activity (Figure 5.34A, yellow spotted bar) in a few of the cell lines with decreased 3A7 epitope levels. Graphical representation of cell surface expression of the 3A7 epitope against the activities of either A transferase (Figure 5.34B) or α1-2 fucosyltransferase (Figure 5.34C) showed that there was no correlation between 3A7 epitope expression and these glycosyltransferase activities in the human colon carcinoma cell lines tested. These data support the hypothesis that cell surface expression of the 3A7 epitope in human colon carcinoma cell lines may be regulated by α3β1 integrin expression, provided that the activities of enzymes regulating
Figure 5.34: Glycosyltransferase activity in blood group A, AB and B positive human colon carcinoma cell lines. A: The specific activities (mU/mg) of the glycosyltransferases α1-3 N-acetylgalactosaminytransferase (A tranferase) (black bar), α1-2 fucosyltransferase (dotted magenta bar), β1-4 galactosyltransferase (Gal T) (dotted yellow bar), N-acetylgalactosaminytransferase I (GnT1) (blue cross hatched bar), β3-galactosyltransferase (Core 1 transferase) (waved red bar) and β6-N-acetylgalactosaminytransferase (Core 2) (brick green bar) were determined in detergent-solubilized microsome fractions isolated from human colon carcinoma cell lines, as described in Section 4.21. The specific activity ± standard error is represented. Note that error bars do not occur on some graphs because the error was below graphic resolution. B: Plot of the mean fluorescence intensity obtained following staining of cells with mAb 3A7 versus the specific activity of A transferase (■) or α1-2 fucosyltransferase (○). C: Plot of mean fluorescence intensity obtained following staining with mAb 7B11 (anti-α3 integrin subunit) versus the specific activity of A transferase (■) or α1-2 fucosyltransferase (○).
the synthesis of the 3A7 epitope are not limiting. Thus, the synthesis of specific polypeptides may play an important role in regulating expression of oncodevelopmental carbohydrate epitopes.

5.12.2 Glycosyltransferase activity in normal and neoplastic rat colon.

The correlation between glycosyltransferase activity and 3A7 epitope expression was also analyzed in the azoxymethane-induced rat model of carcinogenesis. The activities of α1-3-N-acetylgalactosaminyl transferase (A transferase), α1-2 fucosyltransferase, β1-4 galactosyltransferase, Gnt I, Core 1 transferase and Core 2 transferase were assayed in a panel of azoxymethane-induced rat colon tumors of differing histopathological grades and corresponding normal tissues. (Refer to Table 5.2 and Figure 5.3 for expression of the 3A7 epitope in these samples.) The activities of all of the glycosyltransferases tested were greater in the normal tissues examined than in any of the colon tumors (Figure 5.35). These findings are in sharp contrast to the amount of 3A7-immunoreactive glycoproteins in normal colon, which is at least 10 fold lower than in most tumors (Figure 5.3, lanes 1, 2 and 7 compared to lanes 3-6 and 8-21). In particular, A transferase was extremely active in both normal and neoplastic tissues, with the highest activity in the ascending and descending colon (Figure 5.35A) and a moderate level of activity in the caecum. All of the tumors contained significantly less A transferase activity than normal colon samples. It is noteworthy that the A transferase activity was 100 fold higher in rat colon than in the human colon carcinoma cell lines. Among the azoxymethane-induced rat colon tumors examined, samples with elevated levels of 3A7-immunoreactive glycoproteins (e.g. Figure 5.3, lane 17) had high levels of A transferase activity (e.g. Figure 5.35A, sample 26-11), whereas tumors with a low levels of 3A7-immunoreactive glycoproteins (e.g. Figure 5.3, lane 13) had low levels of A transferase activity (e.g. Figure 5.35A, sample 28-13). The A transferase activity present in the detergent lysates of normal and neoplastic colon did not show preference for type 1 or 2
Figure 5.35: Analysis of glycosyltransferase activity in normal rat colon and azoxymethane-induced rat colon tumors. The specific activities (mU/mg) for glycosyltransferases were determined for portions of normal rat colon (caecum, ascending colon (AC) and descending colon (DC)) and azoxymethane-induced rat colon tumors of different histopathological grade, depicted in Table 5.2, as described in Section 4.21. The specific activity ± standard error is represented on the graphs. Note that error bars do not occur on some graphs because the error was below graphic resolution. The specific activities of the ascending and descending colon are mean ± standard error from three separate animals. A: α1-3 N-acetylglactosaminyltransferase (A transferase) specific activities toward the acceptors, H-disaccharide (Fucα1-2Gal-grease, open bar) and H type 2 structure (Fucα1-2Galβ1-4GlcNAc-grease, closed bar). B: α1-2 fucosyltransferase total specific activity (open bar), α1-2 fucosyltransferase activity toward type 1 chain glycans (green cross-hatched bar), α1-2 fucosyltransferase activity toward type 2 chain glycans (red solid bar). C: β1-4 galactosyltransferase (Gal T) specific activity. D: N-acetylglucosaminyltransferase I (GnT I) specific activity. E: β3-galactosyltransferase (Core 1) specific activity. F: β 6-N-acetylglucosaminyltransferase (Core 2) specific activity.
chains since similar specific activities were obtained using Fucα1-2Gal-octyl (Figure 5.35A, open bar) and H type II acceptor (Figure 5.35A, closed bar), respectively. Therefore, the increased presence of 3A7-immunoreactive glycoproteins could not be explained by increased activity of an A transferase specific type 2 chains in azoxymethane-induced rat colon tumors.

Previous studies have indicated that the synthesis of blood group A and B carbohydrate determinants is dependent upon the synthesis of the precursor H determinant by α1-2 fucosyltransferases (refer to Figure 2.12 for ABH blood group synthesis) (Ørntoft et al., 1991). In addition, there are two genes in mammalian tissues which encode α1-2 fucosyltransferase. The Se gene encodes an enzyme which is expressed by epithelia, including colonic mucosa, and preferentially modifies type 1 chain glycans. The H gene encodes an enzyme which is expressed by erythrocytes, and some colon tumors, and preferentially modifies type 2 chain oligosaccharides (Piau et al., 1995). The total activity of α1-2 fucosyltransferase (Figure 5.35B, open bar) in normal colon was found to be greatest in ascending colon, followed by caecum and descending colon. By comparison, the total α1-2 fucosyltransferase activity was significantly lower in all the azoxymethane-induced colon tumors assayed (Figure 5.35B, open bar), suggesting that increased synthesis of precursor is not responsible for increased 3A7 epitope synthesis. However, some of the tumors of the descending colon expressed higher activity of α1-2 fucosyltransferase specific for type 1 chains (the Se enzyme) and fucosyltransferase specific for type 2 chains (the H enzyme) than their normal tissue counterpart (Figure 5.35B, hatched bar and closed bar, respectively). In fact, the activity of the Se enzyme (Figure 5.35B, hatched bar) in both normal and neoplastic colon was consistently higher than the activity of the H enzyme (Figure 5.35B, closed bar).

Measurement of the activities of β1-4 galactosyltransferase (Figure 5.35C), GnTI (Figure 5.35D), Core 1 (Figure 5.35E) and Core 2 (Figure 5.35F) in normal and neoplastic colon revealed specific activities similar to that of A transferase. These
findings suggested that core Asn-linked and O-linked oligosaccharide synthesis did not play a significant role in 3A7 epitope expression.

These data suggested that glycosyltransferase activity alone was not responsible for regulating expression of the 3A7 epitope in normal and neoplastic rat colon, and human colon cancer cell lines -- strengthening the concept that synthesis of specific polypeptides is a regulator of 3A7 epitope expression.

5.13 Possible function of α3β1 integrin in human colon cancer cell lines.

Previous studies have demonstrated that integrins are involved in cell-substratum adhesion and in some cases cell-cell adhesion (reviewed in Hynes, 1992). In addition, recent studies have suggested that migration and invasion of cells is brought about by variation in expression and structure of integrins and other cell surface molecules (Heino, 1996; Lauffenburger and Horwitz, 1996). These findings suggested that perhaps the expression and structure of α3β1 integrin may play a role in colon cancer progression and that the presence of the 3A7 epitope on the integrin complex may influence integrin function. As a first step towards elucidating the role of the 3A7 epitope and α3β1 integrin in these cellular events, the role of α3β1 integrin in human colon cancer cell line adhesion, migration and invasion was determined.

5.13.1 Cell adhesion to extracellular matrix proteins.

Studies from other laboratories have suggested that α3β1 integrin is a promiscuous extracellular matrix protein, since it interacts with a number of ECM proteins including laminin-1, collagen type I, fibronectin and laminin-5 (refer to Section 2.3.2.3.2). In vitro adhesion assays were employed to determine the involvement of α3β1 integrin in adhesion of human colon cancer cell to various extracellular matrix components, as described in Section 4.22. As seen in Figure 5.36, the human colon cancer cells HT29 (panel A), SW480 (panel B), SW620 (panel C) adhered to plastic
Figure 5.36: Adhesion of human colon carcinoma cell lines to extracellular matrix proteins. Aliquots of 1 x 10^5 HT29 (panel A), SW480 (panel B) and SW620 (panel C) cells were incubated for 6 hours at 37°C on 96-well microtitre plates coated with 0.001-5 μg per well laminin-1 (▲), fibronectin (●), collagen type 1 (■), collagen type IV (●) or BSA (○). Plates were washed to remove non-adherent cells and cell adhesion was determined by absorbance following 5 minute incubation with crystal violet, as described in Section 4.22.
wells coated with fibronectin (green circles), laminin-1 (blue triangles), collagen type I (black squares) and collagen type IV (red diamonds) in a concentration-dependent manner. Moreover, the extent of cell adhesion was dependent on tumor cell type. The maximum adhesion was obtained at a concentration of 5 μg ECM protein per well. A concentration of 1 μg ECM protein per well was used in antibody inhibitor studies to maximize sensitivity while minimizing antibody levels needed.

The role of α3β1 integrin in colon cancer cell adhesion was assessed by incubation of cells for adhesion assays in the presence of IgG isolated from 7A8, 7B11 or 8H7 ascites, as described in Section 4.22. Since mAb 7B11 is specific for the ligand binding domain of α3 integrin chain, it was predicted that this antibody would prevent the adhesion of cells if α3β1 integrin was involved in adhesion. Similarly, the presence of 8H7 IgG should prevent the adhesion of cells to the ECM proteins if any receptor in the β1 integrin family is essential to that process. Table 5.9 shows the percentage of tumor cells adhering to the substrate-coated wells in the presence or absence of antibody. Pretreatment of the cells with IgG isolated from mAb 7B11 or 8H7 ascites resulted in a concentration dependent inhibition of tumor cell adhesion (Table 5.9). 8H7 IgG was the most effective inhibitor of adhesion, with considerable inhibition of adhesion of the cells to all four ECM proteins at the highest concentration of IgG (1 μg/well) tested (Table 5.9). The presence of 7A8 IgG did not inhibit adhesion of the cell lines even at 1 μg protein per well. However, it did appear to increase adhesion of the cell lines to collagen IV and to a lesser extent collagen I. 7B11 IgG inhibited cell adhesion to fibronectin and laminin-1 but not to collagens type I or IV, suggesting that α3β1 integrin mediates adhesion of human colon cancer cell lines to fibronectin and laminin-1 but not the collagens.
Table 5.9: Effects of 7A8, 7B11 and 8H7 IgG on human colon cancer cell line adhesion to extracellular matrix proteins.

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<th>ECM Protein</th>
<th>7A8 IgG</th>
<th>7B11 IgG</th>
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<td>0.001</td>
</tr>
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<td>175±1</td>
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<td>CollagenIV</td>
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<td>156±2</td>
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<td>SW480</td>
<td>Fibronectin</td>
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<td></td>
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<tr>
<td></td>
<td>CollagenIV</td>
<td>213±4</td>
<td>154±4</td>
<td>136±1</td>
</tr>
</tbody>
</table>

a Aliquots of 1 x 10^5 HT29, SW480 or SW620 cells were incubated in the absence (control) or presence of 7A8, 7B11 or 8H7 IgG (0.001, 0.1, 10 and 1000 ng/l x 10^5 cells) for 6 hours at 37°C on 96-well microtitre plates coated with 1 μg/well fibronectin, laminin-1, collagen I or collagen IV. Plates were washed to remove non-adherent cells and cell adhesion was determined by absorbance at 565 nm following a 5 minute incubation with crystal violet, as described in Section 4.22. Cell adhesion (percent of control) was calculated as, [(absorbance of control wells) divided by (absorbance of antibody-treated wells)] x 100 ±standard deviation. Values represented are mean ± standard deviation for nine individual wells. Statistical significance was calculated between control (100 percent adhesion) and antibody-treated cell adhesion using a student's t-test. Statistically significant changes in cell adhesion between control and antibody-treated wells are underlined. (p < 0.05)
5.13.2 Cell migration.

*In vitro* migration assays were performed using transwell chambers, as described in Section 4.23, to determine the role of $\alpha_3\beta_1$ integrin in the migration of human colon carcinoma cells to ECM proteins and to determine whether our anti-integrin antibodies could inhibit this migration.

The results showed that the percentage of tumor cells which migrated on ECM substrates was cell-type specific (Table 5.10). For example, HT29 cells displayed a migration rate of approximately 20% toward fibronectin, collagen I and collagen IV but only about 3% toward laminin-1. In contrast, SW620 cells displayed a 35-40% migration rate toward all four ECM proteins tested (Table 5.10). Treatment of cells with 7B11 and 8H7 IgG blocked migration of most of the cell lines toward fibronectin, laminin-1 and collagen I, suggesting that $\alpha_3\beta_1$ integrin may function, at least in part, in migration toward these substrates. MAb 7A8 IgG had no significant effect on migration of cells toward ECM substrates (Table 5.10). Although mAb 8H7 partially blocked migration of the cell lines to collagen type IV substrate, mAb 7B11 had no effect on migration of cells toward collagen type IV, suggesting that $\alpha_3\beta_1$ integrin is not involved in migration of cells to this substrate. Interestingly, mAbs 7B11 and 8H7 did not block migration of LoVo cells to any of the substrates (Table 5.10), suggesting that the $\beta_1$ integrin family is not involved in migration of this cell line. It appears that LoVo cell migration is not mediated by $\alpha_3\beta_1$ integrin. Since the LoVo cell line was established from a metastasis in the supraclavicular region, it is possible that this cell line does not employ the same receptor as cell lines such as HT29 and SW620. These data indicated that $\alpha_3\beta_1$ integrin is involved at least in part in the migration of certain human colon carcinoma cell lines to laminin-1, fibronectin and collagen type I ECM components, however this cannot be generalized to all colon carcinoma cell lines (e.g. LoVo cell line).
Table 5.10: Percent Migration of Human Colon Cancer Cell lines in the presence of 7A8, 7B11 or 8H7 IgG.

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>ECM Protein</th>
<th>Control IgG</th>
<th>7A8 IgG</th>
<th>7B11 IgG</th>
<th>8H7 IgG</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>1000</td>
<td>10</td>
<td>0.1</td>
<td>1000</td>
</tr>
<tr>
<td>HT29</td>
<td>Fibronectin</td>
<td>16.1±3.0</td>
<td>13.8±3.2</td>
<td>14.5±1.4</td>
<td>15.9±1.3</td>
</tr>
<tr>
<td></td>
<td>Laminin-1</td>
<td>2.47±2.1</td>
<td>2.26±1.6</td>
<td>2.32±1.3</td>
<td>2.53±1.4</td>
</tr>
<tr>
<td></td>
<td>Collagen I</td>
<td>20.2±1.1</td>
<td>19.3±0.8</td>
<td>19.7±1.0</td>
<td>19.4±1.2</td>
</tr>
<tr>
<td></td>
<td>Collagen IV</td>
<td>17.4±0.7</td>
<td>17.5±1.6</td>
<td>18.1±2.2</td>
<td>18.1±2.5</td>
</tr>
<tr>
<td>SW480</td>
<td>Fibronectin</td>
<td>8.49±0.4</td>
<td>7.17±0.3</td>
<td>7.93±1.4</td>
<td>8.32±1.0</td>
</tr>
<tr>
<td></td>
<td>Laminin-1</td>
<td>8.46±4.5</td>
<td>9.71±1.6</td>
<td>8.77±1.5</td>
<td>8.41±0.1</td>
</tr>
<tr>
<td></td>
<td>Collagen I</td>
<td>11.6±0.3</td>
<td>9.32±1.2</td>
<td>10.4±1.9</td>
<td>10.9±0.7</td>
</tr>
<tr>
<td></td>
<td>Collagen IV</td>
<td>8.28±0.5</td>
<td>7.78±0.3</td>
<td>7.96±0.1</td>
<td>8.35±0.6</td>
</tr>
<tr>
<td>SW620</td>
<td>Fibronectin</td>
<td>28.3±7.1</td>
<td>21.8±1.2</td>
<td>22.4±0.3</td>
<td>27.2±0.9</td>
</tr>
<tr>
<td></td>
<td>Laminin-1</td>
<td>32.4±3.1</td>
<td>25.6±4.7</td>
<td>27.3±1.6</td>
<td>33.4±0.3</td>
</tr>
<tr>
<td></td>
<td>Collagen I</td>
<td>41.0±4.4</td>
<td>32.9±5.1</td>
<td>34.9±1.4</td>
<td>41.2±1.1</td>
</tr>
<tr>
<td></td>
<td>Collagen IV</td>
<td>34.6±4.4</td>
<td>28.0±7.5</td>
<td>29.4±1.2</td>
<td>34.1±0.4</td>
</tr>
<tr>
<td>KM20C</td>
<td>Fibronectin</td>
<td>18.0±2.6</td>
<td>16.7±2.2</td>
<td>18.4±0.2</td>
<td>19.1±2.4</td>
</tr>
<tr>
<td></td>
<td>Laminin-1</td>
<td>47.7±5.0</td>
<td>35.7±2.0</td>
<td>42.8±1.0</td>
<td>49.9±1.7</td>
</tr>
<tr>
<td></td>
<td>Collagen I</td>
<td>22.6±1.4</td>
<td>22.7±2.5</td>
<td>21.7±2.3</td>
<td>23.6±2.5</td>
</tr>
<tr>
<td></td>
<td>Collagen IV</td>
<td>55.4±9.3</td>
<td>48.5±7.3</td>
<td>52.5±1.4</td>
<td>56.5±2.3</td>
</tr>
<tr>
<td>LOVO</td>
<td>Fibronectin</td>
<td>11.9±2.7</td>
<td>17.0±2.1</td>
<td>12.1±0.5</td>
<td>13.0±1.7</td>
</tr>
<tr>
<td></td>
<td>Laminin-1</td>
<td>40.6±5.0</td>
<td>31.6±5.4</td>
<td>39.7±2.3</td>
<td>41.2±0.4</td>
</tr>
<tr>
<td></td>
<td>Collagen I</td>
<td>61.1±8.2</td>
<td>63.0±1.8</td>
<td>59.7±1.9</td>
<td>61.0±0.5</td>
</tr>
<tr>
<td></td>
<td>Collagen IV</td>
<td>39.1±0.8</td>
<td>38.7±0.8</td>
<td>35.4±1.8</td>
<td>39.8±0.9</td>
</tr>
</tbody>
</table>

a Aliquots of 2 x 10^5 HT29, SW480, SW620, KM20C and LoVo cells were incubated in the absence (Control IgG) or presence of 7A8, 7B11 or 8H7 IgG (0.1, 1 and 1000 ng/2 x 10^5 cells) in Transwell cell culture chambers coated, on the underside of the filter with fibronectin, laminin-1, collagen I or collagen IV, for 24 hours in a 37°C incubator, as described in Section 4.23. Cell migration was determined colorimetrically using the MTT assay. Values represent the Percent Migration (%) calculated as [(absorbance of migrating cells) divided by (absorbance of total cells)] x 100 ± standard deviation. Values represented are mean ± standard deviation from six individual wells. Statistically significant changes in percent migration between control and antibody-treated wells are underlined (p<0.05).
5.13.3 Cell invasion.

Since α3β1 integrin appears to play a role in cancer cell adhesion and migration, two key events in the invasion process, the ability of our anti-α3β1 integrin antibodies to inhibit invasion of cells through a reconstituted basement membrane was also examined. In vitro invasion assays were performed in transwell chambers coated with the commercially available reconstituted basement membrane, Matrigel, as described in Section 4.23. All the cell lines exhibited a significant invasion through the Matrigel, with the cell lines HT29, SW480, SW1417 and KM20C having slightly higher percentages of invasion than cell lines SW620 and LOVO (Figure 5.37, open bar). Overall, the presence of 7A8 (Figure 5.37, solid bar), 7B11 (Figure 5.37, cross hatched bar) or 8H7 IgG (Figure 5.37, hatched bar) had no significant effect on invasion of the cancer cell lines. Interestingly, SW620 and LoVo cells incubated with 8H7 IgG (Figure 5.37, hatched bar) displayed increased invasiveness by approximately 2 fold, suggesting that perhaps, in these cells, blocking receptors of the β1 integrin family may aid in invasion. Further studies will be required to fully understand the role of α3β1 integrin expression in colon cancer.
Figure 5.37: Effects of anti-integrin antibodies on human colon carcinoma cell line invasion. HT29, SW480, SW1417, KM20C, SW620 and LoVo cells were left untreated (open bar) or incubated with mAbs 7A8 (solid green bar), 7B11 (red cross hatched) or 8H7 (blue hatched) for 30 minutes prior to addition to Transwell chambers. Cells were allowed to invade through a Matrigel layer for 72 hours and the percentage of invading cells (Invasion %) was determined using an MTT assay, as described in Section 4.23.
6.0 DISCUSSION

Previous studies in our laboratory demonstrated that mAb 3A7 detects a carbohydrate epitope whose expression in rat intestine is oncodevelopmentally regulated. In the current studies, we have examined further the expression and structure of the 3A7 epitope and 3A7-immunoreactive glycoconjugates in both rat and human colon cancer models. As well, antibodies have been generated against the major 3A7-immunoreactive glycoprotein (gp140) from the human colon cancer cell line, HT29. The major 3A7-binding glycoprotein in human colon carcinoma cell lines was identified as α3β1 integrin. Finally, availability of integrin-specific monoclonal antibodies made it possible to examine α3β1 integrin expression, glycosylation and function in colon cancer cells.

6.1 Structure of the epitope detected by mAb 3A7.

The carbohydrate epitope detected by mAb 3A7 was identified as an epitope containing blood group A (GalNAcα1-3[Fucα1-2]Galβ-R) or B (Galα1-3[Fucα1-2]Galβ-R) structures exclusively on type 2 chains (Galβ1-4GlcNAcβ-R). The structure(s) recognized by mAb 3A7 appears to be distinct from carbohydrate structures recognized by mAbs 2F7, 4F3 and 8D5, which recognize blood group A structures primarily on type 1 chains and mAb 11D4 which recognizes an epitope containing exclusively a blood group A determinant on type 2 chains. These structures were deduced by examining the reactivity of the antibodies to a panel of neoglycoproteins containing blood group A and B oligosaccharide structures and are therefore minimal requirements for antibody binding. The precise oligosaccharide structure recognized by mAb 3A7 in normal and neoplastic colon is unknown, but analysis of 3A7-immunoreactive glycopeptides from normal rat colon has provided some information on
glycans bearing this epitope. In this regard, 3A7-immunoreactive glycopeptides contain tri- and/or tetra-antennary Asn-linked oligosaccharides which are modified by poly-N-acetyllactosamine structures (type 2 chain repeats). Moreover, reactivity of 3A7-immunoreactive glycopeptides to DSA lectin and not LEA lectin suggests that the poly-N-acetyllactosamine repeats are less than four [Galβ1-4GlcNAc] repeats in length (Merkle and Cummings, 1987b). Furthermore, L-PHA blotting of 3A7-immunoreactive glycopeptides indicated that these glycopeptides contain β1-6 branched Asn-linked oligosaccharides. 3A7-immunoreactive glycoproteins isolated from HT29 cells also bind DSA and L-PHA lectins, indicating that 3A7-immunoreactive species from both normal and neoplastic sources have similar carbohydrate structures with respects to poly-N-acetyllactosamine and β1-6 branched Asn-linked oligosaccharide structures. Previous studies have indicated that L-PHA binding glycoconjugates are absent or in very low amounts in normal large intestine. Indeed, the enzyme responsible for initiating synthesis of the β1-6 branch of Asn-linked oligosaccharides, GnT V, is expressed at very low levels in normal colon (Perng et al., 1994). It should be noted that a great deal of sample was used in purification of 3A7-immunoreactive glycopeptides from normal colon due to the limited expression of the 3A7 epitope in normal colon. Therefore, although β1-6 branched Asn-linked oligosaccharides and poly-N-acetyllactosamine repeats are present on 3A7-immunoreactive glycopeptides from normal colon they are present in very low amounts. Thus, it appears that malignancy results in quantitative changes in 3A7-immunoreactive glycoconjugates.

6.2 Characterization of 3A7-immunoreactive glycoproteins.

It is noteworthy that neoplasia also results in qualitative changes in 3A7-immunoreactive glycoproteins. The cancer-associated decrease in the binding of 3A7-immunoreactive glycoproteins to dolichos biflorus lectin suggests that these glycoproteins have different glycan structures compared to glycoproteins isolated from normal colon.
Decreased binding of DBA-lectin to colon carcinomas has been reported previously (Boland et al., 1992; Yuan et al., 1985). Indeed, Bresalier et al. (1985) observed that only a few tumors from distal colon and about 50% of tumors from the proximal colon bind DBA lectin, whereas DBA staining is significant throughout the entire normal colon. Although these changes are well documented, the structural basis for modified DBA binding is unclear. Decreased lectin binding could result from the presence of glycan structures that interfere with lectin binding, as well as the absence of structures required for optimal lectin binding. In this regard, Nakayama et al. (1987) reported that treatment with neuraminidase increases DBA binding, suggesting that sialylation may interfere with DBA-ligand interactions. In other studies, Clausen et al. (1985) demonstrated that A1 erythrocytes, which are preferentially agglutinated by DBA lectin (Bird, 1952) express A type 3 structures (GalNAcα1-3[Fucα1-2]GalNAcα1-3[Fucα1-2]Galβ1-4GlcNAc-R) but A2 erythrocytes neither express A type 3 structures nor bind DBA lectin. Thus, the absence of specific structures, such as A type 3, or the presence of increased sialylation on 3A7-positive glycoproteins may decrease the binding affinity of these glycoproteins to DBA lectin. Indeed, 3A7-immunoreactive α3β1 integrin (gp140) isolated from HT29 cells is a sialialoglycoprotein.

MAb 3A7 appears to recognize different glycoprotein species in normal and neoplastic colon. MAb 3A7 recognizes an epitope on glycoproteins ranging in size from 60-200 kDa. Normal colonic mucosa synthesizes low levels of three to four 3A7-immunoreactive glycoproteins that migrate between 80 and 120 kDa. In contrast, human colon carcinoma cell lines and rat colon tumors synthesize a major 3A7 binding glycoprotein of approximately 140 kDa, suggesting that not only is there increased expression of the 3A7 epitope in neoplastic colon, but also a shift in addition of the 3A7 epitope to a single major glycoprotein species. It is possible that the 140 kDa glycoprotein is the preferred target for addition of the 3A7 epitope. Therefore increased presence of this polypeptide and/or increased synthesis of the 3A7 epitope would result in
preferential addition of the 3A7 determinant to this species. Biochemical and molecular analysis revealed that the major 3A7-immunoreactive glycoprotein in HT29 cells, gp140, corresponds to α3β1 integrin. Attempts to determine the identity of gp140 synthesized in rat colon tumor samples were unsuccessful since our anti-integrin antibodies are specific for human integrin subunits.

6.3 Expression of the 3A7 epitope.

The 3A7 epitope is differentially expressed in rat and human colon tumors compared to normal colon. Specifically, mAb 3A7 stains rodent tumors intensely, whereas staining of normal rat colon is confined to the proliferative zone of the crypts. Moreover, human colon tumors stain moderately to strongly with mAb 3A7, whereas normal human colonic epithelium is devoid of the 3A7 epitope. Significantly, a number of the human tumors isolated from the distal portion of the colon stained with mAb 3A7. Blood group determinants are normally absent in distal adult colon (Yuan et al., 1985) (refer to Figure 2.13). Thus, re-expression of the blood group A/B-like epitope recognized by mAb 3A7 in these tumors is clearly cancer-associated. As well, in both human and rat samples, staining is more pronounced in advanced tumors (i.e. Duke's B and C tumors). However, increased expression of the epitope is also observed in Duke's A tumors and pre-cancerous lesions, suggesting that these changes may be early events in tumor progression. These data suggest that mAb 3A7 could be useful in assessing colon tumors from blood group A, AB and B patients post-operatively. Examination of tumor sections by immunohistochemical staining, in conjunction with current assessment techniques, may be valuable in detecting pre-neoplastic lesions as well as determining the aggressiveness of colon tumors. Significantly, mAb 3A7 detects sites of angioinvasion and pre-neoplastic abnormalities in azoxymethane-induced rat colon tumors (refer to Figure 2.17). Detection of pre-neoplastic lesions and sites of invasion would be
extremely useful in assessing not only new malignant foci, but also the onset of tumor cell invasion and metastatic spread.

Tumor cells within lymph node metastases isolated from human subjects also stained with mAb 3A7 suggesting that the epitope and/or carriers of this epitope may be necessary for the spread of colon cancer cells to secondary sites. In contrast, a liver metastasis did not stain with mAb 3A7 even though the primary tumor isolated from the same patient was 3A7-positive. Furthermore, the SW620 cell line established from a lymph node metastasis, was devoid of the 3A7 epitope, where as SW480 cell line, established from a primary colon tumor expressed the 3A7 epitope. Since the SW480 and SW620 cell lines were established from the same patient, the change in 3A7 epitope expression is cancer associated and not a result of population differences. These data suggest that although increased cell surface expression of the 3A7 epitope or 3A7-immunoreactive glycoproteins is important for primary tumor growth and initial invasion of metastatic cells out of the primary tumor, distant spread may involve a loss and/or modification of the 3A7 epitope and/or 3A7 epitope carrying molecules.

Analysis of a panel of human colon carcinoma cell lines revealed no apparent correlation between levels of the 3A7 epitope and/or the major 3A7-positive glycoprotein (i.e. α3β1 integrin) and tumor stage. However, only a limited number of blood group A, AB and B positive cell lines of differing histopathological origins were available for study and no definitive conclusions regarding a correlation between 3A7 epitope expression and tumor stage could be reached. As well, the properties of tumor cell lines in culture do not always mimic the properties of tumors in vivo. The establishment of cancer cell lines from a tumor is a selective process and often the cells undergo a variety of biochemical changes during this process (DeVita et al., 1993).

It should be noted that in normal and cancerous human colon, endothelial cells of blood vessels stain intensely with mAb 3A7. This drastically contrasts with the staining observed in the rat samples, where the 3A7 epitope is restricted to epithelial cells. Thus,
the expression of 3A7-immunoreactive material is less localized in human colon. Notably, the presence of A type 2 determinants on endothelial cells from human tissues has been documented (Byrne et al., 1993; Gillard et al., 1987). MAb 3A7 may be recognizing type 2 chains bearing A or A-like structures on endothelial cells. The presence of the 3A7 carbohydrate epitope on endothelial cells may influence the interaction of tumor cells with endothelial cells. Indeed, it is known that carbohydrate-carbohydrate interactions are important in cell-cell adhesions. For example, Le^x-Le^x interactions are important in mammalian development (Eggen et al., 1989). It is possible that the glycan structure detected by mAb 3A7 may be involved in homotypic interactions and, thereby, influence the adhesion of tumor cells to endothelium. Furthermore, subsequent loss of the 3A7 epitope in tumor cells of lymph node and liver metastases may aid in detachment of tumor cells from endothelium and invasion of cells into tissues.

Although the reactivity of mAb 3A7 with endothelium does not detract from the significance of increased and/or re-expression of the 3A7 epitope in human colon tumors, it does call into question using this antibody in a clinical setting. MAb 3A7 would be extremely useful in conjunction with histological analysis to determine the extent of tumor progression and perhaps identification of pre-neoplastic lesions. However, this antibody could not be used for the targeting of chemotherapeutic drugs in cancer treatment, since the antibody cross-reacts with normal endothelial and muscle cells. Further study of the precise structure recognized by mAb 3A7 may aid in the development of strategies that use the 3A7 epitope to target colon cancer cells.

6.4 Integrin α3β1.

Integrin α3β1 is the major 3A7-immunoreactive glycoprotein in human colon carcinoma cell lines. As well, α3β1 integrin appears to be the major site for the addition of a number of cancer-associated carbohydrate determinants in human colon carcinomas cell lines. These glycan structures include β1-6 branched Asn-linked oligosaccharides,
poly-N-acetyllactosamine structures, and blood group A or A-like determinants on both type 1 and type 2 chains. Notably, our studies demonstrated the presence of A or A-like structures on Asn-linked oligosaccharides. Most previous studies have reported that ABH determinants expressed by tumor cells are found primarily on glycosphingolipids and/or O-linked oligosaccharides (Itzkowitz et al., 1986; Hakomori, 1985). However, blood group A determinants have been detected on Asn-linked oligosaccharides isolated from hamster pancreatic cancers (Hirota et al., 1992). Significantly, these blood group A determinants are cancer-associated in pancreatic cancer (Egami et al., 1990). The epitopes detected by mAbs 2F7, 4F3 and 8D5, which preferentially detect epitopes containing blood group A determinants on type 1 chains, and mAb 11D4, which detects an epitope exclusively on A type 2 chains, are located on Asn-linked glycan moieties of \( \alpha 3\beta 1 \) integrin. As well, the unique A/B-like epitope on type 2 chains recognized by mAb 3A7 is located on Asn-linked oligosaccharides. However, mAb 3A7 is unique in that it recognizes an epitope which is primarily located on the \( \beta 1-6 \) branch of Asn-linked oligosaccharides. MAbs 8D5 and 11D4 can immunoprecipitate \( \alpha 3\beta 1 \) integrin from HT29 cells treated with swainsonine, an inhibitor which blocks synthesis of the \( \beta 1-6 \) branch, implying that these epitopes are present on another branch of Asn-linked oligosaccharides (Figure 5.8 and 5.28). In contrast, mAb 3A7 is unable to immunoprecipitate \( \alpha 3\beta 1 \) integrin from swainsonine treated HT29 cells. These findings highlight the uniqueness of the 3A7 epitope and suggest that mAb 3A7 may be valuable for studies of the \( \beta 1-6 \) branch of Asn-linked oligosaccharides and extensions to this branch in colon cancer progression.

Analysis of 3A7 epitope and \( \alpha 3 \) integrin subunit co-expression in human colon carcinoma cell lines derived from blood group A, AB or B patients indicated that the expression of \( \alpha 3 \) integrin polypeptide plays an important role in determining cell surface expression of the 3A7 epitope. Indeed, our data indicates that absence or diminished levels of \( \alpha 3\beta 1 \) integrin, such as in the SW620 cell line and SW480-E sub-population, is
accompanied by low cell surface expression of the 3A7 epitope. In contrast, moderate to high levels of \(\alpha 3\beta 1\) integrin on the cell surface, such as in HT29 and the SW480-R subpopulation, is accompanied by high levels of the 3A7 epitope. Thus, it appears that expression of one or a few polypeptides which are preferred substrates for the addition of unique glycans can regulate the level of expression of these glycans. It is possible that the glycosyltransferases responsible for synthesis of the 3A7 epitope specifically recognize certain substrates. However, it is not clear whether these enzymes recognize specific polypeptide sequences, protein conformations or carbohydrate structures.

Examination of glycosyltransferases involved in the synthesis of ABH blood group determinants did not indicate that the activity of these glycosyltransferases alone was regulating 3A7 epitope expression in human colon carcinoma cell or azoxymethane-induced rat colon tumors. Glycosyltransferases are necessary for the synthesis of the 3A7 epitope since this epitope is a carbohydrate structure. Yet the activity of glycosyltransferase, such as A transferase or \(\alpha 1-2\) fucosyltransferase (refer to Figure 2.11 for ABH determinant synthesis), did not appear to be the rate-limiting step in 3A7 epitope synthesis (Figure 5.34). For instance, A transferase and \(\alpha 1-2\) fucosyltransferase are both more active in normal tissues compared to rat colon tumors (Figure 5.35). It should be noted that in some rat colon tumors, increased \(H\) enzyme (\(\alpha 1-2\) fucosyltransferase specific for type 2 chains) activity was observed, suggesting that increased activity of the \(H\) enzyme could explain the increased 3A7 epitope synthesis in some cases. However, this was not a consistent finding in our samples. Nonetheless, this finding agrees with previous reports which suggested that colon adenocarcinomas have increased activity of both type 1 and type 2 chain specific \(\alpha 1-2\) fucosyltransferases but not A or B transferase (Orntoft et al., 1992).

It should be noted that although mAb 3A7 recognizes neoglycoproteins containing type 2 chains modified with blood group A or B structures, the actual structure recognized by mAb 3A7 may not be a 'true' A or B determinant but rather A/B-like. This
being the case, 3A7 epitope expression may be under the control of glycosyltransferases that are not involved in the synthesis of 'true' ABH blood group determinants.

It is also possible that increased activity of extension enzymes such as β1-3 GnT (i) and β1-4 galactosyltransferase, the enzymes responsible for synthesizing type 2 chains, could lead to increased levels of the 3A7 epitope in tumors. No correlation between β1-4 galactosyltransferase and 3A7 epitope levels was observed in our studies. Due to experimental limitations we were unable to determine the activity of β1-3 GnT (i) in these samples. Thus, we cannot rule out the possibility that β1-3 GnT (i) is involved in regulating 3A7 epitope expression. Indeed, studies have demonstrated that β1-4 galactosyltransferase activity remains constant, whereas β1-3 GnT (i) activity is upregulated in neoplastic tissues (Holmes et al., 1987). β1-3 GnT (i) is the rate-limiting enzyme in type 2 chain synthesis (Holmes, 1988) and this enzyme preferentially extends the β1-6 branch of complex type glycans (van den Eijnden et al., 1988).

In light of these facts and data indicating that the 3A7 epitope is preferentially added to the β1-6 branch of Asn-linked oligosaccharides, the activity of GnT V could influence 3A7 epitope synthesis. Inconclusive results were obtained in our experimental situation, therefore it is not yet known if increased activity of GnT V in tumor samples is responsible for increased expression of the 3A7 epitope. However, studies have demonstrated that colon tumors stain more intensely with L-PHA compared to normal colon (Fernandes et al., 1992). As well, increased β1-6 branching, due to increased GnT V activity, is directly linked to metastatic potential in a number of tumor models (Dennis et al., 1987). Furthermore, GnT V activity is significantly lower in normal large intestine compared to GnT V activity in normal small intestine (Perng et al., 1994), which parallels 3A7 epitope levels in these tissues. Thus it is possible that synthesis of the β1-6 branch is involved in determining 3A7 epitope expression, but it is not clear whether this is a result of increased availability of preferred GnT V polypeptide acceptors (e.g. α3β1 integrin) or increased activity of GnT V in these samples.
6.5 Significance of β1-6 branched Asn-linked oligosaccharides on α3β1 integrin.

Surprisingly, the β1-6 branch is added to a select number of glycoproteins including, lamp-1 and -2 (Fukuda, 1991), laminin (Jin et al., 1995), CEA (Benchimol et al., 1989), TAA90/Mac-2 binding protein (Koths et al., 1993) and integrin subunits α3, α5, α6, αv and β1 (Demetriou et al., 1995; Friedman et al., 1993; Chammas et al., 1993; Jasiulionis et al., 1996). It appears that only certain proteins are appropriate substrates for GnT V, the enzyme responsible for synthesis of the β1-6 branch. Increased expression of appropriate polypeptide substrates in cells results in increased presence of β1-6 branched Asn-linked oligosaccharides, when GnT V activity is not limited within the cell (Do et al., 1994). Thus, increased expression of α3 and β1 integrin subunits within human colon cancer cells could result in increased presence of β1-6 branched Asn-linked oligosaccharides. Increased presence of the β1-6 branch and subsequent modification of this branch would provide additional sites for addition of the 3A7 epitope.

Asn-linked oligosaccharides on the integrin subunits appear to be excellent substrates for GnT V. However, it is unclear what structural or conformation factors are important for GnT V recognition and action. Notably, only specific Asn-linked oligosaccharides on lamp-1 and lamp-2 are modified by addition of the β1-6 branch (Chammas et al., 1993). Thus, GnT V only acts on certain N-glycans on certain glycoproteins. Studies by Do et al. (1994) indicated that the transfer of GlcNAc by GnT V is dependent on accessibility of the enzyme to the oligosaccharide acceptor in glycoproteins and not to the specific peptide sequences or conformation of the glycoproteins. In fact, loss of protein conformation increased the number of β1-6 branched Asn-linked oligosaccharides on lamp-1, likely due to increased accessibility of N-glycans to GnT V (Do et al., 1994). Still, accessibility of glycans within a
glycoprotein is ultimately dependent on the conformation of that protein, thus, polypeptide sequence and conformation may indirectly determine whether or not a glycan is an appropriate substrate for GnT V. Knowledge of the structure of glycoproteins bearing β1-6 branched Asn-linked oligosaccharides will be required in order to fully understand the specificity requirements of GnT V.

The contribution of the β1-6 branch and the 3A7 epitope to α3β1 integrin structure and function is not clear. However, the availability of antibodies to both carbohydrate and polypeptide determinants of α3β1 integrin should provide tools to examine the functional significance of glycan moieties to integrin function. Indeed, studies presented in this thesis have demonstrated that α3β1 integrin is involved in adhesion and migration of human colon carcinoma cell lines toward the ECM components laminin and fibronectin. Specifically, antibodies which bind close to the ligand binding region of the α3 integrin subunit (mAb 7B11) specifically inhibited adhesion to laminin and fibronectin and migration toward laminin, fibronectin and collagen type I (Table 5.9 and 5.10). These findings are in agreement with previous studies which demonstrated that α3β1 integrin serves as a receptor for several ECM components including epiligrin (laminin-5), laminin, fibronectin (Takada et al., 1988), collagen (Carter et al., 1991) and entactin (Dedhar et al., 1992). However, neither anti-α3 nor anti-β1 antibodies influenced the invasion of human colon carcinoma cell lines through a basement membrane, suggesting that the invasive process requires the concerted effort of a number of modulators, not α3β1 integrin alone. Recent studies have suggested that integrin expression, and specifically α3β1 integrin expression, is important in colon tumor progression (Boku et al., 1995; Pignatelli et al., 1990; Lindmark et al., 1993). Studies are currently underway in our laboratory to determine the role of the 3A7 epitope and the β1-6 branch of Asn-linked oligosaccharides in α3β1 integrin function.

Recent studies have implicated glycosylation and specifically the β1-6 branch in the function and activity of integrins and other glycoproteins. For example, adhesion of
purified lamp to ECM proteins was shown to depend on glycosylation of the lamp molecules (Laferté and Dennis, 1988). Moreover, deglycosylation of integrin receptors leads to improper assembly of the heterodimer and inhibition of ligand binding (Chammas et al., 1993; Lampe et al., 1992; Zheng et al., 1994). As well, although swainsonine treatment had no effect on α6β1 integrin mediated fibroblast adhesion to laminin, absence of the β1-6 branch did prevent spreading of cells on laminin (Jasiulionis et al., 1996; Chammas et al., 1993). Since spreading of cells on ECM components is a crucial event in cell migration and invasion (Gumbiner, 1996), the presence of the β1-6 branch may aid in the migration of cells out of a primary tumor. Furthermore, transfection of immortalized mink lung epithelial cells with Gnt V, which resulted in addition of β1-6 branched Asn-linked oligosaccharides to lamp and integrin glycoproteins, reduced contact inhibition and ECM substrate adhesion of these cells (Demetrious et al., 1995). These data suggest that glycosylation and specifically the addition of β1-6 branched Asn-linked oligosaccharides to these glycoproteins influences cell-cell and cell-ECM contacts.

It has been speculated that activation of oncogenes and/or loss of tumor suppressor genes may not only influence the expression of cell receptors, but also alter the glycosylation of these receptors and, thus, confer metastatic properties to cells. Interestingly, Gnt V transfection of cells produces cellular responses that are similar to EJ-ras transformation of cells (Demetrious et al., 1995; Jasiulionis et al., 1996), suggesting that increased Gnt V activity may be a result of ras activation. Ras activation is a common and a relatively early event in colon cancer progression in humans (Kinzler and Vogelstein, 1996). Notably, increased 3A7 expression is seen relatively early in colon cancer genesis. It would be of interest to determine if there was a correlation between ras activation and 3A7 epitope expression in colon tumors. As well, ras-mediated and integrin-mediated signaling pathways share common intracellular messengers, suggesting possible links between ras activation and integrin function (Clark and Brugge, 1995).
Still, the true effects of increased β1-6 branching of glycoproteins are not known. The presence of the β1-6 branch on certain glycoproteins may influence the primary function or activity of the glycoproteins. Thus fluctuations in levels of β1-6 branched Asn-linked oligosaccharides on the glycoprotein, may influence the activity of these glycoproteins. In fact, tumor cell mutants with defective GnT V activity and subsequent lack of β1-6 branched Asn-linked glycans, display increased proteolysis inhibitor transcript levels (e.g. TIMP-1), suggesting that the presence of the β1-6 branch may influence gene expression (Korczak and Dennis, 1993). It is possible that β1-6 branched Asn-linked oligosaccharides influences the conformation and subsequently the function of the glycoproteins bearing these structures.

Conversely, since the β1-6 branch is the preferred site for addition of a number of oncodevelopmentally-regulated carbohydrate structures, the presence of this branch may give glycoproteins additional novel carbohydrate ligands and subsequent new functions. In this regard, the β1-6 branch is the preferential site for addition of type 2 chain or poly-N-acetyllactosamine structures, which are the building blocks for blood group antigens, including the 3A7 epitope. Indeed α3β1 integrin does bind DSA lectin, indicating that type 2 chain repeats are present on this glycoprotein. However, α3β1 integrin is not recognized by LEA lectin suggesting that the poly-N-acetyllactosamine repeats are no more than four repeating units in length. L-PHA binding glycoproteins, by virtue of the type 2 chain extension, are ligands for the mammalian lectin, galectin-3 (Sato and Hughes, 1992). EJ-ras transformed fibroblasts typically overexpress galectin-3, in addition to increased synthesis of L-PHA binding glycoproteins. It has been suggested that interactions between galectin-3 and the type 2 chains on L-PHA binding glycoproteins, induce homotypic cell adhesion and cell aggregation analogous to tumor embolization (Inohara and Raz, 1995; Raz et al., 1989).

Similarly, the β1-6 branch may create novel adhesion sites or mask pre-existing adhesion sites through modification of type 2 chains with blood group determinants.
Indeed, sialyl-Le\textsuperscript{x} is a known ligand for the selectin adhesion molecules and the role of these interactions in platelet aggregation and tumor cell-endothelial cell interaction is well described (Lowe, 1990; Phillips \textit{et al.}, 1990). Larkin and associates (1992) have demonstrated that addition of $\alpha 1-3$ GalNAc to the terminal galactose residue of Le\textsuperscript{b} oligosaccharides ($\text{Fuc}\alpha 1-2\text{Gal}\beta 1-3[\text{Fuc}\alpha 1-4]\text{GlcNAc}\beta 1-3\text{Gal}\beta 1-4$-R), which generates a blood group A determinant, inhibited binding of these structures to E-selectin. These data suggest that the addition of ABH determinants, including the 3A7 epitope may influence cell adhesion events. Indeed, the subpopulation SW480-R, which has high levels of $\alpha 3\beta 1$ integrin and the 3A7 epitope, forms larger and more aggressive tumors in nude mice (Tomita \textit{et al.}, 1992). The SW480-E population which has low levels of the 3A7 epitope, forms much smaller tumors. These data suggest that the 3A7 epitope and/or $\alpha 3\beta 1$ integrin may be crucial to tumor cell growth and spread.

Although the role of A and B determinants in cancer and metastasis is not clear, it is known that blood group A individuals are more susceptible to gastric cancer (Doll \textit{et al.}, 1960) and loss of blood group A determinants is associated with poor prognosis in human lung tumors (Matsumoto \textit{et al.}, 1993). Overall, these studies suggest that blood group determinants and A/B-like structures are important in colon cancer progression. Continued examination of the 3A7 epitope and $\alpha 3\beta 1$ integrin will help to understand the complex role glycosylation plays in malignancy.
7.0 CONCLUSIONS

Integrin α3β1 is the major carrier of cancer-related glycosylation changes in human colon carcinoma cell lines. These include β1-6 branched Asn-linked oligosaccharides, poly-N-acetyllactosamine structures, blood group A determinants on type 1 and 2 chains, and the unique carbohydrate epitope defined by mAb 3A7. Furthermore, α3β1 integrin synthesis appears to be important in regulating cell surface expression of the 3A7 epitope in human colon cancer cell lines derived from blood group A, AB and B patients.

The 3A7 epitope is a gastro-intestinal specific, oncodevelopmentally-regulated carbohydrate epitope that contains type 2 chains modified with an A/B-like blood group determinant. Interestingly, the 3A7 epitope is added primarily to the β1-6 branch of Asn-linked oligosaccharides on α3β1 integrin. The presence of the 3A7 epitope on this metastasis-related carbohydrate structure suggests a role for the 3A7 epitope and/or the major 3A7-immunoreactive glycoprotein, α3β1 integrin, in the migration and spread of colon cancer. Further analysis of the 3A7 epitope and α3β1 integrin will help elucidate how cancer cells are capable of leaving one tissue and disseminating thoughout the body and what role glycosylation plays in this process.

To most people, there is nothing as frightening as the diagnosis of cancer. This disease strikes without warning and most often leaves a path of destruction that cannot be repaired. Only with a world-wide, collaborative effort will we be able to fully understand why cells rebel against and ultimately destroy their life source. Only this understanding will allow us to defeat this foe and end a fear that plagues millions. I hope the studies presented within this thesis will one day aid in our fight against cancer and help the people that suffer with this disease.
8.0 LITERATURE CITED


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