GDP-L-fucose: synthesis and role in inflammation

Jaana Niittymäki

Department of Bacteriology and Immunology,
Haartman Institute and Biomedicum Helsinki
and
Division of Biochemistry,
Department of Biological and Environmental Sciences,
Faculty of Biosciences
University of Helsinki
and
Glycoscience Graduate School

Academic dissertation

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Supervised by

Docent Risto Renkonen, M.D.
Department of Bacteriology and Immunology,
Haartman Institute and Biomedicum Helsinki,
University of Helsinki
Helsinki, Finland

Reviewed by

Docent Anne Leppänen, Ph.D.
Division of Biochemistry,
Department of Biological and Environmental Sciences,
University of Helsinki
Helsinki, Finland

and

Docent Jari Helin, Ph.D.
Glykos Finland Oy
Helsinki, Finland

Opponent

Professor Markku Tammi, M.D.
Department of Anatomy,
University of Kuopio
Kuopio, Finland

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To my family

Contents

ORIGINAL PUBLICATIONS

This thesis is based on the following publications, which are referred to in the text by their Roman numerals:

I Niittymäki J., Mattila P., Roos C., Huopaniemi L., Sjöblom S., Renkonen R.
Cloning and expression of murine enzymes involved in the salvage pathway of GDP-L-fucose. *Eur. J Biochem.* 271(1):78-86, 2004.

II Huopaniemi L., Kolmer M., Niittymäki J., Pelto-Huikko M., Renkonen R.
Inflammation-induced transcriptional regulation of Golgi transporters required for the synthesis of sulfo sLex glycan epitopes. *Glycobiology*. 14(12):1285-94, 2004.

III Niittymäki J., Mattila P., Renkonen R.
Cloning and expression of rat fucosyltransferase VII at sites of inflammation. *APMIS*. 113(9):613-20, 2005.

IV Niittymäki J., Mattila P., Renkonen R.
Differential gene expression of GDP-L-fucose-synthesizing enzymes, GDP-fucose transporter and fucosyltransferase VII. *APMIS*. 114(7-8):539-48, 2006.

ABBREVIATIONS

aa	amino acid
bp	base pair
CDG IIc	congenital disorder og glycosylation type IIc
cDNA	complementary deoxyribonucleic acid
CDS	coding determining sequence
CF	cystic fibrosis
CMP-SA	CMP-sialic acid
DTT	dithiothreitol
EDTA	ethylenediaminetetra-acetic acid
EGF	epidermal growth factor
ER	endoplasmic reticulum
EST	expressed sequence tag
FACS	fluorescence-activated cell sorting
Fpgt	fucose-1-phosphate guanylyltransferase
Fuc	fucose
Fuc-T	fucosyltransferase
Fuk	fucokinase
FUT	fucosyltransferase gene
FX	GDP-4-keto-6-deoxymannose 3,5-epimerase-4-reductase
Gal	galactose
GalNAc	*N*-acetylgalactosamine
GDP-L-Fuc	GDP-L-fucose
GlcNAc	*N*-acetylglucosamine
GlcNAc6ST	*N*-acetylglucosamine-6-*O*-sulfotransferase
GlyCAM-1	Glycosylation-Dependent Cell Adhesion Molecule-1
GMD	GDP-mannose-4,6-dehydratase
GPI	glycosylphosphatidylinositol
GST	Gal/GalNAc/GlcNAc-6-*O*-sulfotransferase
HEC	high endothelial cell
HEV	high endothelial venule
HPLC	high-performance liquid chromatography
ICAM	intercellular adhesion molecule
LAD	leukocyte adhesion deficiency
Lea	Lewis a, Galβ1-3(Fucα1-4)GlcNAc
Lex	Lewis x, Galβ1-4(Fucα1-3)GlcNAc
LN	N-acetyllactosamine
LFA-1	leukocyte function antigen-1
LPS	lipopolysaccharide
MAdCAM-1	Mucosal Addressin Cell Adhesion Molecule-1
MALDI-TOF MS	matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry
mAb	monoclonal antibody

NADP+	nicotinamide adenine dinucleotide phosphate (oxidized form)
NADPH	nicotinamide adenine dinucleotide phosphate (reduced form)
Neu5Ac	*N*-acetylneuraminic acid
NK cells	natural killer cells
PAPS	3´-phosphoadenosine 5´-phosphosulfate
PNAd	peripheral node addressin
PSGL-1	P-selectin Glycoprotein Ligand-1
qRT-PCR	quantitative reverse transcription-polymerase chain reaction
RACE	rapid amplification of cDNA ends
RNA	ribonucleic acid
RT-PCR	reverse transcription-polymerase chain reaction
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
sLea	sialyl Lewis a, Neu5Acα2-3Galβ1-3(Fucα1-4)GlcNAc
sLex	sialyl Lewis x, Neu5Acα2-3Galβ1-4(Fucα1-3)GlcNAc
SSEA-1	stage-specific embryonic antigen-1
ST3Gal	sialyltransferase
TEAA	triethylammonium acetate
TNF-α	tumor necrosis factor-α

1. ABSTRACT

The migration of leukocytes from intravascular locations to extravascular sites is essential to the immune responses. The initial attachment of leukocytes to the endothelium and the rolling step of the leukocyte extravasation cascade are mediated by selectins, a family of cell adhesion molecules on cell surfaces. All three selectins, P-, E- and L-selectins, are able to recognize glycoproteins and glycolipids containing the tetrasaccharide sialyl Lewis x (sLex, Neu5Acα2-3Galβ1-4(Fucα1-3)GlcNAc). The biosynthesis of cell surface glycoconjugates involves specific glycosyltransferases utilizing nucleotide sugars as activated donors. The last step in the formation of sLex is the transfer of fucose from GDP-L-fucose to sialylated *N*-acetyllactosamine by α1,3-fucosyltransferase VII.

GDP-L-fucose is synthesized in the cytosol via two distinct pathways. The major, *de novo* pathway involves conversion of GDP-D-mannose to GDP-L-fucose by dehydration, epimerization and reduction reactions performed by two enzymes, GDP-D-mannose-4,6-dehydratase (GMD) and GDP-4-keto-6-deoxy-D-mannose-3,5-epimerase-4-reductase (FX). In the alternative salvage pathway free fucose is converted to GDP-L-fucose by L-fucokinase and GDP-L-fucose pyrophosphorylase. The GDP-fucose transporter translocates GDP-L-fucose from the cytosol to the Golgi for the corresponding fucosyltransferase.

This thesis describes molecular cloning and expression of murine L-fucokinase and GDP-L-fucose pyrophosphorylase involved in the synthesis of GDP-fucose via the salvage pathway. The gene expression levels of these enzymes were found to be relatively high in various tissues; the mRNA levels were highest in brain, ovary and testis. This study also describes molecular cloning of rat fucosyltransferase VII (FUT7) and its expression as a functional enzyme. Gene expression levels of GDP-L-fucose synthesizing enzymes, GDP-fucose transporter and FUT7 were determined in inflamed tissues as well as cancer cells. Our results revealed a clear upregulation of GMD, FX, GDP-fucose transporter and FUT7 in inflamed tissues and in cancer cells. On the contrary, the GDP-L-fucose salvage pathway was found to be irrelevant in inflammation and in tumorigenesis. Furthermore, our results indicated the transcriptional coregulation of Golgi transporters involved in the synthesis of sulfo sLex, *i.e.* CMP-sialic acid, GDP-fucose and 3'phosphoadenosine 5'-phosphosulfate transporters, in inflammation.

2. REVIEW OF THE LITERATURE

2.1 The role of fucosylated glycans in humans

2.1.1 Fucose in human glycan structures

Carbohydrate chains are linked to proteins and lipids on cell surfaces. Protein glycosylation encompasses *N*-glycans, *O*-glycans, glycosaminoglycans and glycosylphosphatidylinositol (GPI) anchor proteins. *N*-glycans are linked to asparagine residues of proteins, specifically within the consensus sequence asparagine-X-serine/threonine (Asn-X-Ser/Thr), where X can be any amino acid residue except for proline (Schachter, 2000; Yan & Lennarz, 2005). *O*-glycans are linked to a subset of serine or threonine residues. Glycosaminoglycans are also attached to serine and threonine, however, they are linear and produced by different biosynthetic pathways, and are often highly sulfated (Esko & Selleck, 2002). GPI is a complex glycolipid that acts as a membrane anchor for many cell surface proteins (Kinoshita & Inoue, 2000). *N*-glycans are constructed in sequential manner by specific glycosyltransferase and glycosidase enzymes in the endoplasmic reticulum (ER) and Golgi apparatus. *O*-glycans are synthesized by glycosyltransferases in Golgi and in the cytoplasm. Glycosyltransferases synthesize glycan chains, whereas glycosidases hydrolyse specific glycan linkages. The expression of mammalian glycans is regulated at both postranscriptional and postranslational levels (Ohtsubo & Marth, 2006). Some glycosyltransferases and glycosidases must be glycosylated themselves to be active. Subsequently, they may be regulated through phosphorylation of their cytoplasmic tails, which may lead to differential substrate access and intracellular trafficking. Hence, the intracellular location of glycosyltransferases and glycosidases can be an effective regulator of glycan formation by controlling access to acceptor substrates. Loss of some chaperones and multiprotein complexes alters glycosyltransferase trafficking between the ER and Golgi (Foulquier *et al.*, 2006; Ju & Cummings, 2005; Wu *et al.*, 2004).

Each class of glycans share a limited number of core structures to which the specific saccharide groups are attached. These terminal groups are usually responsible for the biological functions of carbohydrates. Vertebrate *N*-glycan diversification in the Golgi generates three *N*-glycan subtypes: high mannose, hybrid and complex types that share the same core structure $Man_3GlcNAc_2$-Asn. Most secreted and cell surface *N*-glycans are of the complex type containing a various number of galactose (Gal), *N*-acetylglucosamine (GlcNAc), sialic acid (SA) and fucose (Fuc) residues linked to *N*-glycan core structure. Core fucosylation is a common structural feature among *N*-glycans. Many vertebrate *N*-glycans are modified by fucosyltransferase that adds Fuc via an α1-6 linkage to the GlcNAc residue that is linked to asparagine (Wilson *et al.*, 1976). All mucin-type *O*-glycan structures contain a GalNAcα-residue linked to Ser/Thr. Most mucin-type *O*-glycans contain the core 1 subtype structure formed by the addition of galactose via a β1-3 linkage to the GalNAc by Core 1 β1-3 galactosyltransferase (Core 1 GalT). Core 2-type is generated by addition of GlcNAc to the GalNAc of the core 1 structure via a β1-6 linkage. Hence, the production of core

2 *O*-glycans requires the core 1 structure as a substrate and the activity of Core 2 β1-6 glucosaminyltransferase (Core 2 GlcNAcT). There are also several other *O*-glycan core subtypes, which results in more complex *O*-glycan diversification.

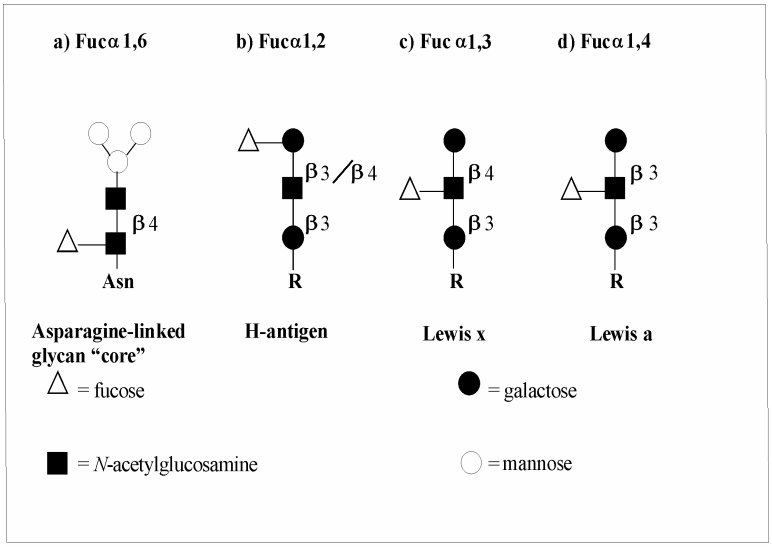

Figure 1. Examples of the common glycan structures with different fucosylation.
a) α1,6-fucosylation is involved in the core structure of vertebrate N-glycans. b) H-antigen is an α1,2-fucosylated structure. c) α1,3-fucosylated Lewis x represents type 2 structure. d) α1,4-fucosylated Lewis a is a type 1 structure.

Fucose has two isomers, L and D, of which the D isomer is rare and not found in mammals. L-fucose (6-deoxy-L-galactose) is an essential component of various mammalian glycan structures. It decorates *N*-and *O*-linked glycoproteins and glycolipids (Walz *et al.*, 1990) or is covalently linked to some serine or threonine residues of proteins (Harris & Spellman, 1993). Fucose can be linked via α1,2-linkage to terminal Gal, α1,3- or α1,4- linkage to subterminal GlcNAc or via α1,6-linkage to the innermost GlcNAc as shown in Figure 1.

Fucosylated glycoproteins and glycolipids can be present in cell membranes or secreted into biological fluids. Various functions in biological processes have been established for fucose residues (Becker & Lowe, 2003; Staudacher *et al.*, 1999). ABO blood group antigens are α1,2-fucosylated glycans (Greenwell, 1997; Lloyd, 2000). α1,3-and α1,4-fucosylated modifications are essential components of sialyl Lewis x (sLex)- and sialyl Lewis a (sLea)- type glycans, respectively, which have roles as selectin ligands in inflammation (Lowe, 1997). Fucosylated glycans also have important roles in fertilization (Johnston *et al.*, 1998; Mori *et al.*, 1998). The Lewis x (Lex) epitope, also known as stage-specific embryonic antigen-1 (SSEA-1), is

expressed during early embryogenesis indicating the essential role of Lex in cell adhesion events in embryonal development (Bird & Kimber, 1984; Eggens *et al.*, 1989). *O*-fucose residues, *i.e.* fucose is directly linked to hydroxyl groups of serine and threonine residues, are present on EGF domains of the mammalian Notch receptors (Moloney *et al.*, 2000b; Rampal *et al.*, 2005). Notch receptors are a family of signaling transmembrane proteins with important roles in normal development of an organism, *e.g* neurogenesis, angiogenesis and lymphoid development (Artavanis-Tsakonas *et al.*, 1999; Halloran *et al.*, 2000). Fucosylation is also involved in programmed cell death, *i.e.* apoptosis (Hiraishi *et al.*, 1993; Russell *et al.*, 1998; Winkler *et al.*, 2004).

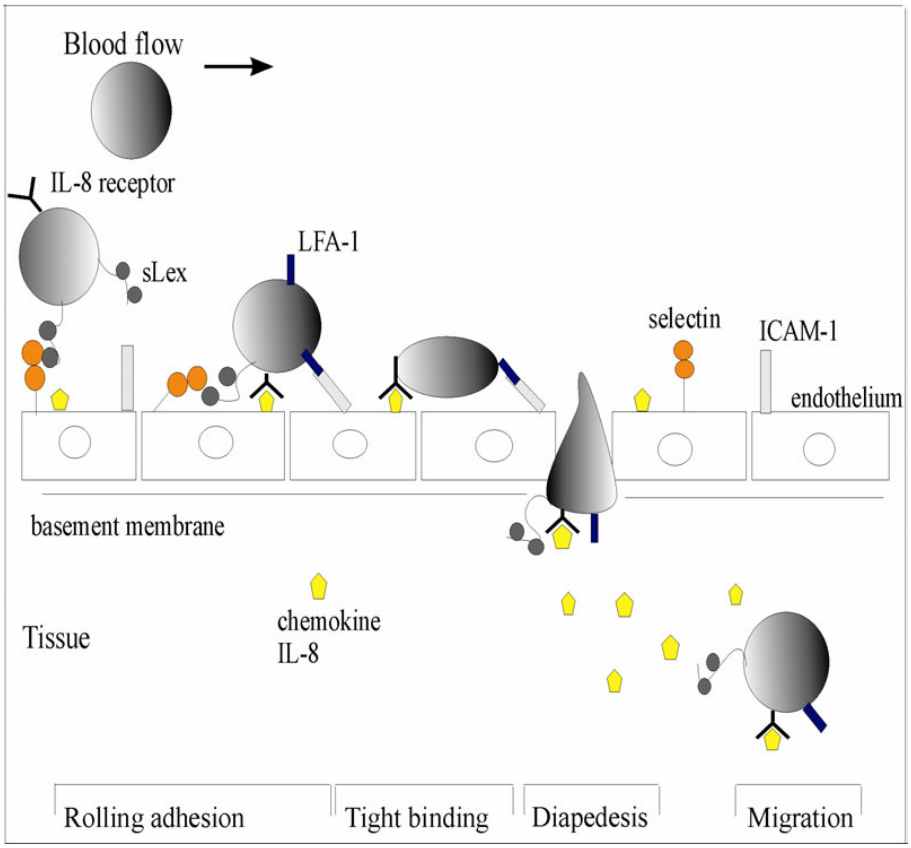

Figure 2. Leukocyte extravasation cascade. The first step involves the reversible binding of leukocyte to vascular endothelium through interactions between selectins and their carbohydrate ligands, such as sLex. This leads leukocyte to roll along the endothelium and to sense activating factors, such as chemokines. Leukocyte integrins (LFA-1) are activated and they bind to their counterreceptors (ICAM-1) on endothelium, which leads to firm adhesion and crossing the endothelial wall. Leukocyte squeezes between endothelial cells and penetrates the basement membrane leading to diapedesis. Finally, the leukocyte migrates through the tissue along the concentration gradient of chemokines secreted by cells at the site of inflammation.

2.1.2 Fucosylated glycans in leukocyte trafficking

The migration of leukocytes from intravascular locations to extravascular sites is an essential component of the the innate and adaptive immune responses. The traffic of leukocytes from blood vessels into the surrounding tissues is termed extravasation (Figure 2). The first step in the extravasation cascade involves a transient adhesive contact between leukocytes and the vascular endothelium mediated by selectins, a family of cell-surface adhesion molecules of leukocytes and activated endothelial cells (Lowe, 1997; Varki, 1997). The adhesion of selectins to their glycosylated counter-receptors, such as sLex, is weak and reversible allowing circulating leukocytes to roll along the endothelium (Etzioni *et al.*, 1999; Vestweber & Blanks, 2000). Binding of leukocytes to the endothelium takes place under dynamic conditions due to blood flow. Recent studies have revealed that this binding occurs more tightly when blood flow is increased (Marshall *et al.*, 2003; Yago *et al.*, 2004). Rolling brings leukocytes into close contact with the endothelium, which leads the endothelial-bound chemokines to bind to their respective chemokine receptors on leukocytes. This phenomenon activates leukocyte-specific β_2-integrins, such as LFA-1, to bind to their counter-receptors on endothelial cells leading to the tight binding of leukocytes to endothelium (Arnaout, 1990; Imhof & Aurrand-Lions, 2004). The counter-receptors of β_2-integrins, such as ICAM-1 or ICAM-2, belong to the immunoglobulin gene superfamily. After tight binding the leukocytes diapedese between endothelial cells and finally migrate through the endothelial cell layer and the underlying basal membrane into tissue to participate in the inflammatory reaction.

Selectins are a family of C-type lectins which contain an N-terminal extracellular lectin domain followed by a domain homologous to epidermal growth factor and two to nine consensus repeats similar to sequences found in complement regulatory proteins. Each of these adhesion receptors is inserted into membranes via a hydrophobic transmembrane domain and possesses a short cytoplasmic tail. Selectins have the ability to recognize and bind to specific carbohydrate determinants on selectin ligands in a calcium-dependent manner (Vestweber & Blanks, 1999). The selectin family consists of three proteins: L-, P-and E-selectins. P-selectin, the largest selectin of size 140 kDa, is the predominant selectin involved in leukocyte rolling on acutely inflamed endothelial cells *in vivo* (Ley *et al.*, 1995). P-selectin is stored in the α-granules of platelets (Berman *et al.*, 1986; Stenberg *et al.*, 1985) and in secretory granules called Weibel-Palade bodies of resting endothelial cells (Bonfanti *et al.*, 1989; McEver *et al.*, 1989). As endothelium is activated by histamine or platelets activated by thrombin, P-selectin is rapidly brought to the cell surface by degranulation of the compartments (Hamburger & McEver, 1990; Larsen *et al.*, 1989; Moore & Thompson, 1992). Under conditions of chronic inflammatory stimulation, P-selectin can be stably expressed on the cell surface (Pan *et al.*, 1998; Yao *et al.*, 1996). Except for skin microvessels, E-selectin is not constitutively expressed on resting vascular endothelium. Expression of E-selectin is induced after activation of endothelium by inflammatory cytokines, such as TNF-α, interleukin (IL)-1β and other stimuli, leading to the *de novo* transcription of E-selectin (Bevilacqua *et al.*, 1987). L-selectin, the smallest of the selectins, is expressed constitutively on circulating leukocytes (McEver, 1994; Rosen & Bertozzi, 1994). L-selectin is rapidly downregulated by sheddases upon cell activation by proteolytic cleavage near the cell surface (Smalley & Ley, 2005)**.** The main function of L-selectin shedding from

lymphocytes is to prevent the re-entry of activated T cells to the secondary lymphoid organs (Galkina *et al.*, 2003).

Leukocyte rolling participates in the recruitment of neutrophils, monocytes, eosinophils, and some effector T cells as well as dendritic cells to sites of acute and chronic inflammation (Sperandio, 2006). This requires the up-regulation of P- and E-selectins and of endothelial L-selectin ligands on inflamed endothelium. Granulocytes and monocytes emigrate from the bloodstream to tissues in response to inflammatory stimuli. The counterreceptors for selectins in the weak adhesive contacts contain sLex and related carbohydrate structures. sLex, Neu5Acα2-3Galβ1-4(Fucα1-3)GlcNAc, is a tetrasaccharide present in many glycoconjugates decorating proteins as well as lipids on cell surfaces. Several glycosyltransferases are involved in the biosynthesis of sLex; sialylation and fucosylation being the terminal glycosylation events (Lowe & Marth, 2003). α2,3-sialyltransferases (ST3Gal) form an α2,3-sialic acid linkage to the terminal galactose of type 2 *N*-acetyllactosamine (LN). As the final step, α1,3-fucosyltransferase (Fuc-T) transfers fucose from GDP-L-fucose to GlcNAc of α2,3-sialylated LN. Sulfated isomers of sLex tetrasaccharide epitopes, being crucial in lymphocyte trafficking, are generated by a family of 6-*O*-sulfotransferases. They are able to sulfate GlcNAc, galactose or both, representing 6-sulfo sLex, 6'-sulfo sLex or 6',6-disulfo sLex, respectively (Hemmerich & Rosen, 2000). Structures of sLex and its sulfated derivatives are presented in Figure 3.

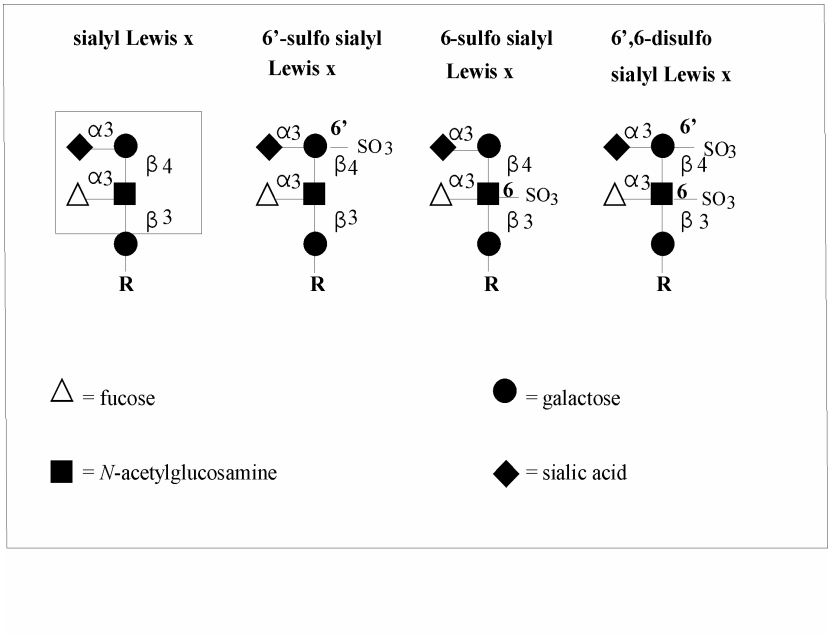

Figure 3. Sialyl Lewis x and its sulfated derivatives.

P-selectin glycoprotein ligand-1 (PSGL-1) is a homodimeric sialomucin, *i.e.* a glycoprotein with multiple mucin-type *O*-glycans and repeating peptide motifs (Moore *et al.*, 1992). It is expressed on most leukocytes and is the best characterized ligand for selectins (McEver & Cummings, 1997). PSGL-1 has been characterized as a predominant ligand for P-selectin during inflammation (Xia *et al.*, 2002; Yang *et al.*, 1999). It is also an important ligand for L-selectin (Guyer *et al.*, 1996; Spertini *et al.*, 1996; Tu *et al.*, 1996; Walcheck *et al.*, 1996). A core 2 based *O*-glycan with sLex epitope as well as three tyrosine sulfates at the N-terminus of PSGL-1 are required for optimal P- and L-selectin recognition (Leppänen *et al.*, 2000; Leppänen *et al.*, 2003; McEver & Cummings, 1997). Studies with PSGL-1 deficient mice confirmed that L-selectin mediated rolling during acute inflammation is mainly dependent on PSGL-1 (Sperandio *et al.*, 2003). Furthermore, the same study demonstrated L-selectin dependent rolling to occur mostly via secondary tethering, *i.e.* rolling occurred via interactions between flowing and adherent leukocytes (Sperandio *et al.*, 2003). Contrary to acute inflammation, in chronic inflammation the L-selectin dependent rolling is mediated via endothelial L-selectin ligands (Rosen, 2004). PSGL-1 binds differently to E-selectin than it does to P- and L-selectin. Core 2, sialylated and fucosylated *O*-glycans are required for binding to E-selectin, but tyrosine sulfation is not required (Li *et al.*, 1996;Goetz *et al.*, 1997). An apparent divergence exists between P- and E-selectin in mediating leukocyte rolling on cytokine-activated endothelium. E-selectin is thought to be responsible for slow rolling interactions and possibly the initiation of firm adhesion (Jung & Ley, 1997; Kunkel & Ley, 1996).

Circulating lymphocytes continuously patrol the body for foreign antigen by recirculating from blood into tissues and lymph and back to blood. Lymphocytes enter the secondary lymphoid organs at specialized endothelium on postcapillary venules in lymphoid tissue (von Andrian & Mempel, 2003). These high endothelial venules (HEVs) express specific vascular addressins called peripheral node addressins (PNAd), which bind subsets of circulating lymphocytes that express complementary homing receptor, L-selectin, on their surface (Rosen, 2004). The ligands of L-selectin are heavily *O*-glycosylated mucins, such as GlyCAM-1, CD34 and MAdCAM-1 bearing sulfated sLex epitopes on core 1- and core 2-type *O*-glycans (Hemmerich *et al.*, 1995; Rosen, 2004; Satomaa *et al.*, 2002; van Zante & Rosen, 2003; Yeh *et al.*, 2001). One of the sulfated epitopes, 6-sulfo sLex has been shown to be important for L-selectin binding. Two GlcNac-6-*O*-sulfotransferases, GlcNAc6ST-1 and GlcNAc6ST-2 act co-operatively to synthesize 6-sulfo sLex epitopes on L-selectin ligands in HEV of lymph nodes in mice (Kawashima *et al.*, 2005; Uchimura *et al.*, 2005). GlcNAc6ST-2 adds sulfate mainly to extended core 1 structures, whereas both GlcNAc6ST-1 and GlcNAc6ST-2 add sulfate to core 2 structures. Although sulfo sLex glycan epitopes are constitutively expressed in lymph node high endothelium, the expression is induced *de novo* in vascular endothelium at sites of inflammation by inflammatory mediators (Toppila *et al.*, 1999; Toppila *et al.*, 2000; Turunen *et al.*, 1995; van Zante & Rosen, 2003). Furthermore, previous studies indicate specific modifications of sLex and sulfo sLex in inflamed tissues of different organs (Renkonen *et al.*, 2002).

2.1.3 Fucosylated glycans in cancer

Cell surface carbohydrate determinants undergo dramatic changes during malignant transformation. Previous studies show that expression of sLex and sLea determinants is markedly enhanced in cancer cells (Kannagi, 1997; Majuri *et al.*, 1995; Renkonen *et al.*, 1997). Leukocyte responses to cancer have many parallels with inflammation, as the same determinants serve as ligands for selectins in inflammation and mediate hematogenous metastasis of cancers (Balkwill & Mantovani, 2001; Coussens & Werb, 2002; Läubli *et al.*, 2006). Leukocytes are considered to have antitumor roles but also to support tumor progression. Natural killer (NK) cells, playing an important role in innate immunity, are able to kill cancer cells without antigen stimulation (Ohyama *et al.*, 2003). On the other hand, during early tumorigenesis inflammatory leukocytes can be efficient tumor promoters, producing chemokines, growth factors and cytokines required for tumor growth, angiogenesis, migration, and differentiation. Later in the tumorigenic process, neoplastic cells also divert inflammatory mechanisms such as selectin-ligand interactions to favour neoplastic spread and metastasis (Coussens & Werb, 2002). The interaction between tumor-associated carbohydrate antigens such as sLex or sLea expressed on cancer cells, and E- or P-selectin on endothelial cells of the target organ, is one of the first and crucial steps in the metastasis cascade (Aigner *et al.*, 1998; Majuri *et al.*, 1992). L-selectin has been proposed to facilitate the recruitment of inflammatory leukocytes to the sites of tumor cell emboli, *i.e.* aggregated platelets and leukocytes, in microvasculature and thus support metastasis (Läubli *et al.*, 2006). Hence, up-regulation of sLex and sLea can be associated with advanced tumor progression and poor prognosis (Inaba *et al.*, 2003; Kannagi, 1997).

Normal epithelial cells express various carbohydrate determinants, some of which have structures more complex than sLex and sLea. For example, 6-sulfo sLex and (2-3, 2-6)-disialyl Lea determinants are further modified forms of sLex and sLea determinants (Kannagi, 2004). These complex determinants are present on non-malignant colonic epithelial cells and mediate interaction of epithelial cells with mucosal mesenchymal cells expressing carbohydrate-binding molecules. Expression of genes responsible for the modification of 6-sulfo sLex and (2-3, 2-6)-disialyl Lea is impaired at the early stages of cancer initiation, which is proposed to occur mainly due to epigenetic silencing through promoter DNA methylation or histone deacetylation (Miyazaki *et al.*, 2004). Later, in more advanced stages, cancer cells accumulate genetic abnormalities, and more malignant cancer cells with higher infiltrative and metastatic activities evolve due to accelerated expression of sLex and sLea determinants (Miyazaki *et al.*, 2004).

2.1.4 Fucosylated glycans in other diseases

Altered glycosylation is involved in several diseases. Various autoimmune diseases and other human afflictions are conditions that result when the inflammatory response is uncontrolled, self-directed or in the wrong place at the wrong time. Cystic fibrosis (CF) is an autosomal genetic disease resulting in the accumulation of mucus in exocrine organs. CF mucins have high *O*-glycans content (Rhim *et al.*, 2001; Scanlin & Glick, 1999); furthermore, comparison of CF mucins with mucins of healthy individuals, revealed significantly increased levels in sulfated and sialylated *O*-glycans

of CF mucins (Xia *et al.*, 2005). Increased expression of fucosylated glycans on serum immunoglobulins have been detected in rheumatoid arthritis patients (Gornik *et al.*, 1999). Several pathological processes, such as reperfusion injury following ischemia, astma, and inflammatory skin diseases, are conditions that result from the excessive and uncontrolled recruitment of leukocytes (Boyman *et al.*, 2007; Lefer *et al.*, 1994; Toppila *et al.*, 2000).

Organ transplant rejection is caused by immune responses to alloantigens on the graft, proteins that vary from individual to individual within a species and are therefore perceived as foreign by the recipient. The pathogenesis of rejection results from the highly efficient extravasation of lymphocytes into the transplanted organ (Kirveskari *et al.*, 2000; Turunen *et al.*, 1995).

Deficiency of fucosylated glycans has been observed in a rare human disease, leukocyte adhesion deficiency type II (LAD II), which results from a defective GDP-fucose transporter (Lubke *et al.*, 2001). In this genetic disease patients suffer from recurrent infections, persistent leukocytosis and severe growth and mental retardation (Becker & Lowe, 1999; Etzioni *et al.*, 1992).

2.2 The synthesis of GDP-L-fucose

Fucosylation requires a nucleotide-activated form of fucose, GDP-L-fucose, as a fucose donor for fucosyltransferases. GDP-L-fucose is synthesized *in vivo* via two different metabolic pathways, which both take place in cytosol. The pathways are shown in Figure 4.

2.2.1 GDP-L-fucose synthesis via the de novo pathway

The major, constitutively active, *de novo* pathway is evolutionarily conserved. It was first identified in bacteria (Ginsburg, 1960) and then described in plants (Liao & Barber, 1971), mammals (Overton & Serif, 1981), and invertebrates (Bulet *et al.*, 1984). The enzymes of the pathway have been cloned from several bacteria (Tonetti *et al.*, 1998), plants (Bonin *et al.*, 1997), and mammals (Reitman *et al.*, 1980). Furthermore, the pathway has been characterized *in silico* in *Drosophila melanogaster* (Roos *et al.*, 2002).

The *de novo* pathway involves conversion of GDP-α-D-mannose to GDP-β-L-fucose via three enzymatic reactions catalyzed by two enzymes, GDP-D-mannose-4,6-dehydratase (gmds, GMD, EC 4.2.1.47) and GDP-4-keto-6-deoxy-D-mannose-3,5-epimerase-4-reductase (Tsta3, in human designated as FX, EC 1.1.1.187) (Sullivan *et al.*, 1998; Tonetti *et al.*, 1996; Tonetti *et al.*, 1998). In the first step, GMD converts GDP-D-mannose to an unstable intermediate, GDP-4-keto-6-deoxy-D-mannose, by a dehydration reaction. This enzyme reaction, oxidation of a hydroxyl group at C-4 of mannose to a keto group and reduction of a hydroxyl group at C-6 of mannose to a methyl residue, requires the cofactor nicotinamide adenine dinucleotide phosphate

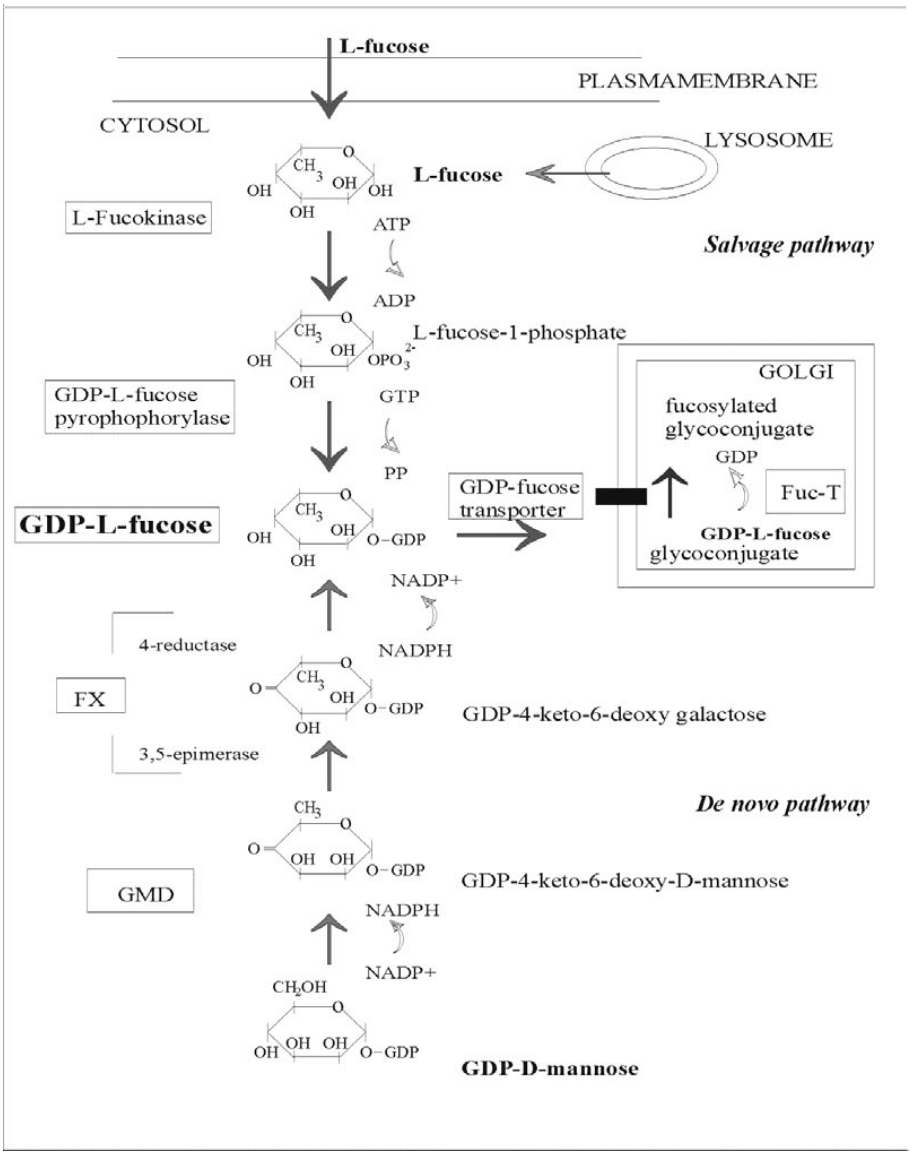

Figure 4. The biosynthesis of GDP-L-fucose. *De novo* pathway, the constitutively active pathway, converts GDP-D-mannose to GDP-4-keto–6-deoxy-D-mannose via oxidation by GDP-mannose-4,6-dehydratase (GMD). The resulting intermediate is epimerized to GDP-4-keto-6-deoxy galactose and further reduced to GDP-L-fucose by FX, an enzyme, which consists of 3,5-epimerase and 4-reductase. In the alternative salvage pathway, free fucose, obtained from extracellular sources or from lysosomal degradation of glycoconjugates, is phosphorylated by L-fucokinase to form L-fucose-1-phosphate, which is further converted to GDP-L-fucose by GDP-L-fucose pyrophosphorylase. GDP-fucose transporter translocates GDP-L-fucose to the Golgi, where it serves as a donor of fucose in a reaction catalyzed by a fucosyltransferase (Fuc-T).

(NADP+), which is reduced to NADPH during the reaction (Chang *et al.*, 1985; Oths *et al.*, 1990; Sullivan *et al.*, 1998). The enzyme cascade from GDP-D-mannose to GDP-L-fucose is inhibited by GDP-L-fucose as it is a potent competitive inhibitor of GMD (Bisso *et al.*, 1999; Sturla *et al.*, 1997).

Dual functional epimerase-reductase enzyme FX converts GDP-4-keto-6-deoxymannose to GDP-L-fucose (Chang *et al.*, 1988; Tonetti *et al.*, 1996). In the first FX reaction, GDP-4-keto-6-deoxy-D-mannose is epimerized at C-3 and C-5, which causes a change from D- to L-configuration and yields GDP-4-keto-6-deoxy-L-galactose. The 4-reductase activity of the FX protein catalyzes a H+ transfer from the NADPH cofactor to a keto group yielding GDP-L-fucose and NADP+ (Menon *et al.*, 1999).

GMD and FX transcripts are ubiquitously expressed in most human tissues (Sullivan *et al.*, 1998). A study of an induced mutation in the locus encoding mouse FX revealed the requirement for GDP-L-fucose in fertility, growth and development as well as in leukocyte adhesion (Smith *et al.*, 2002). Fucosylated glycan deficiency has been corrected by dietary fucose supplementation, which restored the synthesis of GDP-L-fucose through a normally quiescent salvage pathway (Smith *et al.*, 2002).

2.2.2 GDP-L-fucose salvage pathway

The enzymes of the alternative, "salvage", biosynthetic pathway of GDP-L-fucose synthesis, L-fucokinase (Fuk, EC 2.7.1.52) and GDP-L-fucose pyrophosphorylase (Fpgt, EC 2.7.7.30), were first discovered in pig liver in the 1960's (Ishihara & Heath, 1968a; Ishihara *et al.*, 1968b). To date, L-fucokinase has been partially purified and characterized from porcine liver (Ishihara & Heath, 1968a) and thyroid gland (Kilker *et al.*, 1979), and purified to apparent homogeneity from pig kidney (Park *et al.*, 1998). Furthermore, the human amino acid sequence has been identified (Hinderlich *et al.*, 2002). GDP-L-fucose pyrophosphorylase has been purified from porcine kidney and the corresponding gene has been cloned from man (Pastuszak *et al.*, 1998).

In the GDP-L-fucose salvage pathway, L-fucokinase synthesizes L-fucose-1-phosphate from L-fucose and ATP (Ishihara *et al.*, 1968b; Park *et al.*, 1998). L-fucokinase activity is feedback-inhibited by GDP-L-fucose (Park *et al.*, 1998). The second step involves GDP-L-fucose pyrophosphorylase, also designated as fucose-1-phosphate guanylyltransferase, which catalyzes the formation of GDP-L-fucose from L-fucose-1-phosphate and GTP (Ishihara & Heath, 1968a; Pastuszak *et al.*, 1998). Free fucose for the salvage pathway is obtained from the diet, or in the case of cultured cells from culture medium, and is transported across the plasma membrane into the cytosol. Furthermore, free fucose can be obtained from intracellular degradation of glycoproteins and glycolipids in lysosomes by fucosidases (Michalski & Klein, 1999).

Both Fuk (Miller *et al.*, 2005; Park *et al.*, 1998) and Fpgt activity (Pastuszak *et al.*, 1998) have been detected in various tissues indicating that salvage metabolism is a common phenomenon in eukaryotes. Previous studies indicate that fucokinase activity is regulated in response to stimulation of the brain by dopaminergic pathways and

exposure of aortic endothelial cells to nicotine (Hocher *et al.*, 1993; Ricken *et al.*, 1990).

2.3 Golgi transporters required for the synthesis of sulfo sialyl Lewis x glycan epitopes

The biosynthesis of glycans is dependent on the activity of nucleotide sugar donor synthesis and transport to Golgi as well as the expression of specific glycosyltransferases. The activated nucleotide sugar is transported from cytosol to the Golgi via a donor-specific transporter. The nucleotide sugar transporter family, the solute carrier family SLC35, consists of at least 17 members in humans (Ishida & Kawakita, 2004). Nucleotide sugar transporters are 320-400 amino acid residues long and are structurally conserved hydrophobic multi-spanning transmembrane proteins (Gerardy-Schahn *et al.*, 2001). They translocate nucleotide sugars from the cytosol, their site of synthesis, into the Golgi apparatus and the ER, where the activated sugars serve as substrates for a variety of glycosyltransferases.

Lymphocyte homing is mediated by specific interactions between L-selectin on lymphocytes and sulfated carbohydrates restricted to HEVs in lymph nodes. Three modifications: sialylation, fucosylation and carbohydrate sulfation, are required for optimal L-selectin binding (Maly *et al.*, 1996). The synthesis of sulfo sLex requires energy carrying donors, CMP-sialic acid (CMP-SA), GDP-L-fucose (GDP-L-Fuc) and 3'-phosphoadenosine 5'-phosphosulfate (PAPS) for donation of SA, Fuc and sulfate, respectively. These sugar nucleotide donors are synthesized in the cytosol or nucleus and transported by specific transporters to the Golgi apparatus, where the corresponding transferases and acceptor molecules reside. CMP-SA is synthesized in the nucleus by CMP-SA synthase (Eckhardt *et al.*, 1996; Lansdon *et al.*, 2004) and is transported to the Golgi via CMP-SA transporter (SLC35A1) (Aoki *et al.*, 2001; Eckhardt & Gerardy-Schahn, 1997; Eckhardt *et al.*, 1999; Eckhardt *et al.*, 1996).

Sulfation of all macromolecules in higher organisms requires the high-energy donor PAPS, which is synthesized by the sequential actions of two cytoplasmic enzymes, ATP sulfurylase and APS kinase, and is subsequently transferred across the Golgi membrane for utilization by lumenal sulfotransferases (Ozeran *et al.*, 1996). The specific PAPS transporter (SLC35B2) acts through an antiport mechanism with adenosine 3´, 5´ -bisphosphate as the returning ligand (Kamiyama *et al.*, 2006; Kamiyama *et al.*, 2003).

GDP-fucose transporter (FUCT1, SLC35C1) is responsible for the uptake of GDP-L-fucose by Golgi (Lubke *et al.*, 2001; Luhn *et al.*, 2001). GDP-fucose transporter is a protein with 10 predicted transmembrane domains spanning the Golgi membrane (Luhn *et al.*, 2004). As with other sugar nucleotide transporters, the C- and N-terminal regions of GDP-fucose transporter are exposed to the cytosol. GDP-fucose transporter is a antiporter, which transports GDP-L-fucose from the cytosol into the Golgi lumen in exchange for guanidine monophosphate (GMP) (Puglielli & Hirschberg, 1999). LAD II, also known as a congenital disorder of glycosylation type IIc (CDG IIc) is caused by a defect in GDP-L-fucose transport. A missense mutation in the GDP-fucose

transporter leads to partially defective function and is responsible for the defective fucosylation in LAD II patients (Lubke *et al.*, 2001; Luhn *et al.*, 2001). In this very rare human disease patients suffer from recurrent infections and persistent leukocytosis due to the loss of particular selectin ligands (Becker & Lowe, 1999; Etzioni *et al.*, 1992). In addition to immunodeficiency, LAD II patients show severe mental and growth retardation (Becker & Lowe, 1999; Wild *et al.*, 2002). Fucosylation can be restored with oral fucose, indicating that salvage metabolism is responsible for the generation of GDP-L-fucose (Marquardt *et al.*, 1999).

2.4 Fucosyltransferases

Fucose is transferred to acceptor oligosaccharides by various fucosyltransferases (Fuc-T) via α1,2-, α1,3-, α1,4-, α1,6-linkages (Becker & Lowe, 2003). Examples of fucosylated structures are illustrated in Figure 1. Certain proteins can also be fucosylated directly by protein *O*-fucosyltransferase (*O*-Fuc-TI).

α1,3-fucosyltransferases catalyze the transfer of fucose from GDP-L-fucose in an α1,3-linkage to an appropriate acceptor substrate. This transfer is the final step in the synthesis of fucosylated glycoconjugates. Like other glycosyltransferases in the Golgi, fucosyltransferases consist of a lumenally oriented C-terminus with a large catalytic domain, a stem region, a single membrane spanning region and a short cytoplasmic *N*-terminus. The *N*-terminal regions, *i.e.* cytoplasmic, transmembrane and stem regions, have the highest sequence heterogeneity (de Vries *et al.*, 2001a). The *N*-terminal transmembrane domain has a role in the localization of this protein to the Golgi and in the retention of the enzyme. Secreted forms are produced by proteolysis in the Golgi apparatus at multiple protease sensitive sites within the stem region of the protein (Paulson & Colley, 1989). These soluble enzymes are responsible for the enzyme activities detected in milk and in body fluids such as serum or saliva (Mollicone *et al.*, 1990).

Six human fucosyltransferase genes *(FUT)* encoding α1,3-fucosyltransferases have been cloned to date (de Vries *et al.*, 2001a). *FUT3* encodes the Lewis-type fucosyltransferase Fuc-TIII (Kukowska-Latallo *et al.*, 1990) and *FUT4* the myeloid-type enzyme Fuc-TIV (Goelz *et al.*, 1990; Kumar *et al.*, 1991; Lowe *et al.*, 1991). *FUT5* encodes the enzyme Fuc-TV (Weston *et al.*, 1992a) and *FUT6* the plasma type enzyme Fuc-TVI (Koszdin & Bowen, 1992; Weston *et al.*, 1992b). *FUT7* encodes the leukocyte expressed Fuc-TVII (Natsuka *et al.*, 1994; Sasaki *et al.*, 1994) and *FUT9* the Fuc-TIX (Kaneko *et al.*, 1999). Furthermore, two additional putative α1,3-fucosyltransferase genes, *FUT10* and *FUT11*, have been identified in the human genome *in silico* by comparison with the fucosyltransferase sequences in the *Drosophila melanogaster* genome (Roos *et al.*, 2002). The *FUT3, FUT5* and *FUT6* genes form a cluster on chromosome 19p13.3 (McCurley *et al.*, 1995). Furthermore, the sequences of these genes are highly homologous to each other (Koszdin & Bowen, 1992; Kukowska-Latallo *et al.*, 1990; Weston *et al.*, 1992a; Weston *et al.*, 1992b) suggesting a common ancestor (Oulmouden *et al.*, 1997). *FUT4, FUT7* and *FUT9* are less similar to each other and to the former group.

In general, each glycosyltransferase recognizes only one type of sugar nucleotide. All fucosyltransferases utilize GDP-L-fucose as a L-fucose donor, hence, their specificities reside in the specific type of linkage formed and in the acceptors they recognize (Niemelä *et al.*, 1998; Toivonen *et al.*, 2002). Fuc-TIII catalyzes the formation of α1,3- and α1,4-linkages, being able to fucosylate both type 2 (Galβ1,4GlcNAc) and type 1 (Galβ1,3GlcNAc) *N*-acetyllactosamines forming Lex and Lea, respectively (Kukowska-Latallo *et al.*, 1990; Weston *et al.*, 1992a). Nevertheless, Fuc-TIII strongly prefers type 1 lactosamine acceptors to type 2 acceptors (Kukowska-Latallo *et al.*, 1990; Weston *et al.*, 1992a). The three homologous enzymes Fuc-TIII, Fuc-TV and Fuc-TVI act on both sialylated and non-sialylated acceptors (Koszdin & Bowen, 1992; Weston *et al.*, 1992a; Weston *et al.*, 1992b). Fuc-TV and Fuc-TVI prefer type 2 lactosamine acceptors to type 1 acceptors. Fuc-TIV and Fuc-TIX act primarily on nonsialylated type 2 *N*-acetyllactosamine, which results in the formation of Lex (Britten *et al.*, 1998; Kaneko *et al.*, 1999; Niemelä *et al.*, 1998). On the contrary, Fuc-TVII prefers to fucosylate the α2,3-sialylated type 2 *N*-acetyllactosamine (Natsuka *et al.*, 1994; Sasaki *et al.*, 1994).

α1,3-fucosyltransferases show complex tissue and cell type specific expression patterns, which vary during development and malignant transformation. Fuc-TIII is abundantly expressed in epithelial cells and gastrointestinal tissues, specifically stomach, jejunum and colon, but is not detected in brain, liver or peripheral blood leukocytes (Cameron *et al.*, 1995). Fuc-TV is only minimally expressed in spleen, liver, colon and testis (Cameron *et al.*, 1995). The tissue distribution of Fuc-TVI is similar to that of Fuc-TIII, except that Fuc-TVI is also expressed at high levels in liver and kidney (Cameron *et al.*, 1995; Kaneko *et al.*, 1999). Fuc-TIV is widely expressed in various tissues and cells (Gersten *et al.*, 1995) whereas the expression of Fuc-TIX is restricted to brain, stomach, spleen and peripheral blood cells (Kaneko *et al.*, 1999). Fuc-TIX is expressed as transcripts of different lengths in different tissues and developmental stages (Cailleau-Thomas *et al.*, 2000; Kaneko *et al.*, 1999). Fuc-TVII is expressed abundantly in leukocytes and in high endothelial cells of venules (Natsuka *et al.*, 1994; Sasaki *et al.*, 1994). Fuc-TIV and Fuc-TVII complimentarily synthesize fucosylated cell surface epitopes recognized by selectins and thus are responsible for normal leukocyte trafficking and function (Natsuka *et al.*, 1994; Niemelä *et al.*, 1998; Smith *et al.*, 1996; Weninger *et al.*, 2000). The essential role of Fuc-TVII has been demonstrated in gene ablation experiments. Fuc-TVII-/- mice showed not only blood leukocytosis but also impaired lymphocyte homing and leukocyte extravasation to sites of inflammation (Maly *et al.*, 1996; Smithson *et al.*, 2001). In contrast to Fuc-TVII-/- mice, studies with Fuc-TIV-/- mice demonstrated a minor significance for Fuc-TIV in neutrophil and lymphocyte recruitment (Homeister *et al.*, 2001; Smithson *et al.*, 2001). Nevertheless, Fuc-TIV was indicated to be the major regulatory enzyme in the synthesis of selectin ligands in eosinophils (Satoh *et al.*, 2005).

α1,2-fucosyltransferases H and Se, encoded by FUT1 (Larsen *et al.*, 1990) and FUT2, add fucose via α1,2-linkage to the terminal Gal of *N*-acetyllactosamine types 2 and 1, respectively, to make H antigens (Apoil *et al.*, 2000). The H enzyme is responsible for the expression of H antigen in the red cell lineage and vascular endothelium, whereas the Se enzyme is responsible for the synthesis of H antigen in secretory epithelia (Kelly *et al.*, 1995).

α1,6-fucosyltransferase Fuc-TVIII catalyzes the transfer of a fucose residue from GDP-L-fucose to the innermost GlcNAc residue of *N*-linked oligosaccharides in glycoproteins to form core fucosylation in mammals (Yanagidani *et al.*, 1997). Fuc-TVIII is widely distributed in mammalian tissues and human cancer cell lines (Struppe & Staudacher, 2000); Miyoshi *et al.*, 1999). The catalytic mechanism of FucTVIII is distinct from α1,3/1,4-fucosyltransferases and is more similar to that for α1,2-fucosyltransferases (Ihara *et al.*, 2006; Takahashi *et al.*, 2000). The common motifs between α1,2-and α1,6-fucosyltransferases and sequence similarity analysis suggest a common genetic origin for these families of enzymes (Breton *et al.*, 1998; Oriol *et al.*, 1999).

Protein *O*-fucosyltransferase I adds fucose directly to serine or threonine in EGF-like repeats of certain glycoproteins (Wang *et al.*, 2001). The *O*-FUT1 gene sequence is highly conserved in mammals and is expressed in various mammalian tissues indicating that *O*-fucose modification has a significant roles in different contexts (Wang *et al.*, 2001). *O*-fucose glycans on EGF-like repeats of several cell surface and secreted proteins play important roles in ligand-induced receptor signalling (Bruckner *et al.*, 2000; Harris & Spellman, 1993; Moloney *et al.*, 2000a; Okajima *et al.*, 2005). A short region associated with donor binding is conserved among α1,2-, α1,6- and protein *O*-fucosyltransferases, suggesting that *O*-FUT1 belongs to the same superfamily as α1,2-and α1,6-fucosyltransferases (Martinez-Duncker *et al.*, 2003).

The basic steps of the *N*-glycosylation pathway are evolutionally conserved in animals and plants. However, the structures of mature *N*-glycans differ between mammals and plants because of the major differences in the final steps of the biosynthetic pathways. While mature mammalian *N*-glycans are primarily of the complex type, mature plant *N*-glycans are mainly truncated $Man_3GlcNAc_2$-structures containing core α1,3-fucose and β1,2-xylose residues (Bondili *et al.*, 2006). Comparing the porcine and human core α1,6-fucosyltransferases with the mung bean core α1,3-fucosyltransferase (Fuc-T C3), there are no significant sequence similarities despite similar substrate specificities. Subsequently, the amino acid sequence of cloned FucT C3 from mung beans showed only a low degree of overall homology with mammalian Lewis blood group α1,3/4-fucosyltransferases. However, a few highly conserved amino acid residues were identified between mammalian Lewis and plant core fucosyltransferases (Leiter *et al.*, 1999). The plant glycan epitopes β1,2-xylose and core α1,3-fucose are not present in humans and therefore constitute epitopes for carbohydrate reactive antibodies, which represent a limitation for the therapeutical use of recombinant mammalian glycoproteins produced in transgenic plants (Strasser *et al.*, 2004). Core α1,3-linked fucosylated glycans have also been identified in insects, but β1,2-xylose has not been detected in this class of organism. Instead, *N*-glycans of *Drosophila melanogaster* have been found to carry both core α1,3- and α1,6-linked fucose (Fabini *et al.*, 2001). Taken together, the substitution of the asparagine-linked GlcNAc by α1,3-linked fucose is a widespread feature of plant and insect glycoproteins.

3. AIMS OF THE PRESENT STUDY

● to clone and express the murine enzymes of the GDP-L-fucose salvage pathway

● to determine the expression levels of the salvage matabolism enzymes in different tissues

● to clone and express rat fucosyltransferase VII

● to compare the gene expression levels of GDP-L-fucose synthesizing enzymes, GDP-fucose transporter and Fuc-TVII in normal as well as inflamed tissue and in cancer cells

● to investigate the possible transcriptional co-regulation of the different Golgi transporters required for the synthesis of sulfo sLex glycan epitopes

4. METHODS

	ORIGINAL PUBLICATION
Cell cultures	I, III, IV
Cloning and gene expression techniques	
DNA cloning	I, III
Sequence analysis	I, III
cDNA synthesis	I, II, III, IV
PCR	I, III
RACE	I, III
qRT-PCR	I, II, III, IV
RNA extraction	I, II, III, IV
Northern blot	III
Protein expression methods	
Expression of fusion proteins	I, III
SDS-PAGE	I, III
Immunological methods	
Flow cytometry	III
Western blot	I
Tissue sample processing	
Tissue extraction	II, III, IV
In situ hybridization	II, III
Enzyme activity assays	
Fucosyltransferase assay	III
Fucokinase assay	I
Methods of carbohydrate chemistry	
DEAE anion exchange	I, III
Solid phase extraction	I (described in Räbinä et al.,2001)
Ion-pair reversed-phase HPLC	I
MALDI-TOF mass spectrometry	I
***In silico* analyse**	I, II

5. RESULTS AND DISCUSSION

5.1 The GDP-L-fucose salvage pathway (I)

5.1.1 Cloning of murine L-fucokinase and GDP-L-fucose pyrophosphorylase

In the GDP-L-fucose salvage pathway free cytosolic fucose is phosphorylated by L-fucokinase (Fuk) to form L-fucose-1-phosphate, which is further converted to GDP-L-fucose in the reaction catalyzed by GDP-L-fucose pyrophosphorylase (Fpgt), as shown in Figure 3.

A part of the putative murine L-fucokinase sequence was identified from mouse genomic sequence from the EMBL/Genbank/DDJB databases using the three known pig fucokinase peptide sequences (Park *et al.*, 1998) as probes. The region corresponding to the putative L-fucokinase was cloned from mouse kidney cDNA and used as a query for further sequence database searches. An IMAGE clone containing the full coding sequence (CDS) of the putative L-fucokinase was identified and used for designing the primers for reverse transcription PCR (RT-PCR). Two cDNAs of sizes 3270 bp and 3057 bp were cloned from mouse kidney total RNA (Figure 3/I). These putative splice variants of L-fucokinase encoded proteins of 1090 amino acids (aa) and 1019 aa, respectively. Protein sequence variants were identical in the amino-terminal end up to the splice area, as well as in the carboxy-terminal end after the alternative splice area, hence maintaining the same reading frame (Figure 2/I). While this work was under way, the gene encoding human L-fucokinase was reported (Hinderlich *et al.*, 2002). A high degree of similarity exists between the human and mouse cDNAs, the human cDNA resembling the long splice variant of mouse L-fucokinase at the splice area.

The cloned human GDP-L-fucose pyrophosphorylase (Pastuszak *et al.*, 1998) was used as a query in BLAST searches to find mouse expressed sequence tags (ESTs) corresponding to the putative Fpgt. By using the mouse EST as a probe, the 3'end of Fpgt was cloned by screening a mouse kidney cDNA library. The 5'end of the gene was resolved by the RACE-PCR method. The isolated cDNA consisted of 3480 bp predicting a protein of 591 aa (Figure 6/I).

5.1.2 Expression of the enzymes involved in the GDP-L-fucose salvage pathway

To determine the enzymatic activities of the proteins, the cloned genes were expressed in mammalian cells. The molecular masses of L-fucokinase proteins determined by Western blot analysis were 125 kDa and 115 kDa (Figure 4/I). The production of L-fucose-1-phosphate from L-[^{3}H]fucose and ATP was measured in order to determine the enzymatic activity of both L-fucokinase splice variants. The specific enzyme activity of the long splice variant was 598.5 pmol mg^{-1} h^{-1} in transfected COS-7 cells, whereas that of the short splice variant (13.7 pmol $mg^{-1}h^{-1}$) was at the same level as the mock control (11.4 $pmolmg^{-1}h^{-1}$) (Figure 5/I).

The product of the GDP-L-fucose pyrophosphorylase assay was identified by ion-pair reverse-phase high-performance liquid chromatography (HPLC). This method was developed for analysis of nucleotide sugars in our laboratory by Räbinä *et al.* (2001). Nucleotide sugars were first purified from cell lysate using solid-phase extraction (SPE) columns containing graphitized carbon. To remove impurities the samples were further treated with alkaline phosphatase, which degrades nucleotides but leaves nucleotide sugars intact. Purified negatively charged nucleotide sugars bound to ion-pairing reagent TEAA and were retained in the HPLC column. They were eluted with a gradient of increasing acetonitrile concentration, and the synthesized nucleotide sugars were identified by comparison of retention times to those of nucleotide sugar standards. The analysis revealed a peak with the same retention time as the commercial GDP-L-fucose standard at 29.6 min (Figure 7/I). The peak was purified and subjected to further analysis by MALDI-TOF MS, which gave a single peak at 588.08 m/z, being identical to the GDP-L-fucose control.

5.1.3 Tissue distribution of L-fucokinase and GDP-L-fucose pyrophosphorylase

Quantitative studies of fucose metabolism in HeLa cells demonstrate that over 90% of GDP-L-fucose is derived from the *de novo* pathway (Yurchenco & Atkinson, 1975). Nevertheless, as detected by quantitative real-time RT-PCR (qRT-PCR), various mouse tissues expressed considerably high mRNA levels of L-fucokinase and GDP-L-fucose pyrophosphorylase. The mRNA expression of L-fucokinase was highest in brain, ovary and testis. Furthermore, significant expression could also be detected in kidney, liver and lung (Figure 8, panels A and B/I). A study by Park *et al.* (1998) demonstrated high fucokinase activities in pig kidney, liver and brain. Moreover, a study of fucokinase activities in various rodent tissues by Miller *et al.* (2005) indicated that L-fucokinase activity was highest in brain. Fucokinase is upregulated in response to stimulation of the brain by dopaminenergic pathways and exposure of aortic endothelial cells to nicotine (Hocher *et al.*, 1993; Ricken *et al.*, 1990). Hence, L-fucokinase may have a role in neuronal tissues.

Gene expression of GDP-L-fucose pyrophosphorylase in various tissues resembles the gene expression pattern of L-fucokinase (Figure 8, panel C/I). High levels of GDP-L-fucose pyrophosphorylase mRNA was detected in brain, testis, ovary and kidney. Expression levels were lower in liver, spleen, heart and kidney.

The GDP-L-fucose salvage pathway is not universal and has been identified only in mammals (Park *et al.*, 1998; Pastuszak *et al.*, 1998). Salvage metabolism is used for GDP-L-fucose synthesis if the *de novo* pathway is blocked (Smith *et al.*, 2002) or GDP-L-fucose import to the Golgi apparatus is impaired as in the case of the LAD II disorder (Lubke *et al.*, 2001; Luhn *et al.*, 2001). Nevertheless, GDP-L-fucose is synthesized via the salvage pathway only if extra fucose is supplied (Marquardt *et al.*, 1999). The distinct role of the GDP-L-fucose salvage pathway is still unelucidated, although the enzymes are relatively abundantly expressed in mammals. The specific salvage pathways also exist for other nucleotide sugars such as UDP-galactose, UDP-glucuronic acid and UDP-*N*-acetylgalactosamine (Bulter & Elling, 1999).

5.2 Inflammation-induced transcriptional regulation of Golgi transporters involved in the synthesis of sulfo sLex glycan epitopes (II)

Sulfo sLex epitopes are known to be induced *de novo* in vascular endothelium at sites of inflammation (Fukuda, 2002; Turunen *et al.*, 1995; van Zante & Rosen, 2003). The decoration of *N*-acetyllactosamine, *i.e*. sialylation, fucosylation and sulfation, is carried out by specific transferases in the Golgi, to where the high-energy nucleotide derivates CMP-SA, GDP-L-Fuc and PAPS are transported from cytosol or nucleus to act as donors in the synthesis of sulfo sLex. In order to elucidate the possible coregulation of CMP-SA, GDP-Fuc and PAPS transporters, their gene expression was studied by *in situ* hybridization and qRT-PCR during rat organ allograft rejection.

Peritubular capillaries in kidney have previously been shown to express sulfo sLex *de novo*, to display lymphocyte-specific adhesion and to display endothelial morphology during acute rejection episodes (Kirveskari *et al.*, 2000; Renkonen *et al.*, 1990; Turunen *et al.*, 1994). A clear upregulation of transcription of the GDP-Fuc transporter was detected three days after transplantation by *in situ* labeling of kidney cortex and outer medulla, the site of leukocyte infiltration during early phases of acute rejection. Subsequently, the signal was significantly strong at the corticomedullary junction. At day four after transplantation, a strong signal was evenly distributed in the cortex, outer medulla and transitional epithelium, after which at the fifth day the signal was diminished (Figure 2A/II). The transcription of the transporters were also analyzed in rat heart allografts. No signal for GDP-Fuc transporter was evident in control hearts, but a specific signal was detected under the epicardium three days after transplantation, subsequently, at day four the signal was even stronger, but at day five only a low diffuse labeling was detected (Figure 2E/II).

As with the GDP-Fuc transporter, no signal for the PAPS transporter was detected in control kidneys. Three days after transplantation a signal was present in the cortex and outer medulla, in the same area where rejection occurred as well as where the GDP-Fuc transporter signal was induced. The signal was strongest in the corticomedullary junction and in the transitional epithelium of the renal pelvis. Four days after transplantation both the cortex and inner medulla showed a strong signal for the PAPS transporter, which was decreased at day five (Figure 3A/II). In control heart, no PAPS transporter signal was detected and only a low signal was seen under the pericardium three days after heart transplantation. At day four a moderate signal was evident at the right ventricular wall and subsequently, at day five the signal had diminished (Figure 3E/II).

The induction of expression of the transporter transcripts were confirmed by qRT-PCR, which is a more sensitive method than *in situ* hybridization. All the transporters for GDP-Fuc, CMP-SA and PAPS were clearly induced in a time-dependent manner during the rejection episodes after kidney transplantation. The expression of all transporter transcripts were highest at day three after which they started to decline (Figure 4A/II). The expression pattern of transporters in heart allografts were slightly different from those in kidney allografts. The transcript levels of transporters increased in a time-dependent manner and the expression was highest at day five (Figure 4B/II). Allograft rejection in kidney usually affects larger areas of tissue than in heart, which

could explain the difference between the levels of expression of the transporter mRNAs in kidney and heart.

Previous results indicated that GDP-Fuc, CMP-SA and PAPS transporters are coregulated upon inflammatory stimuli caused by allograft rejection. The expression of CMP-SA and GDP-Fuc transporters were further investigated by *in silico* analysis to elucidate whether these genes are coregulated in a large number of experiments from various cell types and over a broad range of physiological and experimental conditions. Publically available transcriptome data from human gene chip analyses containing probes for GDP-Fuc and CMP-SA transporters was used to extract the data of interest. The gene chip data indicated that GDP-Fuc and CMP-SA transporters were not significantly coregulated under the conditions used in those experiments (Figure 5/II). Our data, however, suggests that there is a temporospatial induction of GDP-Fuc and PAPS transporters and at least a time-dependent induction of the CMP-SA transporter at the time the sulfo sLex-dependent leukocyte extravasation begins into the sites of tissue inflammation.

5.3 Rat fucosyltransferase VII (III)

Rat Fuc-TVII was cloned from rat lymph node by RT-PCR using the sequence details of human Fuc-TVII (Lowe *et al.*, 1991). The 3' end of the sequence was resolved by the RACE-PCR method. The two clones isolated, of sizes 2013 and 2100 bp, were predicted to encode proteins of 341 and 370 aa, respectively, thus representing two splice variants of Fuc-TVII (Figure 1/III). The deduced amino acid sequences predicted a classical glycosyltransferase, *i.e.* a type II transmembrane protein composed of a short cytoplasmic tail, a membrane spanning region, a short stem area, and a catalytic domain. The sequence had three potential *N*-glycosylation sites whereas the previously cloned human Fuc-TVII had two sites of which both have been reported to be occupied (de Vries *et al.*, 2001b). There is evidence that *N*-glycans of fucosyltransferases are required for optimal enzyme activity (Baboval *et al.*, 2000; Christensen *et al.*, 2000; Holmes *et al.*, 2000). The cloned rat Fuc-TVII had the α1,3-Fuc-T motif, FxL/VxFENS/TxxxxYxTEK, a highly conserved stretch of 17 aa´s found in α1,3-fucosyltransferases (Martin *et al.*, 1997). It has been speculated that the α1,3-Fuc-T motif is involved in GDP-L-fucose binding (de Vries *et al.*, 2001a).

Fuc-TVII activity was analyzed by measuring the radiolabeled GDP-L-fucose incorporation to sialylated *N*-acetyllactosamine in a reaction catalyzed by Fuc-TVII in transfected COS-7 cells (Figure 3/III). The specific enzyme activity of the long splice variant of Fuc-TVII was high, determined to be 1030 pmol mg^{-1} h^{-1}, whereas the specificity of the short splice variant was the same as the mock control (50 pmol mg^{-1} h^{-1}). The acceptor specificity was also detected using neutral acceptor substrate. The long variant of cloned cDNA showed no α1,3-Fuc-T- activity with the neutral acceptor, which was consistent with previous studies of Fuc-TVII, where it acted only on sialylated acceptors (Natsuka *et al.*, 1994; Sasaki *et al.*, 1994). When Fuc-TVII transfected cells were treated with the *N*-glycosylation inhibitor tunicamycin, the α1,3-fucosyltransferase activity was decreased significantly, indicating the requirement of *N*-glycosylation for optimal enzyme activity. Flow cytometric analysis was also used

to detect the enzymatic activity of Fuc-TVII in transfected COS-7 cells that express endogenously sialylated type 2 glycans (Kukowska-Latallo *et al.*, 1990). COS-7 cells transfected with the long splice variant of Fuc-TVII expressed sLex glycans at the cell surface, as detected with the anti-sLex monoclonal antibodies (mAb) KM93, CSLEX and sLex ab. In contrast to the long splice variant, the short variant and the negative vector control showed only minor signal or remained totally negative depending on the mAb used (Figure 4/III). Hence, the flow cytometry analysis confirmed the enzyme assay results of the long splice variant being the functionally active Fuc-TVII.

5.4 Gene expression of GDP-L-fucose synthesizing enzymes, GDP-fucose transporter and fucosyltransferase VII in inflamed tissues and cancer cells (IV)

The formation of fucosylated glycans depends on the concerted activity of GDP-L-fucose synthesis and transport into Golgi as well as expression of the appropriate fucosyltransferase. The levels of fucosylated epitopes, *e.g.* sialyl Lewis x, increases during the early events of inflammation and in the progress of cancer (Inaba *et al.*, 2003; Lowe, 2003). The regulation of gene expression of GDP-L-fucose synthesizing enzymes, GDP-fucose transporter and fucosyltransferase VII was studied by qRT-PCR in inflamed tissues. Kidney allograft rejection after transplantation between major histocompatibility complex-incompatible inbred rat strains was chosen as a model of acute inflammation, since *de novo* expressed sulfo sLex epitopes on the graft endothelium have been shown to be crucial in the recruitment of leukocytes to the site of inflammation (Kirveskari *et al.*, 2000; Toppila *et al.*, 1999; Turunen *et al.*, 1995). The mRNA levels of the enzymes of the *de novo* pathway of GDP-L-fucose synthesis, GDP-fucose transporter and Fuc-TVII were upregulated significantly in kidney allografts when compared to normal rat kidney (Figure 3/IV). The upregulation of Fuc-TVII in kidney allografts could be detected also by the *in situ* hybridization assay (Figure 6/III). The transcript levels of the enzymes of the salvage pathway were not elevated in acute inflammation, suggesting a minor role for them in inflammation (Figure 3/IV).

The gene expression of the same set of enzymes was studied in normal mouse endothelial cells and lymphoid tumor cells. Elevated FX expression and increased production of GDP-L-fucose have been reported previously in human hepatocellular carcinoma (Noda *et al.*, 2003). Our results demonstrated the upregulation of both enzymes of the *de novo* route, FX and GMD, in lymphoid tumor cells as compared to non-induced endothelial cells (Figure 2/IV). We subsequently found elevated mRNA levels of GDP-fucose transporter and Fuc-TVII in tumor cells, which is consistent with the elevated GDP-L-fucose level in cytosol and thus indicates the increased expression of fucosylated glycans in tumor cells.

6. SUMMARY

The migration of leukocytes from the blood vessels into inflamed tissue is the essential step in the process of inflammation. Binding of leukocytes to the blood vessel wall is mediated by cell adhesion molecules and leukocyte-activating factors. Emigration of leukocytes from the blood is initiated by the capture of leukocytes from the bloodstream followed by their rolling along the endothelial cell surface. The initial binding leads to recognition of inflammatory mediators, *e.g.* chemokines, on endothelial cells by leukocytes and subsequently activation of leukocyte integrins. β_2-integrins mediate the tight binding of leukocytes to endothelium by binding to their counter-receptors on endothelial cells. Leukocytes diapedese between endothelial cells and transmigrate through the endothelial cell layer and the underlying basal membrane into the tissue.

The rolling step is mediated by P-, E- and L-selectins, which bind to their specific carbohydrate ligands on proteins or lipids. All selectins recognize a tetrasaccharide structure, sialyl Lewis x (sLex), Neu5Acα2-3Galβ1-4(Fucα1-3)GlcNAc on their glycoconjugate ligands. Several glycosyltransferases are involved in the biosynthesis of sLex, fucosyltransferase VII (Fuc-TVII) being the last enzyme to modify the sLex structure. Fuc-TVII transfers L-fucose from GDP-L-fucose to sialylated *N*-acetyllactosamine. GDP-L-fucose is synthesized in the cytosol via two different metabolic pathways. The major, constitutively active *de novo* pathway involves conversion of GDP-α-D-mannose to GDP-β-L-fucose. In the alternative "salvage" pathway, L-fucokinase synthesizes from free fucose L-fucose-1-phosphate, which is further converted to GDP-L-fucose by GDP-L-fucose pyrophosphorylase. GDP-L-fucose is translocated from the cytosol to Golgi for fucosylation via the GDP-fucose transporter.

This thesis involved the study of the synthesis of GDP-L-fucose via the salvage pathway: cloning of the murine L-fucokinase and GDP-L-fucose pyrophosphorylase as well as their expression as functional enzymes. Furthermore, the gene expression levels of both enzymes were analyzed in various tissues. Golgi transporters involved in the synthesis of sulfo sLex, *i.e.* CMP-SA, GDP-L-Fuc and PAPS transporters, were investigated in regard to their transcriptional regulation during inflammation. This study also involved the cloning and characterization of rat fucosyltransferase VII, an essential enzyme in the formation of sLex. Moreover, the gene expression of GDP-L-fucose synthesizing enzymes, GDP-fucose transporter and Fuc-TVII was studied in acute inflammation and in cancer cells.

Although the salvage metabolism is the minor pathway of GDP-L-fucose synthesis, L-fucokinase and GDP-L-fucose pyrophosphorylase are expressed at considerably high levels in various tissues. We found the mRNA expression to be highest in brain, ovary, testis, and kidney. Nevertheless, these enzymes were not upregulated in acute inflammation, nor was their expression significant in cancer cells. Hence, our results indicated the salvage pathway of GDP-L-fucose to be irrelevant in tumorigenesis and in inflammation. The gene expression of the enzymes of the *de novo* pathway, GMD

and FX, were upregulated significantly in inflammation as well as in cancer cells leading to increased amount of GDP-L-fucose in cytosol.

The mRNA expression level of the GDP-fucose transporter was elevated in inflamed tissues as well as in cancer cells, which is consistent with the increased cytosolic GDP-L-fucose amount. In addition to GDP-fucose transporter, we detected the transcriptional regulation of CMP-SA and PAPS transporters in acute inflammation. Our results indicate that these transporters have coordinated transcriptional regulation during the induction of the sulfo sLex glycan biosynthesis.

Our results revealed a high gene expression of FUT7 in lymph nodes, which is consistent with the constitutive expression of sulfo sLex glycan epitopes in lymph node high endothelium and hence their important role in lymphocyte trafficking. In non-induced endothelial cells the FUT7 expression was extremely low, but a strong upregulation of FUT7 transcripts was detected in acute inflammation as well as in cancer cells. Our study indicates that there is transcriptional regulation of GDP-L-fucose synthesizing enzymes of the *de novo* pathway, GDP-fucose transporter and FUT7, which correlates to the induced expression of sLex glycans in inflammation and in tumorigenesis.

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