Enzymatic synthesis of known and novel oligosaccharides

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Academic Dissertation

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CONTENTS

ORIGINAL PUBLICATIONS

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

I Jari Natunen, Ritva Niemelä, Leena Penttilä, Antti Seppo, Terhi Ruohtula, and Ossi Renkonen (1994) Enzymatic synthesis of two lacto-N-neohexaose-related Lewis x heptasaccharides and their separation by chromatography on immobilized wheat germ agglutinin.
Glycobiology, **4**, 577-583.[a]

II Jari Natunen, Pinja Parmanne, Jari Helin, Olli Aitio, Marja-Leena Majuri, Ritva Niemelä, Risto Renkonen, and Ossi Renkonen (1999) Biosynthesis of sialylated fucosylated selectin ligands of HL-60 cells *in vitro:* Midchain α3-fucose inhibit terminal α6-sialylation but not α3-sialylation of polylactosamines.
FEBS Lett., **452**, 272-276.[b]

III Jari Natunen, Antti Seppo, Jari Helin, Bruce B. Reinhold, Jarkko Räbinä, Catherine C. Costello, and Ossi Renkonen (1997) Enzymatic transfer of a β1,6-linked N-acetylglucosamine to the α-galactose of globo-N-tetraose: In vitro synthesis of a novel hybrid pentasaccharide of lacto-globo type.
Glycobiology, **7**, 711-718.[a]

IV Jari Natunen, Olli Aitio, Jari Helin, Hannu Maaheimo, Ritva Niemelä, Suvi Toivonen, Minna Ekström, Sami Heikkinen, and Ossi Renkonen (1999) Human α1,3-fucosyltransferases convert N-acetylchito-oligosaccharides into products of GlcNAcβ1-4(Fucα1-3)GlcNAcβ1-OR type.
Submitted for publication.

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ABBREVIATIONS

cIGnT6	"Centrally acting" β1-6-GlcNAc-transferase (EC 2.4.1.-)
Chito-Lex	GlcNAcβ1-4(Fucα1-3)GlcNAc
Cer	Ceramide
dIGnT6	"Peridistally acting" β1-6-GlcNAc-transferase (EC 2.4.1.-)
DQF-COSY	Double quantum filtered correlated spectroscopy
ESI-CID MS	Electrospray ionization - Collision-induced decomposition mass spectrometry
Fuc, F	L-Fucose
Fuc-T	Fucosyltransferase (α1-3/4Fuc-Ts, EC 2.4.1.65; α1-3FucT-s, EC 2.4.1.152)
Fuc-Thm	Partially purified fucosyltransferases of human milk
Fuc-TIII-VII, -TIX, -TX	Recombinant α1-3/4fucosyltransferases III-VII, IX, X
Gal	D-Galactose
GalGb4	Galβ1-3Globoside, Fig.2
Gb4	Globoside, Table 1/Fig. 2
GDP-Fuc	GDP-Fucose
Gg3	Gangliotriosylceramide (asialo-GM2), Fig.2
Glc	D-Glucose
GM3	Sialyl-lactosylceramide, Fig. 2
GlcNAc, GN	N-Acetyl-D-glucosamine
GlcA	D-Glucuronic acid
HEV	High endothelial venules
HPAEC-PAD	High pH anion exchange chromatography - pulsed amperometric detection
HPLC	High performance liquid chromatography
IdoA	L-Idouronic acid
L	Lactose, Galβ1-4Glc
LacdiNAc	N-acetyllactosdiamine, GalNAcβ1-4GlcNAc
LacNAc, LN	N-acetyllactosamine, Galβ1-4GlcNAc
Lea	Lewis a, Galβ1-3(Fucα1-4)GlcNAc
Leb	Lewis b, Fucα1-2Galβ1-3(Fucα1-4)GlcNAc
Lex	Lewis x, Galβ1-4(Fucα1-3)GlcNAc
LexNAc	GalNAcβ1-4(Fucα1-3)GlcNAc
Ley	Lewis y, Fucα1-2Galβ1-4(Fucα1-3)GlcNAc
MALDI-TOF MS	Matrix assisted laser desorption/ionization time-of-flight mass spectrometry
Man	D-Mannose
ManNAc	N-acetyl-D-mannosamine
m/z	Mass to charge ratio
NDV	Newcastle disease virus
nLc4	Lacto-N-neotetraosylceramide, Table 1/Fig 2
NMR	Nuclear magnetic resonance
NeuNAc/Neu5Ac	N-acetyl-D-neuraminic acid
PSGL-1	P-selectin glycoprotein ligand-1
SE	Sulfate ester
sLea	Sialyl-Lewis a, NeuNAcα2-3Galβ1-3(Fucα1-4)GlcNAc
sLex	Sialyl-Lewis x, NeuNAcα2-3Galβ1-4(Fucα1-3)GlcNAc
TOCSY	Total correlation spectroscopy
WGA	Wheat germ agglutinin
ZP	Zona pellucida

Introduction

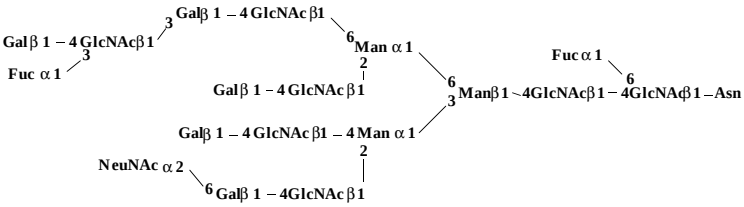
Animal cell surfaces are covered by carbohydrates. These oligosaccharides have several functions for example in inflammation, fertilization and adhesion of bacteria to tissues (Varki, 1993). The progress in understanding these processes has created an increasing interest in studies of saccharide chains and their biological activities. A major obstacle in the isolation of the structures from natural sources has been their heterogeneity. The variation of structures results from the presence of different glycoforms, glycan chains at different levels of elongation and branching, and from the large chemical versatility in the monosaccharide residues. The monosaccharide building blocks can be linked to each other from several hydroxyl groups with the potential of branching and with either α- or β-forms of linkages. The utilization of seven or even more monosaccharides with possible chemical modifications makes the theoretical number of possible structures astronomical even in a short oligosaccharide (Laine, 1994).

In contrast to proteins, glycans are neither directly coded by genes nor readable from them. The actual glycan structures in animal cells are defined by the co-operation of the glycosyltransferase machinery and their cofactors (Van den Eijnden and Joziasse, 1993). Tens of mammalian glycosyltransferases have been cloned. The enzymes previously known for their activities in making specific linkages are increasingly being shown to be actually families of homologous enzymes making the linkage on a specific site or type of possible acceptor glycans (Natsuka et al., 1994; Sears and Wong, 1998; Tsuji, 1996). Glycosyltransferase knock-out studies are revealing highly specific functions of individual glycosyltransferases (Ellies et al., 1998; Lowe, 1998; Maly et al., 1996). Major factors determining glycosylations are the acceptor and donor specificities of the glycosyltransferases. Acceptor specificity may also include protein specificity (Tikkanen et al., 1997; Yeh and Cummings, 1997; Zöllner and Vestweber, 1996), reviewed in (Sears and Wong, 1998). The organization of the glycosyltransferases in membranes (Colley, 1997), their regulation (Ma et al., 1999; Van den Eijnden and Joziasse, 1993) and the availability of acceptor and donor substrates are important as well (Berninsone and Hirschberg, 1998). Defective glycosylations or deglycosylations by glycosidases cause severe and even lethal human diseases (Reuter and Gabius, 1999).

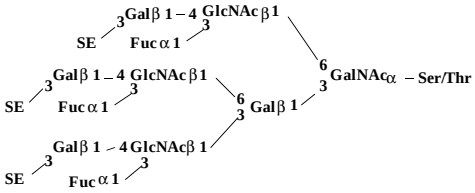
In vitro enzymatic synthesis offers a possibility to study the biosynthesis of glycans and also an effective way to produce model molecules for bioactivity studies. Recently, large scale enzymatic synthesis has made it possible to produce and test glycomedicines for inflammatory disorders (Lowe and Ward, 1997) and bacterial infections (Zopf and Roth, 1996; Zopf et al., 1996).

Table 1. Introduction to glycan structures. SE is sulfate ester.

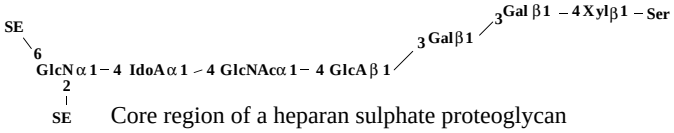
Examples of saccharide chains on animal glycoproteins



A tetraantennary complex type N-glycan

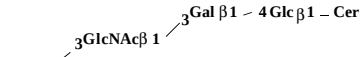


Trivalent 3'sulfo-Lex O-glycan from human colon carcinoma cells, Capon, C. et al. (1997)

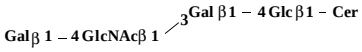


Core region of a heparan sulphate proteoglycan

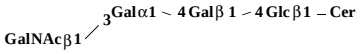
Examples of common oligosaccharide glycolipids of mammals



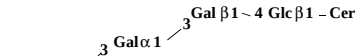
Lacto-series:
lacto-N-tetraosylceramide



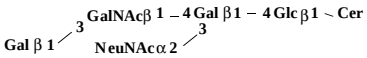
Neolacto-series:
lacto-N-neotetraosylceramide



Globo-series: globoside

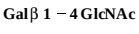


Isoglobo-series: isogloboside

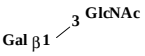


Ganglio-series: GM1

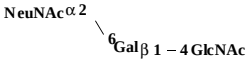
Terminal lactosamine epitopes



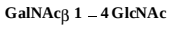
Type 2 N-acetyllactosamine, LN



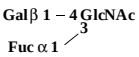
Type 1 N-acetyllactosamine,LNB



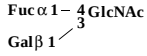
α6-sialyl-LN



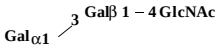
LacdiNAc, LdN



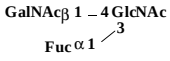
Lewis x, Lex



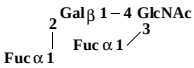
Lewis a, Lea



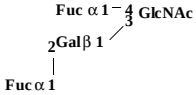
Gal-alfa antigen



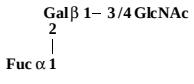
LexNAc



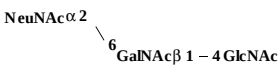
Lewis y, Ley



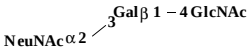
Lewis b, Leb



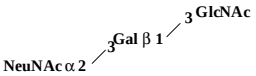
Blood group H



α6-sialyl-LdN



α3-sialyl-LN



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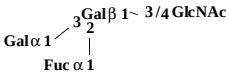
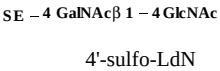
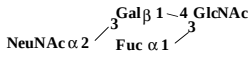


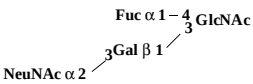
Blood group B



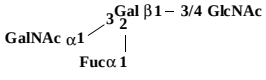
4'-sulfo-LdN



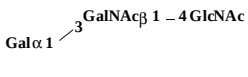
sialyl-Lewis x, sLex



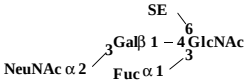
sialyl-Lewis a, sLea



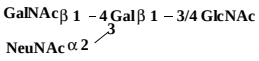
Blood group A



Gal-alfa-LdN



6-sulfo-sLex



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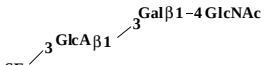

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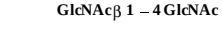
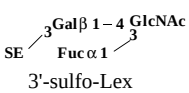
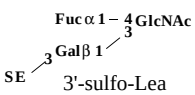


Chitobiose



3'-sulfo-Lex



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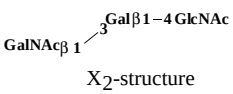
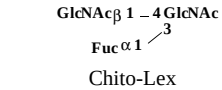


X_2-structure



Chito-Lex

1. Review of the literature

1.1. Structures of animal glycans

1.1.1. The basic types of oligosaccharide chains on animal cells

Most extra cellular and cell surface proteins carry O- and N-linked oligo- or polysaccharide structures. Novel glycosylation variants linked to proteins are reviewed by (Reuter and Gabius, 1999). The common O-glycosidic structures are built on an α-N-acetylgalactosamine or in glycosaminoglycans often on a β-linked xylose, which are linked to either the serine or threonine of the protein (Van den Steen et al., 1998). Various N-glycans are based on the branched pentasaccharide core on asparagine of the protein backbone (Kobata, 1992). The common lipid linked glycosylations include five families of oligosaccharide glycosphingolipids containing the lactosylceramide core: ganglio-, globo-, isoglobo, lacto- and neolactoseries (Hakomori, 1993; Ichikawa and Hirabayashi, 1998) and glycosylphosphoinositol-lipids (GPI-anchors) linking certain proteins to the plasma membrane (Yeh et al., 1994). The glycosaminoglycan hyaluronan forms a class of its own: the large polysaccharide does not have a core protein and it is synthesized on plasma membrane (Weigel et al., 1997). Yet another class of glycans is the free oligosaccharides especially found from human milk (Yamashita et al., 1982) and urine (Parkkinen and Finne, 1983) (Examples of structures are given in Table 1).

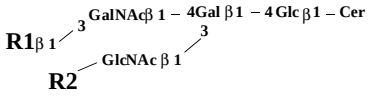
1.1.2. N-acetyllactosaminoglycans

N-acetyllactosamines are a major family of bioactive glycans with a large variability of structures. They consist of disaccharide units, Galβ1-4GlcNAc (type 2) and Galβ1-3GlcNAc (type 1), which can be linked in polylactosamines with β1-3- and/or β1-6-linkages. N-acetyllactosamines are found on the core structures of N-glycans (Krusius et al., 1978), reviewed in (Kobata, 1992), and in many O-glycans (Van den Steen et al., 1998), in lacto(type 1)- and neolacto(type 2)- glycolipids (Hakomori, 1993), in the scaffolds of human milk oligosaccharides (Yamashita et al., 1982). The type 2 disaccharide is the repeating unit of the glycosaminoglycan keratan sulfate (Brown et al., 1996). The lactosamines are often substituted with terminal structures like α2-3- or α2-6-linked sialic acids, ABO-blood group antigens, α1-3-linked galactose (not in humans or old world primates), see Table 1. A homolog of lactosamines, N-acetyllactosdiamine GalNAcβ1-4GlcNAc (LacdiNAc), and its modifications are present as terminal modifications on several glycoproteins (van den Eijnden et al., 1997). In addition to these terminal variations there are different N- and O-glycan core structures, branches in lactosamine chains, subterminal or midchain α1-3/4fucosylations to GlcNAc residues forming the Lewis structures and sulfates that can be terminal (on C3 or C6 of Gal) or midchain (on C6 of Gal or GlcNAc). The nomenclature used for the terminal epitopes is given in Table 1. With Lewis structures the common superscripts are not used, e.g. Lewis x/Lex instead of $Lewis^x$ /Le^x. The terms LexNAc and Chito-Lex are novel names applied here for the first time.

Table 2. Hybrid type glycolipids

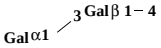
Hybrids of lacto- and ganglio-structures

1. Branched lacto-ganglio hybrids



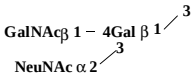
R1=R2= no substituents

or R1= none, R2=



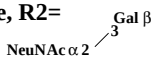
Hybrid glycolipids from murine leukemia cells,
Kannagi, R. et at. (1984)

R1= none or Gal , R2=

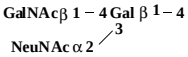


Lacto-ganglio hybrids from bovine brain,
Nakao, T. et al. (1993)

R1= none, R2=

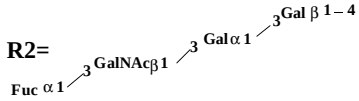


or



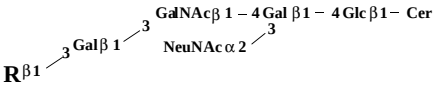
Lacto-ganglio hybrids from the roe of
striped mullet (fish), DeGasperi, R. et at. (1987)

R1= none, R2=

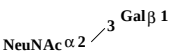


Isoglobo-ganglio-lacto hybrid from the liver of
English sole (fish), Ostrander, G.K. et al. (1988)

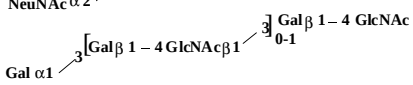
2. Elongated GM1-structures



R=

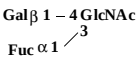


or R=



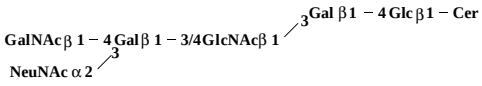
Lactosaminyl-GM1:s from B-lymphocytes
of rat spleen, Nohara, K. et al. (1994)

R=



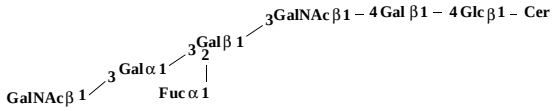
1. Lex-GM1 from chicken intestine:
Hirabayashi, Y. et al. (1991)
Similar structures: Probably in
2. the intestine of guinea pig: Breimer, M.E. et al. (1983)
and in **3.** brains of a human INCL-patient:
Svennerholm, L. et al. (1987)

**3. Cad-epitope, GalNAcβ1-4(NeuNAcα2-3)Gal,
on lacto/neolacto-series**

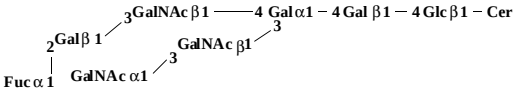


On neolacto-series:
1. A Cad antigen of human erythrocytes
Gillard, B.K. et al. (1988)
2. The fundic gland specific ganglioside of
human stomach, Dohi, T. et al. (1991)
3. A glycolipid from the roe of striped mullet (fish)
DeGasperi, R. et at. (1987)
On lacto-series: A hybrid glycolipid
of human meconium: Fredman, P. et al. (1989)

Hybrids of ganglio- and globo-structures

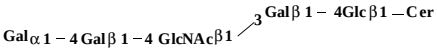


Isoglobo- B-blood group- ganglio hybrid
from rat testis, Teneberg, S.et al. (1994)

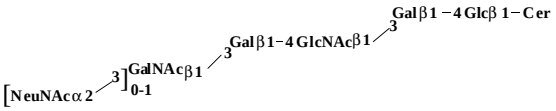


A possible ganglio-globo hybrid from
dog gastric mucosa Slomiany, B.L. .et al. (1978)

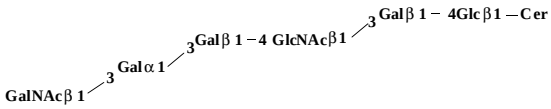
Hybrids of lacto- and globo-structures



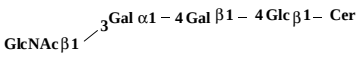
Human P_1-antigen, Naiki, M. et al. (1975)



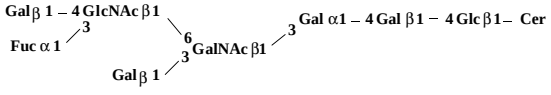
X_2-structures of human erythrocytes,
Thorn, J.J. et al. (1992), Teneberg, S. et al (1996)



Isoglobo-lacto glycolipid from tumors of rat colon
Thurin, J. et al. (1989)



Glycolipid from human meconium, Larson, G. (1986)



Glycolipid from mouse kidney, Sekine, M. et al. (1990),
Lanne, B. et al. (1995), and Osanai, T. et al. (1996)

The free oligosaccharides of milk, especially human milk, contain polylactosamine structures bound to a lactose core and decorated with sialic acid and/or fucose residues (Yamashita et al., 1982). Tens of structures with branched and linear lactosamines ranging from tetrasaccharides to tridecasaccharides have been characterized and MALDI-TOF mass spectrometric studies indicate the presence of hundreds of structures with molecular weights up to 8000, containing more than 40 monosaccharide residues (Stahl et al., 1994). A biological function of these saccharides may be to inhibit the adhesion of pathogenic bacteria (Ashkenazi, 1996).

1.1.3. Hybrid types of glycosylations

Several animal glycan structures have been described which represent mixtures of the commonly found saccharide sequences. In Table 2, 20 hybrid structures between the traditional glycolipid families are summarized. Several of these structures are of human origin and some are found in two or more species. The glycolipids are immunogenic and may cause autoimmune diseases as described for lacto-ganglio hybrids from bovine brain which caused an amyotrophic lateral sclerosis (aml) - like motor neuron disorder in a human patient (Nakao et al., 1993). Some of them may also present differentiation antigens like the branched lacto-ganglio hybrids of murine leukemia cells (Kannagi et al., 1984), the fundic gland specific Cad-glycosylation present only in non-malignant tissue (Dohi et al., 1991), and the isoglobo-lacto glycolipid specific for tumors of the dog colon (Thurin et al., 1989). Part **III** of this thesis deals with the synthesis of a novel branched lacto-globo-structures from globo-N-tetraose. One related glycan structure based on the galactosylgloboside backbone has been described**.** The branched glycolipid of mouse kidney Galβ1-4(Fucα1-3)GlcNAcβ1-6(Galβ1-3)GalNAcβ1-3Galα1-4Galβ1-4Glc-Cer (Lanne et al., 1995; Sekine et al., 1994) binds effectively to E-selectin (Osanai et al., 1996). In addition to these, hybrids of lacto- and globo-series have been reported by (Larson, 1986; Naiki et al., 1975; Teneberg et al., 1996; Thorn et al., 1992), and hybrids of lacto- and ganglio-series by (Breimer et al., 1983; DeGasperi et al., 1987; Fredman et al., 1989; Gillard et al., 1988; Hirabayashi et al., 1991; Nohara et al., 1994; Ostrander et al., 1988; Svennerholm et al., 1987), Table 2. Examples of hybrids of ganglio- and globo-series include a glycolipid from rat testis (Teneberg et al., 1994) and a possible glycolipid from the dog (Slomiany and Slomiany, 1978), Table 2.

1.1.4. α1-3Fucosylated glycans

Fucα1-3 structures of animals are usually parts of Lewis x type glycans in the sequence Galβ1-4(Fucα1-3)GlcNAc (Hakomori, 1992) or in fucosylated LacdiNAc GalNAcβ1-4(Fucα1-3)GlcNAc (Manzella et al., 1996; van den Eijnden et al., 1995). However, glycosylations similar to these bioactive structures (see section 1.4) occur in many types of organisms. In plants and insects an α1-3linked fucose residue has been frequently reported on asparagine linked GlcNAc of core chitobiose in N-linked glycans, in the sequence -GlcNAcβ1-4(Fucα1-3)GlcNAcβ1-Asn. This α1-3fucosylation makes the structure a very potent and cross reactive allergen for humans (Wilson et al., 1998). The parasite *Haemonchus contortus* has both of N-glycan the core GlcNAcs α1-3fucosylated (Haslam et al., 1996). In the case of human fucosidosis the core GlcNAc-Asn of urinary glycopeptides carried some α1-3linked fucose (Yamashita et al., 1979) while NMR-studies of N-glycans of another fucosidosis

patient showed only α1-6linked fucose (Michalski et al., 1991). Most recently *Mesorhizobium loti*, a symbiotic bacteria, was reported to synthesize the non-reducing terminal sequence GlcNAcylβ1-4(Fucα1-3)GlcNAcβ1- on a secreted lipochitooligosaccharide (Olsthoorn et al., 1998). Novel saccharides having terminal Fucα1-3GlcNAc-units have been reported from the zona pellucida of porcine eggs (Mori et al., 1998), and from the urinary N-glycopeptides of a fucosidosis patient (Michalski et al., 1991).

1.2. Biosynthesis of the oligosaccharide chains

1.2.1. Glycosyltransferases

The animal oligosaccharide chains are synthesized by the sequential action of glycosyltransferase enzymes and in the case of N-glycans also glycosidases are involved (Schachter, 1991; Sears and Wong, 1998; Van den Steen et al., 1998). The enzymes are mainly located in the Golgi complex but the biosynthesis of N-glycans begins on the membranes of the endoplasmic reticulum (Sears and Wong, 1998). Glycosyltransferases transfer monosaccharide from a donor nucleotide sugar to a certain hydroxyl group of the acceptor structure (Leloir, 1971). In general the enzyme specifically recognizes the donor and the acceptor making only one type of linkage between them. One enzyme-one linkage is the major rule controlling the biosynthesis of glycans. More than a hundred glycosyltransferases are needed for the synthesis of the known complex saccharides in humans (Drickamer and Taylor, 1998). Some enzymes are able to recognize two or even several similar natural type acceptor structures and perhaps to participate in the synthesis of different kinds of saccharides, for examples in α1-3fucosyl- and β1-6-GlcNAc-transferases, see below. It must be noted that there may be differences between the saccharides acting as acceptors *in vitro* and *in vivo*, and only a very limited amount of data has been obtained under *in vivo* conditions (Grabenhorst et al., 1998).

1.2.2. Biosynthesis of α2-3/6sialylated and/or α1-3fucosylated structures

Synthesis of the terminal epitopes. The biosynthetic pathways to known terminal type 2 lactosamine epitopes having either α2-3-, α2-6-linked sialic acid or α1-3linked fucose, and to sialyl-Lewis x are shown in Figure 1. The α1-3fucosylation of the distal LN unit probably occurs after the α2-3sialylation (Holmes et al., 1986), because no α3-sialylation of terminal Lex-unit has been demonstrated. α2-6-Sialylation and α1-3fucosylation are mutually exclusive: α1-3fucosylation of α2-6sialylated N-acetyllactosamines has not been achieved using by the known fucosyltransferases (de Vries et al., 1997; de Vries et al., 1995; Johnson et al., 1992; Paulson et al., 1978); a possible weak reaction by Fuc-TV was reported by (Wong et al., 1992). Only one family of α2-6sialyltransferases reacting with LacNAc is known, ST6Gal I (Tsuji, 1996), and the α2-6sialylation attempts of terminal Lewis x structures have failed (Paulson et al., 1978).

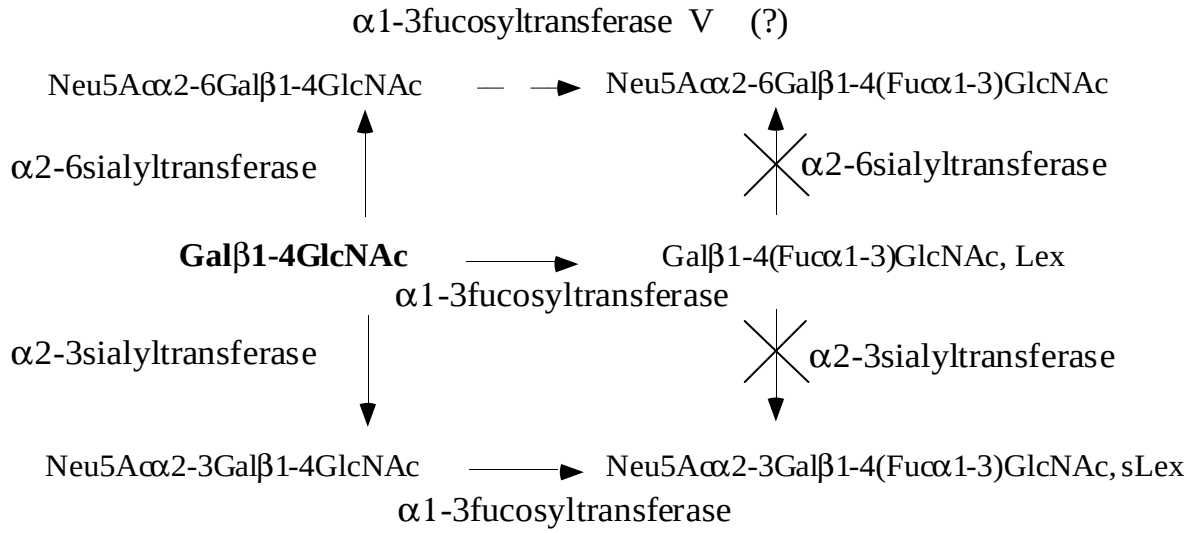


Fig. 1. The known *in vitro* biosynthetic pathways for sialylation and α1-3fucosylation of N-acetyllactosamine. The crossed pathways indicate reactions, which have not been demonstrated *in vitro*, and the dashed arrow indicates a possible weak reaction.

The order of internal fucosylation and terminal α2-3sialylation in polylactosamines. Sialylated and fucosylated polylactosamines are potential selectin ligands. The fucosylation events in their biosynthesis are well characterized. Pre-α3-sialylated linear LacNAc(β1-3LacNAc)$_n$ (i-chains) of polylactosamines can be α3-fucosylated both at distal and internal positions by Fuc-TVII and Fuc-TIV (Britten et al., 1998; de Vries et al., 1995; Easton et al., 1993; Lowe et al., 1991; Niemelä et al., 1998; Sueyoshi et al., 1994), and the major fucosyltransferases of leukocytes (Clarke and Watkins, 1996; Sasaki et al., 1994). However, Fuc-TIV transfers also to all LN units of non-sialylated i-chains of polylactosamines (Niemelä et al., 1998; Sueyoshi et al., 1994), raising the possibility of *internal* fucosylation prior to the sialylation step, either during the elongation of the backbone or right afterwards. The sialylation of non-fucosylated and internally fucosylated polylactosamines was studied in part **II** of this thesis.

α1-3Fucosylation of chito-type oligosaccharides. In plants and insects an α1-3fucosyltransferase activity transfers to the asparagine linked GlcNAc of core chitobiose in N-linked glycans of proteins e.g. to form GlcNAc$_2$Man$_2$-Manβ1-4GlcNAcβ1-4(Fucα1-3)GlcNAcβ1-Asn-protein (Staudacher et al., 1995; Staudacher et al., 1992). The known core Asn-GlcNAc α1-3fucosyltransferases do not transfer to chitobiose, -triose or lactosamines, the acceptor need to have the N-glycan core with an unsubstituted GlcNAcβ1-2Manα1-3-branch (Staudacher et al., 1995). The human Fuc-TVI was recently shown to have weak activities for the synthesis of the saccharides Galβ1-3GlcNAcβ1-4(Fucα1-3)GlcNAcβ1-4GlcNAc and Galβ1-4(Fucα1-3)GlcNAcβ1-4GlcNAcβ1-4(Fucα1-3)GlcNAc from corresponding galactosylated chitooligosaccharides (Nimtz et al., 1998).

1.2.3. Biosynthesis of the hybrid glycolipid structures

The biosynthesis of the elongated GM1 epitopes, Table 2, requires a β1-3GlcNAc-transferase capable of glycosylating the terminal Gal of GM1. Such an enzyme, also transferring to lactosylceramide and neolactotetraosylceramide, has been characterized from developing rat brain (Chou and Jungalwala, 1993). The second type of hybrids have the Cad-structure, GalNAcβ1-4(NeuNAcα2-3)Galβ, on neolacto/lacto glycolipids. β1-4GalNAc-transferase preferring NeuNAcα2-3neolactotetraosyl-ceramide over NeuNAcα2-3lactosylceramide has been described from human kidney and fundic mucosa (Dohi et al., 1991). A β1-4GalNAc-transferase has been described to synthesize the branched lacto-ganglio, GalNAcβ1-4(GlcNAcβ1-3)Galβ1-4Glc-Cer, structures from lactotriosylceramide GlcNAcβ1-3Galβ1-4Glc-Cer (Kannagi et al., 1984).
A β1-6-GlcNAc-transferase of mouse kidney is homologous to the human core 2 β1-6-GlcNAc-transferase. This was found to transfer GlcNAc to the GalNAc of a linear galactosylgloboside and to controll the synthesis of the branched lacto-globoglycolipid of the mouse kidney (Sekine et al., 1994). The enzyme generates branches also to asialo-GM_1 and an O-glycosidic core 1 analog, but globoside, lactotriosylceramide, and an O-glycosidic core 3 analog are not acceptors. All these types of saccharides, excluding globoside, are also *in vitro*-acceptors for the broad specificity β1-6GlcNAc-transferases of hog gastric mucosa and rat intestine (Brockhausen et al., 1986; Gu et al., 1992).

1.2.4. The use of *in vitro* biosynthesis to search for novel glycosylations

Many studies of the acceptor specificities of glycosyltransferases have shown that usually enzymes tolerate modifications of certain hydroxyl groups in the acceptor or donor structures (possibly leading to lower activity) while a few of them are essential key polar groups for recognition (Palcic and Hindsgaul, 1991; Palcic and Hindsgaul, 1996). Most of the studies are accomplished with specific deoxy analogs of acceptor saccharides. There may be differences between the reactivities of the deoxysaccharides and the natural saccharides with glycosylation or other modification on the hydroxyl positions studied (van Dorst et al., 1996). The studies using natural saccharides, which are a little different from the known acceptors of the transferases, include for example galactosylations of glucose disaccharides (Yoon and Laine, 1992) and fucosylation of galactosylated chito-oligosaccharides (Nimtz et al., 1998). An interesting example is α2-6sialylation of the oligosaccharides Manβ1-4GlcNAc and Manβ1-4GlcNAcβ1-4GlcNAc (van Pelt et al., 1989). The sialylated products were later identified in certain mannosidosis patients (van Pelt et al., 1990). In part **III** of this thesis the broad specificity β1-6GlcNAc-transferase of hog gastric microsomes was used to branch globo-tetraose forming a novel biosynthetically possible saccharide sequence. The fucosylation of chito-oligosaccharides by human fucosyltransferases (part **IV**) may reflect an evolutionary conserved side activity of fucosyltransferases. α1-3fucosyltransferases related to human transferases are known even in the bacterial kingdom (Oriol et al., 1999). *Mesorhizobium loti*, a bacter living in symbiosis with leguminous plants, produces an α1-3fucosylation on a chito-type Nod-factor oligosaccharide which is similar to the chito-Lex epitope synthesized in this work (Olsthoorn et al., 1998).

1.3. The glycosyltransferases of this study

1.3.1. Enzyme preparations and reaction conditions

Enzyme preparations. For *in vitro* enzymatic synthesis of pure oligosaccharide structures often very crude sources of enzymatic activities like human serum, preparations of milk or placenta or hog gastric mucosal microsomes are efficient tools. Purification of an enzyme to homogeneity is a demanding task. In the case of very homologous enzymes, like the fucosyltransferases of human milk even $1.8*10^6$-fold purification using modern HPLC-technology did not give absolutely pure enzyme (Eppenberger-Castori et al., 1989). The purification requires a large amount of material and often does not yield enough enzyme activity for preparative synthesis of saccharide samples, so that definitive structural analysis of the product could be achieved. By contrast, the production of recombinant enzymes usually gives significant amounts of pure and specific enzymes. The recombinant technologies also give the possibility for the engineering of the enzymes to create novel specificities (Nguyen et al., 1998; Seto et al., 1997). Pure enzymes or preparations containing a single transferase are especially useful for *in vitro* enzyme assays and enzymatic synthesis.

Enzymatic synthesis and enzyme assays. The aim of preparative enzymatic synthesis is usually to achieve as complete a reaction as possible and produce large amounts of products. In preparative work, the amounts of products of ten nmol or higher allow structural characterization by NMR- and/or mass spectrometric methods like those in articles **I**, **III** and **IV**. For preparative synthesis, a high-activity enzyme source is required and contaminating hydrolytic activities may have to be controlled by inhibitors, like galactonolactone against galactosidases, or ATP against enzymes degrading donor nucleotides (Yates and Watkins, 1983). The nucleotide released from the donor substrate may inhibit the reactions (Weinstein et al., 1982). This can be prevented by using alkaline phosphatase or donor nucleotide recycling in the reaction mixtures (Ichikawa et al., 1992).
The enzymatic assays can be performed with lower amounts of enzymes by using radioactive donor nucleotides. Structural characterization of the products may be possible even with very low amounts of products. For instance, in article **II**, 10-15 picomoles of product saccharides allowed MALDI-mass spectrometry, glycosidase degradation, and chromatographic analysis of the products. To achieve comparable results with different acceptors the reactions are limited to their initial phase so that the consumption of the substrate does not limit the reaction.

1.3.2. α1-3/4Fucosyltransferases

The cloned human α1-3/4fucosyltransferases (α1-3/4fucosyltransferases, EC 2.4.1.65 and α1-3fucosyltransferases, EC 2.4.1.152), have been recently reviewed by (Lowe, 1997; Niemelä, 1999) and human milk α1-3/4fucosyltransferases see (Niemelä, 1999). Five human α1-3fucosyltransferases have been cloned. The mouse homologs of two of these, Fuc-TIV and Fuc-TVII, have been strongly associated with the biosynthesis of selectin ligands by knock-out studies in mice (Lowe, 1998; Maly et al., 1996). The Fuc-TIV fucosylates preferentially non-sialylated polylactosamines at both terminal and mid-chain positions. Fuc-

TVII is highly specific for the terminal Neu5Acα2-3LacNAc-sequence on polylactosamines, non-sialylated lactosamines are very weak acceptors. The complementary action of the two enzymes in the synthesis of sialyl-triLex like selectin ligands is shown in (Niemelä et al., 1998). The other three fucosyltransferases, Fuc-TIII, Fuc-TV and Fuc-TVI are very homologous enzymes coded by a cluster of genes in chromosome 19, but their quite distinct specificities indicate specialized functions. Of disaccharide acceptors the Fuc-TIII strongly prefers Galβ1-3GlcNAc (type 1) while Fuc-TV strongly prefers Galβ1-4GlcNAc (type 2). Fuc-TVI solely uses type 2 acceptors (de Vries et al., 1997; Natsuka and Lowe, 1994). The transferases Fuc-TIII (Lewis enzyme) and Fuc-TVI (plasma enzyme) do not seem to have very crucial functions, because the lack of the enzymes in certain populations does not cause any obvious defects (Mollicone et al., 1994a; Mollicone et al., 1994b). Human Fuc-TVI fucosylates acute phase proteins secreted by the liver, possibly have an immunomodulatory function (Brinkman-Van der Linden et al., 1996). Recently, the sixth human fucosyltransferase was cloned and named, Fuc-TIX (Kaneko et al., 1999). The extremely conserved structures of human Fuc-TIX (Kaneko et al., 1999) and mouse Fuc-TIX (Kudo et al., 1998) and their restricted expression in few tissues except the brain may indicate specific developmental functions.

The activity profile of human milk fucosyltransferases has been shown to be similar to a mixture of Fuc-TIII and Fuc-TVI (de Vries et al., 1997). There is some controversy about the specificity of the Fuc-TIII discussed above. A highly purified preparation of the putative Fuc-TIII (Lewis enzyme) from human milk did not effectively fucosylate the non-reducing terminal lactosamine of Galβ1-4GlcNAcβ1-3Galβ1-4Glc or the LacNAcs of the glycoproteins α1-acid glycoprotein, fetuin, and transferrin in either the native or in asialo-form (Johnson et al., 1992). Similarily, LacNAc was not an acceptor *in vitro* with a recombinant form of the enzyme (de Vries et al., 1995). By contrast other *in vitro* studies with recombinant enzymes and higher acceptor concentrations show reactivity also with LacNAc (Weston et al., 1992), and the enzyme expressed in cells could synthesize Lex and sLex-epitopes on the cell surface (Sueyoshi et al., 1994), and on biantennary N-glycans of secreted β-trace protein (Grabenhorst et al., 1998) under *in vivo* conditions. The results may reflect differences in the enzyme constructs and reaction conditions, or a high specificity of the enzyme for certain of the acceptor lactosamine sites in the acceptor glycans. Possibly also specific recognition of protein is involved, as observed with Fuc-TIV and Fuc-TVII in fucosylation of selectin ligand glycoproteins (Zöllner and Vestweber, 1996). A natural mutant form with a very low activity *in vitro* has been shown by antibodies to produce Lewis a, sialyl-Lewis a and sialyl-Lewis x but, in contrast to non-mutated form, not Lewis x, which could explain some of the controversy (Elmgren et al., 1997). Similar results were also obtained by (Dupuy et al., 1999; Nishihara et al., 1999). The Lewis enzyme, Fuc-TIII, fucosylates effectively the reducing lactose unit of linear human milk oligosaccharides (Johnson et al., 1992), while Fuc-TVI has no or low activity with the lactose residue (de Vries et al., 1997).

Partially purified fucosyltransferases from human milk have been used for *in vitro* synthesis of various oligosaccharides containing Lex (de Vries et al., 1993; Niemelä et al., 1995; Niemelä et al., 1999), VIM-2 (de Vries and van den Eijnden, 1994; Kashem et al., 1993; Räbinä et al.,

1998), sLex (de Vries and van den Eijnden, 1994; Turunen et al., 1995) and sLea structures (Natunen et al., 1997; Palcic et al., 1989).

1.3.3. α2-3Sialyltransferases

The family of α2-3sialyltransferases consists of six enzymes. The corresponding members of the sialyltransferase families are highly homologous between species (Tsuji, 1996). Two of these, ST3Gal I and ST3Gal II utilize Galβ1-3GalNAc acceptor sequences (EC 2.4.99.2/4) on glycolipids (Tsuji, 1996). The transferase ST3Gal V utilizes lactosylceramide as acceptor (EC 2.4.99.9) (Fukumoto et al., 1999). The enzymes ST3Gal III and ST3Gal IV (Kono et al., 1997) and ST3Gal VI (Okajima et al., 1999) effectively tranfer to LacNAc-carrying acceptors (EC 2.4.99.6) (Kono et al., 1997). The ST3Gal III was originally purified from rat liver and cloned on the basis of a partial amino acid sequence obtained from the purified protein by mass spectrometry (Weinstein et al., 1982; Wen et al., 1992). It was considered to be specific for N-glycans because it does not use Galβ1-3GalNAc- on O-glycans of mucin glycoproteins, but the inactivity has not been demonstrated with LacNAcs carried on O-glycan (Weinstein et al., 1982). The enzyme more effectively uses type 1 than type 2 lactosamines on small oligosaccharides (Weinstein et al., 1982). ST3Gal IV (Kitagawa and Paulson, 1994) and VI (Okajima et al., 1999) are specific for type 2 lactosamines. ST3Gal VI has a preference for elongated lacto-N-neohexaosylceramide over lacto-N-neotetraosylceramide (Okajima et al., 1999). The α2-3sialyltransferase activity used in article **II** of this study may be ST3Gal IV and/or ST3Gal VI, which are both expressed in HL-60 cells at least on the RNA level (Kitagawa and Paulson, 1994; Okajima et al., 1999), and the enzyme(s) reacts with type 2 but not with type 1 lactosamines (Chandrasekaran et al., 1995).

ST3Gal IV and VI are also expressed in human placenta (Kitagawa and Paulson, 1994; Okajima et al., 1999), and probably one or both of these correspond to the placental enzymatic activity, which has also been used for *in vitro* synthesis of sialylated polylactosamines (de Vries and van den Eijnden, 1994; Räbinä et al., 1998; Turunen et al., 1995). Purified α2-3sialyltransferase from rat liver (ST3Gal III) have been also used for *in vitro* synthesis e.g. in (Kashem et al., 1993).

1.3.4. β1-6-N-acetylglucosminyltransferases

The β1-6-GlcNAc-transferase activity (EC 2.4.1.-) of hog gastric mucosa has a broad acceptor specificity. It has the peri<u>d</u>istally polylactosamine branching (biosynthesis of I-blood group) activity, <u>d</u>IGNT6, for branching terminal polylactosamine epitope GlcNAcβ1-3**Gal**βR, and "mucin type" O-glycan branching activity transferring to both core 1 (Galβ1-3**GalNAc**αR) (EC 2.4.1.102) and core 3 (GlcNAcβ1-3**GalNAc**αR) (Brockhausen et al., 1986; Piller et al., 1984) saccharides (EC 2.4.1.148) (acceptor residue in bold font). Preparations of hog gastric mucosa has been efficiently used for *in vitro* synthesis of branched polylactosamines (Seppo et al., 1995) and O-glycan type oligosaccharides (Maaheimo et al., 1994). A similar broad specificity β1-6-GlcNAc-transferase has been purified to homogeneity from bovine tracheal epithelium (Ropp et al., 1991) and related enzymes are present in Novikoff ascites tumor cells (Koenderman et al., 1987), and rat intestine (Gu et al., 1992). These enzymes use the penultimate 3-substituted α- or β-Gal(NAc)-units next to the non-reducing end as branching

sites and tolerate both GlcNAc- and Gal-configuration at the terminal residue. A recently cloned human enzyme has also broad specificity with mucin type O-glycan branching activities (Schwientek et al., 1999; Yeh et al., 1999) and it also has the dIGNT6-activity (Yeh et al., 1999). This enzyme has a strong preference for the core 1 over core 3 of O-glycan-type acceptors and has only weak activity for the dIGNT6 type reaction *in vitro*, but transfection of CHO cells with the enzyme leads to the synthesis of I-antigens (branched polylactosamines) on the cell surface (Yeh et al., 1999).

Two other β1-6-GlcNAc-transferases involved in the biosynthesis of lactosamines have been described. The terminally acting enzyme transfers to the C6 of galactose in **Gal**β1-4GlcNAc at the non-reducing end (EC 2.4.1.150) (Van den Eijnden and Joziasse, 1993). The centrally branching enzyme (cIGNT6) requires polylactosamine acceptors like Galβ1-4GlcNAcβ1-3**Gal**β1-4GlcNAc. It was first described from human serum and almost simultaneuosly from various tissues of rat (Gu et al., 1992; Leppänen et al., 1991) (acceptor galactose in bold font). Recently a similar enzymatic activity was described from the human embryonic carcinoma cell line PA-1 (Leppänen et al., 1998). An I-type polylactosamine branching enzyme cloned from these cells (Bierhuizen et al., 1993) was shown to have specific cIGNT6 activity (Mattila et al., 1998). With a different acceptor also some dIGNT6 activity was detected (Yeh et al., 1999). A related enzyme, human leukocyte type β1-6-GlcNAc-tranferase, is specific for the O-glycosidic core 1 acceptors and synthesizes the core 2 structure, Galβ1-3(GlcNAcβ1-6)GalNAcα1-OSer/Thr. The cloned enzyme (Bierhuizen and Fukuda, 1992), is homologous to another enzyme from mouse kidney which transfers GlcNAc-branches to galactosylgloboside (Sekine et al., 1994).

1.4. Biology of Carbohydrates

A novel science, studying the biological functions of carbohydrates, glycobiology, is developing together with molecular biology, biotechnology, and analytical methods. The field is rapidly increasing and extends from chemistry to nutrition, agriculture and clinical medicine. Already in 1993 Ajit Varki had more than 1000 references in his review of the biological roles of glycans (Varki, 1993), today more than 1000 references per year can be found in Medline about a single family of carbohydrate recognizing proteins, the selectins, only. Our knowledge of primary glycan structures is similarily increasing (Sears and Wong, 1996; Van den Steen et al., 1998) and gradually revealing the species-specificities of glycosylations (Kobata, 1992; Manzella et al., 1995). Methods like NMR-spectroscopy to reveal the three dimensional structures of glycans (Petrescu et al., 1997) and their interactions with proteins (Meyer et al., 1997) are another essential part of the picture. Views of evolutionary relations are being shaped by our increasing understanding about glycosylations/glycosyltransferases and the functions of saccharides including ideas about the evolution of the lectin families (Drickamer and Taylor, 1998; Gabius and Romero, 1998). Here, some of the recent developments in understanding the functions of animal carbohydrates are summarized.

1.4.1. Mechanisms of carbohydrate interactions

Protein-Carbohydrate interactions. The most well-known of carbohydrate-mediated interactions involve the specific binding of proteins to their saccharide counterreceptors or ligands. Sugar binding proteins of a non-immune origin are called lectins (Sharon and Lis, 1998). Proteins binding specifically glycosaminoglycans can be considered as a separate group of carbohydrate binding proteins. Enzymes that modify sugar chains, like glycosyltransferases or glycosidases, are generally not considered as lectins, although they can act as lectins like β1-4galactosyltransferase discussed below or some other glycosylation enzymes (Rauvala and Hakomori, 1981; Rauvala et al., 1983). The lectin activity of ricin protein was first found by Hermann Stillmark at Tartu University. This started studies of plant lectins, which later led to the discovery of the binding of human blood group antigens by plant lectins, simultaneously by K.O. Renkonen at the University of Helsinki and W.C. Boyd at Boston University School of Medicine (Sharon and Lis, 1987; Sharon and Lis, 1998). The plant lectins have much use as tools for biochemistry and medicine, but their actual biological roles are still mostly unknown (Rüdiger and Rougé, 1998).
Five major families of animal lectins have been described: C-type, I-type, galectins, pentraxins and P-type lectins, for review see (Gabius, 1997; Gabius and Romero, 1998). Beside the actions of the traditional lectins numerous other protein-carbohydrate interactions have been reported including ones involving glycosaminoglycans, such as heparan sulfates reviewed by (Salmivirta et al., 1996) or hyaluronan (Hardingham and Muir, 1972). Lectin like, catalytically inactive variants of glycosylation enzymes review in (Gabius and Romero, 1998). On the other hand, proteins homologous to lectins may participate in protein-protein interactions (Gabius and Romero, 1998). Some functions of lectins that probably bind saccharides similar to the ones in this study are described in 1.4.2. and 1.4.3.

Carbohydrate-carbohydrate interactions. These interactions are weak in general and require multivalent binding (Spillmann and Burger, 1996). The studies of adhesion between oligosaccharide chains started with observations of cryptic glycolipids which were not available for antibodies or galactose oxidase on the cell surface (Hakomori et al., 1998; Lampio et al., 1986). Most of the glycolipid mediated interactions have been characterized first by Hakomori's group (Hakomori, 1992; Song et al., 1998). These include the Ca^{2+} dependent adhesions between Lewis x (Lex)-epitopes (for structures, see Figure 2) (Boubelik et al., 1998; Eggens et al., 1989; Kumar and Sarkar, 1996), between Ley- and H-antigens (type 1 and 2) (Zhu et al., 1995), and between GM3 and either Gg3, Lactosylceramide, or Gb4 (Kojima and Hakomori, 1991). The interactions of GM3 have been shown to be active even under dynamic flow conditions (Kojima et al., 1992b). The Ca^{2+} dependent Lex-Lex-interaction has also been demonstrated with mass spectrometry (Siuzdak et al., 1993) and recently also by NMR-spectroscopy (Geyer et al., 1999). Ca^{2+}-ions can aggregate Lex-embryoglycan-glycopeptides (Kojima et al., 1994), in which Lewis x epitopes are most probably carried by branched polylactosamines (Kamada et al., 1987; Renkonen, 1983) and a trivalent Lex-lysyllysine-conjugate inhibits the compaction of mouse embryo possibly by disturbing Lex-Lex-interactions (Fenderson et al., 1984; Toyokuni and Hakomori, 1994). The adhesion between Gal-Ceramide and sulfatide (Hakomori et al., 1991) is dependent on divalent cations and glycolipid ceramide compositions (Stewart and Boggs, 1993) and was also

reported from mass spectrometric experiments (Koshy and Boggs, 1996). Recently it was shown that Ca^{2+} independent adhesion between Gb4 and either Lc4Cer, GalGb4 or Lex-epitopes was able to aggregate human embryonal carcinoma cells (Song et al., 1998).

Beside the interactions between glycolipids, carbohydrate-carbohydrate interactions have been reported between polysaccharides. The most traditional example of these is the interactions between glucose chains in cellulose fibres as noted in the review (Spillmann and Burger, 1996). The interaction has also been indicated to be present between cellulose and hemicellulose (Carpita and Gibeaut, 1993) and between the polysaccharides of hemicellulose (Carpita and Gibeaut, 1993), between bacterial (Tzianabos et al., 1992) or plant and bacterial polysaccharides (Cairns et al., 1986), between specific epitopes of polysaccharides from marine sponges (Spillmann and Burger, 1996), and between animal glycosaminoglycans hyaluronic acid and chondroitin sulfate (Turley and Roth, 1980). Adhesions also occur between the saccharides of glycoproteins (Gupta et al., 1994), the saccharides of a glycoprotein and a glycolipid (Endo et al., 1982), and yeast glucan and the glycolipid lactocylceramide (Zimmerman et al., 1998), indicating that numerous types of saccharides can adhere to same or different carbohydrate sequence on various types of carrier structures.

1.4.2. Cell adhesion

Vascular cell adhesions mediated by selectins.

The interactions between the selectin family of calcium dependent (C-type) lectins and various carbohydrates are the most studied biomedical model in glycobiology. In addition to this, there is an increasing number of other vascular protein-carbohydrate interactions, like interactions mediated by non-selectin C-type lectins (Weis et al., 1998). Interestingly the sialic acids (Salmi and Jalkanen, 1996) of the vascular adhesion protein 1 (VAP-1) could participate in specific lymphocyte homing even in peripheral lymph nodes and independently of L-selectin mediated adhesion, which previously was considered to be solely responsible for the adhesion (Salmi et al., 1997).

The three selectin proteins, E-, L-, and P-selectin, bind various glycoconjugates and mediate the primary adherence phase called rolling in the adhesion between leukocytes and the inflamed endothelium of blood vessels, review in (Kansas, 1996; Vestweber and Blanks, 1999). L-selectin participates in the homing of lymphocytes to high endothelial venules (HEV) of peripheral lymph nodes. The structures of the selectin proteins consist of a carboxyterminal C-type carbohydrate recognition domain, an epidermal growth factor like domain, 2-9 complement binding protein like domains (also known as short consensus repeats or Sushi domains), transmembrane and a cytoplasmic domain (Kansas, 1996; Vestweber and Blanks, 1999). Selectins have been shown to bind many glycoproteins *in vitro*. The binding between P-selectin and P-selectin glycoprotein ligand-1 (PSGL-1) of leukocytes is characterized most thoroughly (McEver and Cummings, 1997) and considered to function *in vivo*, too (Varki, 1997b). Further experiments may still be required to reveal the actual roles of the other putative ligands (Varki, 1997b). The interactions require correct glycosylations and other

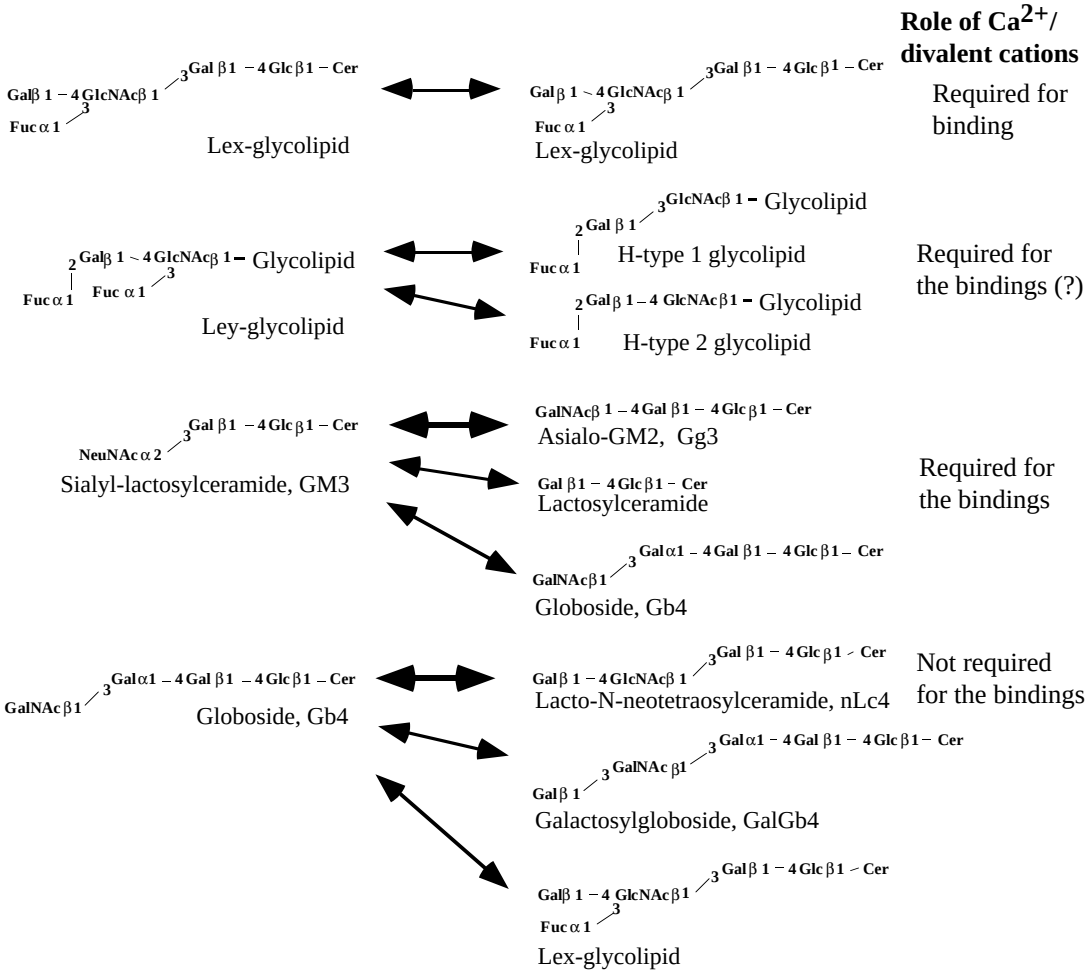
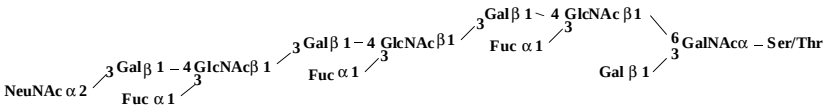
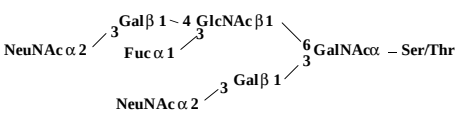


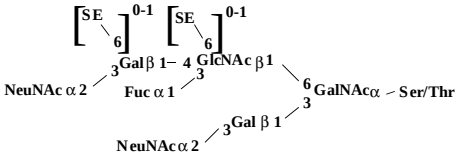
Fig. 2. Examples of carbohydrate-carbohydrate interactions.
For references see 1.4.1. The strongest ones of the related bindings are indicated by bold arrows.



The major fucosylated oligosaccharide of P-selectin glycoprotein ligand-1 from human HL-60 cells, Wilkins, P.P. et al. (1996)



The other fucosylated oligosaccharide of PSGL-1, Wilkins, P.P. et al. (1996)



The sulfo-sLex saccharides of GLYCAM-1 from murine lymph nodes, Hemmerich, S. et al. (1996). Sulfate ester (SE) is on either Gal or GlcNAc or possibly on both.

Fig. 3. The SLex-oligosaccharides sequenced from selectin ligand glycoproteins.

posttranslational modifications, like tyrosine sulfations of PSGL-1, of the glycoprotein ligands (Varki, 1997b; Vestweber and Blanks, 1999).

Potential glycoprotein ligands for L-selectin. Five glycoproteins binding to L-selectin in the lymph nodes have been described: GlyCAM-1 (known from mouse only), CD34, sgp200 and MAdCAM-1, reviewed in (Varki, 1997b; Vestweber and Blanks, 1999) and podocalyxin like glycoprotein (Sassetti et al., 1998). A specific glycoform of endothelial CD34 is suggested to cause the L-selectin ligand activity of CD34 in active lymph nodes and HEV-like venules of inflamed pancreas of nonobese diabetic (NOD) mice (Baumheuter et al., 1994). Adhesion between L-selectin and PNAd (peripheral lymph node addresin, collective name of L-selectin ligands of peripheral lymph nodes)-type material also partially mediates the binding of leukocytes to the inflamed pancreas of NOD mice (Hänninen et al., 1993). CD34 deficient mice did not show any defect in lymphocyte homing but a lower accumulation of eosinophils in their lungs in an allergy model was observed (Suzuki et al., 1996). Most recently podocalyxin like glycoprotein was shown to be a ligand for L-selectin in human lymph nodes (Sassetti et al., 1998). This mucin type protein is widely expressed on luminal surfaces of various vascular endothelia (reviewed in (Sassetti et al., 1998)) and, interestingly, also on platelets and early hematopoietic progenitors (McNagny et al., 1997). A novel type of L-selectin ligand activity was observed on activated endothelial cells (HUVEC) or similar cells transfected by Fuc-TVII, which were used as a model of inflamed vascular endothelium (Tu et al., 1999). The adhesion was inhibitable by the HECA-452 antibody (known to recognize cutaneous lymphocyte antigen, CLA) but not by the MECA-79 antibody (Tu et al., 1999). The MECA-79 antibody recognizes L-selectin ligands in HEV and in a HEV-like inflamed endothelium of inflamed pancreas (Hänninen et al., 1993). The binding depends on sialylation, sulfate, and Fuc-TVII but is not inhibitable by O-sialoglycoprotease (Tu et al., 1999). This ligand is obviously not PSGL-1 or CD34 (Tu et al., 1999).

L-selectin and adhesion between leukocytes. The CD34 of hematopoietic progenitor KG1a cells has been observed to be a low affinity L-selectin ligand (Puri et al., 1995) but according to an another report (and unpublished data discussed in (Puri et al., 1995), CD34 is probably not a major ligand for L-selectin in the cell line (Oxley and Sackstein, 1994). L-selectin also binds to PSGL-1 although with lower affinity than P-selectin, of which the EGF-domain takes part in the high affinity recognition (Tu et al., 1996). According to unpublished data in (Tu et al., 1999), L-selectin - PSGl-1 binding is also mediated by a CLA-antigen (like E-selectin, see below). Human promyelocytic HL-60 cells contain another mucin like L-selectin binding protein besides PSGL-1 (Ramos et al., 1998).

Glycoproteins binding to E-selectin. E-selectin has a specific E-selectin glycoprotein ligand-1 (ESL-I), variant of cysteine rich fibroblast growth factor receptor, on myeloid cells (Steegmaier et al., 1995). Different sets of E-selectin binding proteins are present on human and bovine lymphocytes (Jones et al., 1997). E-selectin also binds cutaneous lymphocyte antigen, a possibly Fuc-TVII dependent glycoform of PSGL-1, which mediates the skin homing of T lymphocytes (Borges et al., 1997; Fuhlbrigge et al., 1997). The physiological role of L-selectin as a sLex presenting ligand for E-selectin has raised some controversy (Jones et al., 1997; Kansas, 1996; Picker et al., 1991; Vestweber and Blanks, 1999). The binding of

E-selectin to the leukocyte integrins CD11/CD18 may activate the integrins (Kotovuori et al., 1993).

Selectins and cancer. The selectins and the corresponding glycosylated ligands also have roles in the spreading of cancer by metastasis (Kim et al., 1998; Ohyama et al., 1999). Recently, specific selectin binding glycoproteins from cancer cells have been reported. In K5 breast cancer cells CD24, a GPI-anchored protein, is a ligand for P- but not L-selectin under flow conditions (Aigner et al., 1998). The human melanoma cells line NKI-4 carries nonsulfated P-selectin ligand glycoproteins of molecular weights 250, 110 and 100 kDa, which were not recognized by anti-PSGL-1 antibodies (Kaytes and Geng, 1998).

The saccharide structures that bind to the selectins. The exact saccharide structures which bind to selectins are not known in most cases. Lactosamine structures with sialic acid and fucose, possibly in sialyl-Lewis x (sLex)-structure, are indicated most commonly, but for example heparan sulfates, HNK-1 antigens or sulfatide may be involved (Varki, 1994). The "prototype" selectin ligand, sLex-sequence, was first described from gangliosides of human kidney (Rauvala, 1976) and it occurs frequently on human glycoproteins and glycolipids indicating that a specific type of sLex or specific saccharide cluster epitope containing sLex-type saccharides may be required for specific adhesions (Varki, 1994). A mouse L-selectin counterreceptor, GlyCAM-1, carries a sLex structure sulfated at position 6 of the galactose or N-acetylglucosamine on the O-glycosidic core 2 and some larger glycans, which could not be accurately analyzed, Figure 3 (Hemmerich et al., 1995). SLex-structures sulfated at position 6 of GlcNAc have been suggested to be L-selectin counterreceptors on human HEV on the basis of the specificities of antibodies (Mitsuoka et al., 1998). The specific expression of the sulfotransferase modifying position 6 of GlcNAc but not Gal has been reported to be present in HEV (Bistrup et al., 1999). In human HL-60 cells PSGL-1 carries two types of core 2 O-glycans, one with sialyltrimeric-Lex (sLexβ1-3Lexβ1-3Lex) and a smaller one with sLex in the GlcNAcβ1-6 branch and sialic acid on Galβ1-3branch (Wilkins et al., 1996), Figure 3. The binding of E-selectin to ESL-1 has been reported to depend on N-linked glycans (Lenter et al., 1994), and several complex N-glycans having a sLex-3Lexβ1-4Man-sequence bind to E-selectin affinity column with micromolar affinities (Patel et al., 1994). Interestingly, a knock-out mouse lacking O-glycosidic core 2-structures has also defects in E-selectin mediated cell adhesion similarily to L- and P-selectin functions (Ellies et al., 1998). Long chain gangliosides of the HL-60 cells with terminal VIM-2-type sequence are also able to bind E-selectin at least *in vitro* under static and flow conditions (Stroud et al., 1996).

Unknown sialylations in selectin ligand saccharides. The glycosylations vary species and tissue specifically (Kobata, 1992), especially on leukocytes (Ito et al., 1994; Thorpe and Feizi, 1984). This may indicate the presence of other types of selectin ligands different from sLex-saccharides. Several reports using specific sialidases have indicated that some of the sialic acids binding selectins is possibly not α2-3-linked in the HL-60 promyelocytic cell line (Corral et al., 1990; Larsen et al., 1992; Lenter et al., 1994) even under flow assay conditions mimicking blood circulation (Kojima et al., 1992a). In one report the L-selectin ligand activity partly depending on PSGL-1 was destroyed by sialidase from *Vibrio cholerae* while P-selectin binding is reduced only by 30% (Ramos et al., 1998). Some anomalous sialylations could be

explained by a cyclic form of sialic acid on 6-sulfo-Lex reported recently. The epitope is formed by a leukocyte enzyme possibly making an amide bond between the amine and carboxylic acid of de-N-acetyl-sialic acid. The structure formed does not bind selectins at least under static conditions (Mitsuoka et al., 1999). P-selectin mediated adhesion has been inhibited by a lectin from *Sambucus nigra* which is specific for α2-6-linked sialic acids suggesting this type of sialylation on the selectin ligand (Larsen et al., 1992). However, above the lectin and the *Trichosanthes japonicum* agglutinin (TJA-1) have been shown to recognize 6'-sulfo-N-acetyllactosamines on the desialylated GlyCAM-1, a L-selectin binding protein of mouse (Hemmerich and Rosen, 1994). Obviously, some of the commonly used HL-60 cells carry quite unusual selectin ligand glycoproteins or glycolipids like the cells containing a specific 150 kD glycoprotein binding P-selectin with N-glycans (Lenter et al., 1994). A variant of HL-60 cells has a low sialylation level and a very low reactivity with anti-sLex antibodies but has high levels of adhesion to E- and P-selectins (Wagers et al., 1998).

Other saccharide epitopes that bind selectins. In leukocyte adhesion deficient (LAD)-patients with a defect in the production of fucosylated glycans the leukocyte adhesion to inflamed endothelium outside of lymph nodes seems to be normal which could indicate the presence of non-fucosylated L-selecting ligands (Karsan et al., 1998). Resting platelets have E- and P-selectin ligand activity which is independent of Fuc-TIV and Fuc-TVII (Frenette et al., 1998). Sulfate is not required for all L-selectin binding proteins, e.g. (Puri et al., 1995; Sackstein et al., 1997) and it is not crucial for all proposed P-selectin ligands either (Kaytes and Geng, 1998). One potential class of non-lactosamine selectin ligands are heparan sulfate proteoglycans of endothelial cells, especially aortic endothelial cells (Giuffrè et al., 1997). Heparan sulfates are possible high affinity ligands for L- (Giuffrè et al., 1997; Norgard-Sumnicht et al., 1993) and P-selectins (Koenig et al., 1998) and unfractionated heparins used for anticoagulation effectively inhibit the binding of the selectins to HL-60 cells (Koenig et al., 1998).

Branched polylactosamines as selectin antagonists. In vitro sLex-sequences carried by branched polylactosamines are among the most effective inhibitors of L-selectin mediated adhesion (Renkonen et al., 1997; Toppila et al., 1997; Toppila et al., 1999; Turunen et al., 1995). The branched polylactosamines may be analogs of some natural selectin ligands: I-antigens (branched polylactosamines) together with sLex have been shown on activated B-lymphocytes and on certain lymphoma cells (Ohmori et al., 1993) and trivalent O-glycosidic 3'sulfosLex has been found on colon cancer mucins (Capon et al., 1997), Table 1. L-selectin molecules cluster on the tips of the microvilli of leukocytes (von Andrian et al., 1995). L-selectin molecules in dimerized form were shown to increase sevenfold the rolling of lymphocytes on vascular endothelium and the dimers were considered to be the activated form of L-selectin present after leukocyte activation (Li et al., 1998). The tetravalent sLex molecules (Renkonen et al., 1997; Toppila et al., 1997; Turunen et al., 1995) may be especially effective inhibitors of dimerized L-selectins as each lectin domain contains a binding site for sLex and another site binding acidic epitopes (Malhotra et al., 1996).

Gamete adhesion in fertilization

There are three major steps in mammalian fertilization before the fusion of the sperm and the egg: 1. Binding of sperm to the egg extracellular coat called the zona pellucida, 2. the acrosome reaction, which is a exocytosis of large vesicle called the acrosome below the plasma membrane in the head of the sperm and 3. penetration of the zona pellucida by the sperm (Wassarman, 1999). Primary adhesion between sperm and the zona pellucida layer of egg of many animals has been shown to depend on carbohydrates with numerous indications of different saccharides and lectins or binding glycosylation enzymes (Töpfer-Petersen and Calvete, 1996). The primary adhesion is dependent on at least two receptor systems with dissociation constants of 50 nM and 0.72 nM, the measurements were made with fixed sperm as the acrosome reaction disturbs assays with live sperm (Thaler and Cardullo, 1996). The O-glycans of porcine zona pellucida contain linear polylactosamines based on the O-glycosidic core 1 with terminal α2-3sialylations or α1-3galactosylations and some smaller glycans with terminal GlcNAcs (Hokke et al., 1994). Interestingly, knocking out of the gene for calmegin, a testis specific ER-protein homologous to calnexin, caused male infertility of homozygous mutant mice by preventing the binding the sperm to the egg. Calmegin was proposed to be a specific chaperone for primary egg receptor(s) on sperm (Ikawa et al., 1997). The evolution of gamete recognition proteins of animals has been reviewed in (Vacquier, 1998).

The binding of mammalian sperm to galactosylated glycans of the zona pellucida. Wassarman and colleagues have shown that an α-galactosylated O-glycan of a major protein in the zona pellucida, ZP3, from mouse with a molecular weight of about 3900 is the primary sperm receptor on eggs (Wassarman, 1999). Recently, Wassarman and colleagues showed by site directed mutagenesis, that two serines in the carboxy terminal serine cluster of ZP3 are necessary for the sperm-egg adhesion of mouse. Interestingly, these putative glycosylation sites are, in contrast to other amino acids of the region, conserved in mouse hamster and human ZP3 (Chen et al., 1998). Oligosaccharides with terminal Galα1-3'LacNAcs carried on branched polylactosamines were shown to be effective micromolar inhibitors of the adhesion, while glycans with terminal lactosamine have a lower activity and glycans with terminal GlcNAc were inactive (Litscher et al., 1995). Recently similar results were obtained with monovalent saccharides, showing also that α1-3fucosylation of the epitopes increased inhibitory activity (Johnston et al., 1998). The α1-3galactosyltransferase is specifically expressed in female but not male germ cells of mouse (Johnston et al., 1995). However, knock-out mice deficient in α1-3galactosyltransferase are fertile (Thall et al., 1995). Binding to β-linked galactoses may be able compensate for the lack of Galα-structures.

Candidates for the primary egg receptor of sperm. Recent studies have revealed some controversies about the most well characterized mammalian sperm-egg binding proteins. A 56 kDa-protein (sp56) from mouse sperm was found by cross-linking to the oligosaccharide domain of ZP3 and could be purified by ZP3 affinity chromatography (Cheng et al., 1994). It has been also reported to bind to galactose- but not GlcNAc-affinity columns (Bleil and Wassarman, 1989). The protein was cloned and showed not to contain any known lectin domains but six "complement binding" repeats or Sushi domains and two unique domains of 44 and 70 amino acids (Bookbinder et al., 1995). sp56 was recently shown to be localized

inside the acrosome and therefore it is not so likely to mediate the primary binding. However, it was suggested to get to the sperm surface through pores in the membrane before or at the beginning of the acrosomal reaction (Foster et al., 1997). One generally accepted theory of mammalian gamete adhesion is that the β1-4galactosyltransferase of sperm binds to GlcNAc containing saccharides in zona pellucida (Miller et al., 1992a). The knocking-out of a gene for β1-4-galactosyltransferase did not affect the fertility of mice (Asano et al., 1997; Lu and Shur, 1997), but the acrosomal reaction and penetration of zona pellucida by the sperm was inhibited (Lu and Shur, 1997). A human sperm surface 95-kD protein Hu9 with a sequence similar to protein tyrosine kinases is still an other possible receptor in the binding of sperm to egg (Burks et al., 1995). It has been argued that Hu9 is a cloning artefact corresponding to the widely expressed human proto-oncogene c-mer (Bork, 1996; Tsai and Silver, 1996) or a really unique sperm-egg receptor (Saling et al., 1996). Zonadhesins are mammalian transmembrane proteins from mammalian sperm, which bind species-specifically to zona pellucidas of eggs (Gao and Garbers, 1998; Hardy and Garbers, 1994; Hardy and Garbers, 1995).

Potential sperm receptors for β-linked galactoses. Several other studies besides the inhibition studies with oligosaccharides (Johnston et al., 1998; Litscher et al., 1995) have implicated β-linked galactoses in fertilization. Adhesion between the mouse sperm and egg has been inhibited by treatment with β- but not α-galactosidase and incubation with asialofetuin coupled beads (Mori et al., 1997). A C-type lectin with homology to hepatic Gal-binding lectin and a molecular weight of 17 kDa has been cloned from rabbit. The protein is present on sperm surface only after the acrosomal reaction has begun. The authors suggest that in rabbit the acrosomal reaction could begin in the cumulus oophurus-layer surrounding the zona pellucida (Richardson et al., 1994). The pig spermadhesins are 12-16 kD proteins found in the seminal plasma and/or peripherally associated with the sperm surface (Töpfer-Petersen et al., 1998). They may participate in adhesion to egg surface, though the amount of the associated proteins is reduced during the maturation of sperm (Töpfer-Petersen and Calvete, 1996; Töpfer-Petersen et al., 1998). The spermadhesins AQN-1 and AQN-3 bind the saccharide sequences Galβ1-3GalNAc and LacNAc on asialoglycoproteins with submicromolar dissociation constants (Calvette et al., 1996a) while the spermadhesin AWN-1 prefers the O-glycan type sequence and sialylated saccharides on glycoproteins (Dostálová et al., 1995). The porcine sperm adhesins bind heparin, too (Calvette et al., 1996b). The spermadhesin protein structures from porcine PSPS-I/PSP-II and bovine aSFP-spermadhesins were determined to contain a single CUB-domain (Romero et al., 1997; Varela et al., 1997). The CUB protein module consists of beta-sheets, so that it is different from the carbohydrate recognition domains of the known lectins (Romero et al., 1997; Varela et al., 1997). It is expressed as a part of numerous developmentally regulated proteins including bone morphogenetic proteins (Bork and Beckmann, 1993). The aSFP bovine spermadhesin was reported to have growth factor like mitogenic activity (Einspanier et al., 1991; Wempe et al., 1992).

"Selectin- like" adhesions between the sperm and egg. A selectin like adhesion involving fucosylated and probably acidic molecules has been suggested by many studies (Oehninger et al., 1998). Using specific antibodies porcine sperm were shown to carry a P-selectin like molecule on the acrosomal membrane and porcine eggs contained a PSGL-1 like protein in zona pellucida (Geng et al., 1997). The adhesion between the sperm and egg by P-selectin and

PSGL-1 occurs after an acrosomal reaction belonging to the later phase of the adhesion cascade (Geng et al., 1997). Glycodelin protein, which has an inhibitory activity for human sperm-egg adhesion, carries specific LacdiNAc-type glycosylations when isolated from amniotic fluid (Dell et al., 1995), but "the male form" of the same protein from seminal plasma has LacNAc-type glycosylations, and no anti-fertilization activity (Morris et al., 1996). Interestingly, the human LacdiNAc molecules in fucosylated form similar to ones present in glycodelin have been shown to bind selectins including P-selectin (Jain et al., 1998). Sialyl-Lewis x is a millimolar inhibitor of binding between human sperm and oocyte (egg) (Clark et al., 1995), but no selectins were observed in human sperm according to data discussed in (Clark et al., 1995; Oehninger et al., 1998). Other reports using antibodies indicate the presence of L-selectin on human sperm (Lucas et al., 1995) and interestingly on human oocytes, too (Campbell et al., 1995). If selectins participate in adhesions they seem not to be indispensable because all the L-, P-, and E- selectin deficient mice are fertile (Arbones et al., 1994; Labow et al., 1994; Mayadas et al., 1993).

Glycosylation defects and glycosyltransferase knock-out mice

Knock-out mice lacking complex and hybrid type N-glycans. The importance of glycosylation in mammalian development was dramatically demonstrated by the fact that knocking out GlcNAc-transferase I blocked the synthesis of complex and hybrid type N-linked glycans (Ioffe and Stanley, 1994; Metzler et al., 1994). The mice died by embryonal day 10.5 with serious defects in the development of the neural tube, vasculature and the left-right asymmetry (Ioffe and Stanley, 1994; Metzler et al., 1994). The mice could survive at least until embryonal day 4.5, through compaction and implantation, by maternal RNA of the transferase (Ioffe et al., 1997). The block in the N-glycosylation pathway also causes the absence of epithelial layer of bronchi in the lungs (Ioffe et al., 1996).

Deficiencies of GPI-anchored proteins. A human disease called paroxymal nocturnal hemoglobinuria is caused by the deficiency of GPI-anchored proteins in a population of blood cells. It leads to the deficiency of GPI-anchored complement inhibitors and hemolysis. The patients lack GPI-proteins because of a somatic mutation in the *PIG-A* gene (Takeda et al., 1993), which codes a putative GlcNAc-transferase required in the beginning of the biosynthesis of GPI-linkers (Watanabe et al., 1998). The knock-out of the corresponding murine gene pig-a is lethal causing multiple developmental defects and end of development at the ninth day of gestation. A partial knock-out with the lack of GPI-anchors only in half of its cells developed until 19 days post coitum with defects in the closure of the neural tube and the cleft palate. The severity of the knock-outs can be understood by the lack of a class of developmentally important cell surface proteins (Nozaki et al., 1999). A skin specific knock-out of *Pig-a* caused severely altered skin and death within a few days after birth. The defect was suggested to be caused by the lack of ceramide and cholesterol in the extracellular space of the skin, when deficiency of GPI-proteins, normally transported together with ceramide, prevents its natural secretion (Tarutani et al., 1997). Milder mutant forms were also generated to study the *Pig-a* knock-outs in blood cells (Rosti et al., 1997) and specifically on T-lymphocytes (Takahama et al., 1998).

Specific and local defects caused by altered glycosyltransferase expression. Several glycosyltransferase knock-outs lead to specific immunologic defects. The deficiencies of mice in fucosyltransferases Fuc-TIV, Fuc-TVII (Lowe, 1998; Maly et al., 1996) and the core 2 β1-6-N-GlcNAc-transferase (Ellies et al., 1998) correlate with defects in selectin ligand saccharides (discussed above) causing defects in the adhesion of leukocytes and cancer cells. The knock-out of the α2-6sialyltransferase ST6Gal I causes reduced serum IgM levels and attenuates the activation of B-lymphocytes. Interestingly the defects are more severe than in the knock-out mice missing the lectin CD22, which is a B-cell receptor for α2-6-sialylated glycans (Hennet et al., 1998). Another sialyltransferase of mouse, ST3Gal I, is essential for maintenance of the cytotoxic T-cell line (Marth, 1998). The function of the transferase sialylating Galβ1-3GalNAc of O-glycans could be related to the transport of O-glycan as discussed in 1.4.3.

The ablation of the gene for the galactosyltransferase making galactosylceramide in mouse leads to demyelination and death by 3 months of age. The defect is a lack of galactosylceramide, galactosyldiglyseride and sulfatide, a sulfated derivative of galactosylceramide (Bosio et al., 1998). The sulfatide and galactosylceramide are necessary for the compact and insulating structure of myelin, the knock-out animals are trying to escape the defect by making more glucose-analogs of galactosylceramice and sulfatide (Bosio et al., 1998). The compact structure may be partially related to carbohydrate-carbohydrate adhesion between the two galactolipids (Koshy and Boggs, 1996), see 1.4.1. Not all glycosyltransferase knock-outs lead to dramatic defects e.g. in the case of β1-4- (Asano et al., 1997; Lu and Shur, 1997) and α1-3- (Thall et al., 1995) galactosyltrasferases, as discussed above. Neuron development seem to be normal in embryonal cells with knock-out of sialyltransferase which makes the glycolipid GD3 previously indicated to be important for neuronal development (Kawai et al., 1998). These could mean the presence of an escape glycosylation pathway making a replacing glycosylation.

The functions of the glycans, like the adhesions of pathogenic bacteria to tissues, may also not be observable in healthy animals. The versatility of natural glycoforms may be partially a consequence of evolution to avoid harmful bindings (Varki, 1997a). Not all of the natural glycosylation defects can be analysed by knock-out technology. A natural mutant mouse has a bleeding disorder similar to human von Willebrand disease with low levels of von Willebrand factor. This was shown to be caused by a remarkable change of expression of GalNAc-transferase from intestinal epithelia to vascular endothelial cells. The enzyme probably synthesizes the Cad/Sd^a-epitope [GalNAcβ1-4(NeuNAcα2-3)Gal-] on von Willebrand factor secreted by the endothelia, and the GalNAc residue causes rapid clearance of the protein by the asialoglycoprotein receptor of the liver (Mohlke et al., 1999).

N-acetyllactosaminoglycans and development

Development and fucosylated glycans. Specific Lewis x like epitopes (SSEA-1, CD15, L5 of the brain (Streit et al., 1996)) may participate in early embryonal differentiation (Gooi et al., 1981), in the development of the brain (Dodd and Jessel, 1986; Oudega et al., 1992) and kidneys (Candelier et al., 1993). A recently cloned fucosyltransferase of mice, Fuc-TIX, is

specifically expressed in the kidney and in hippocampal neurons of the brains. It synthesizes Lex epitopes *in vitro* (Kudo et al., 1998). The embryonal Lex-epitopes may adhere by carbohydrate-carbohydrate interaction as demonstrated by aggregation of embryoglycan glycopeptides with Ca^{2+} (Toyokuni and Hakomori, 1994) and inhibition of the embryonal compaction by trimeric Lex-conjugates (Toyokuni and Hakomori, 1994). Monovalent Lex-trisaccharide has been reported to trimerize fibroblast growth factor 2 (FGF-2) and to have mitogenic effects independent of FGF-2 at micromolar concentrations (Dvorák et al., 1998). Interestingly, knock-out mice, which have a deficiency of the glycoprotein basigin, a carrier of Lex glycans on embryonal cells (Kamada et al., 1987; Miyauchi et al., 1990), show male infertility related to the development of sperm (Igakura et al., 1998), female infertility related to problems in development of oocytes and implantation (Kuno et al., 1998), and defects of sensory and memory functions (Naruhashi et al., 1997). Also, anti Ley-antibodies can also partially inhibit implantation in the mouse (Zhu et al., 1995).

Lactosamines in development. Embryonal carcinoma cells synthesize large polylactosamines on glycoproteins. These are present on mouse F9 cells induced to primitive endoderm, but not on a permanently differentiated parietal endoderm cell line (Spillmann and Finne, 1994). Interestingly, endo-β-galactosidase enzyme, which cleaves polylactosamines, can disturb the development of mouse preimplantation embryo (Rastan et al., 1985). Inhibitors of cell surface β1-4galactosyltransferase also inhibit the compaction of embryo (Shur, 1983) and outgrowth of neurites from PC12 cells on laminin (Begovac and Shur, 1990). Overexpression of β1-4galactosyltransferase in a transgenic animal caused impairment of mammary gland branching morphogenesis and lactation, possibly due to defects in the binding of mammary gland cells to the extracellular matrix (Hathaway and Shur, 1996).

Developmental lectins. The C-terminal part of the lectican proteoglycans contains one or two epidermal growth factor like domains, a C-type lectin domain, and a complement regulatory protein domain (Ruoslahti, 1996). Recently, brevican, a nervous system specific proteoglycan of this family was shown to bind the glycolipid sulfatide and also sulfated HNK-1 epitope on glycolipids but not effectively on glycoproteins (Miura et al., 1999). The lectin domains from lecticans versican (Aspberg et al., 1995; Ujita et al., 1994) and aggrecan (Halberg et al., 1988) have been demonstrated to bind Ca^{2+}-dependently several mono/disaccharides including fucose. In *Drosophila* a protein homologous to human C-type lectins and having ten complement binding repeats similarily to those of P-selectin is important for the development of the eye and mechanosensory bristles (Leshko-Lindsay and Corces, 1997). Galectin-1, recognizing polylactosamines, has been shown to mediate apoptosis of T-cells (Perillo et al., 1995). Amelogenin, a lectin involved in the development of teeth, recognizes terminal GlcNAc-residues with high affinity. The protein has homology to the secondary-GlcNAc binding site of the plant lectin wheat germ agglutinin. The natural ligands of the lectin, and their relation to the potential ligands of the β1-4galactosyltransferase also recognizing terminal GlcNAc discussed above, are not known (Ravindranath et al., 1999). β1-4-Gal-transferase would also be an effective tool to study the possible ligands with terminal GlcNAc.

Bacterial adhesion

Many pathogenic bacteria have been shown to bind saccharides, especially glycolipids, on human cells (Karlsson, 1998). Obviously also many of the beneficial bacteria bind to saccharides of the host tissues. In some cases both the adhesin protein and the target saccharides have been characterized, like with papG-proteins on P-fimbriae of uropathogenic *E. coli* (Korhonen et al., 1982; Strömberg et al., 1990) and the P_N and P_O adhesins of *Streptococcus suis* (Haataja et al., 1994) which bind Galα1-4Gal-sequence present on globoseries glycolipids (Strömberg et al., 1990). Recently an adhesin binding Lewis b-structures was found in *Helicobacter pylori*, the bacteria which causes gastric ulcers and cancer (Ilver et al., 1998). The sialic acid binding specificity of the neutrophil-activating protein of *H. pylori* has been characterized, too, but at least two other receptors for sialylated saccharides are still to be found (Karlsson, 1998; Teneberg et al., 1997). The free polylactosamine oligosaccharides of human milk may act as adhesion inhibitors for pathogenic bacteria. Phase 2 clinical trials to prevent middle ear infection, *otitis media*, and gastric ulcers are on going by the Neose Corporation (Zopf and Roth, 1996; Zopf et al., 1996).

1.4.3. Protein folding and targetting

Protein folding and targetting to lysosomes

In the endoplasmic reticulum the lectins recognizing monoglucosylated N-glycans, calnexin and calreticulin, belong to the machinery that controls the correct folding of proteins (Trombetta and Helenius, 1998). Later, in the Golgi complex, lysosomal proteins can be specifically marked by mannose 6-phosphates (Tikkanen et al., 1997) and routed to lysosomes by specific lectins, mannose 6-phosphate receptors (Kornfeld, 1992).

The rafts

For the correct targeting of proteins and lipids as well as signal transduction on plasma membrane at least some of these are directed in the Golgi to specific domains of the membrane called rafts (Simons and Ikonen, 1997). The rafts are membranes insoluble in detergents like Triton X-100 and enriched with specific glycolipids, cholesterol, GPI-anchored and certain transmembrane proteins together with palmityl-anchored src-family tyrosine kinases enriched on the cytoplasmic site of the raft membrane (Iwabuchi et al., 1998b; Rietveld and Simons, 1998). The cholesterol dependent rafts of GPI-proteins contain 15 or more (Friedrichson and Kurzchalia, 1998) but less than 50 of the GPI-proteins within a diameter of under 70 nm (Varma and Mayor, 1998). The lectin VIP-36 is specifically associated with raft-structures (Fiedler et al., 1994). It binds glycopeptides which can be labelled with radiolabelled galactose but not mannose indicating O-glycans as possible ligands. This binding is inhibitable by N-acetylgalactosamine (Fiedler and Simons, 1995). GalNAcα1-Benzyl, a traditional inhibitor of O-glycosylation has been shown to be galactosylated to form Galβ1-3GalNAcα1-Benzyl and inhibit α2-3-sialylation (Huet et al., 1995), and to stop apical but not basolateral secretion of proteins (Huet et al., 1998). It is not known if the defect is caused by underglycosylation of proteins or by inhibition of VIP-36 or related lectins. N-glycans are able to target soluble proteins to the apical cell surface of the polarized MDCK-cells (Scheiffele et al., 1995). ERCIG-53, a protein with homology with VIP-36 and certain plant lectins (Fiedler and Simons, 1994), may also have a lectin activity and target proteins intracellularily.

Evidence for different types of glycolipid rafts

Recent studies indicate that several raft type domains can exist on cell surfaces (Hakomori et al., 1998). GM3-glycolipid (structures of the glycolipids, Fig.2, Table 1) and src-kinase containing low density membranes, "glycosphingolipid enriched domain - GEM", were separated from caveolin containing low density membranes by anti-GM3 antibodies. The GM3 enriched domain remained active in signaling and in glycolipid-glycolipid adhesion after cholesterol depletion which is known to destroy caveolins (Iwabuchi et al., 1998a). A low density membrane fraction separable from the caveolins contains G-proteins and adenylcyclase in S49 lymphoma cells (Huang et al., 1997), GPI-proteins of several cell types (Mayor et al., 1994; Schnitzer et al., 1995) and the ganglioside glycolipid GM1 of rat lung endothelial plasma membranes (Schnitzer et al., 1995). On human embryonal carcinoma cells the Gb4-glycolipid domains, but not the Gg3-gangliosides, mediated signal transduction even though both of the cell surface glycolipids were active in carbohydrate-carbohydrate interactions (Song et al., 1998). Exogenous radiolabeled glycolipids (GM3, Gb4 and GM1) can be incorporated to GEM-domains of mouse neuroblastoma Neuro2a cells. GM3 but not the control glycolipid lactosylceramide caused neuritogenesis of Neuro2a by phosphorylation of c-Src- and decrease of Csk-signal transduction molecules in GEM (Prinetti et al., 1999). Patching of crosslinked protein/glycolipids indicate several types of microdomains which can aggregate with each other or segregate (Harder et al., 1998).

Direct glycolipid-protein interactions in rafts or more specifically in GEMs / glycolipid signaling domains (GSDs) may also explain in part the specific effects of glycolipids to signaling by cell surface tyrosine kinase receptors, reviewed in (Hakomori et al., 1998). A pioneering study by lectins and anti-carbohydrate antibodies shows that there probably exists carbohydrate-carbohydrate interaction between glycophorin and globoseries glycolipids on cholesterol-phospholipid micelles (Endo et al., 1982), which could be important for the raft/GEM-structures. This may be related to observation of increased membrane fluidity in erythrocyte membranes lacking glycophorin A (Jansson et al., 1981). Sialic acids of glycophorin A have been also shown to stabilize membrane bilayers of phosphaditylethanolamine liposomes indicating even a sugar phospholipid interaction (Pinnaduwage and Huang, 1989).

2. Aims of the study

The specific aims of the thesis were:
1. To study the fucosylations producing the natural type Lex-glycans of human milk (**I**) and the final sialylation steps of *in vitro* biosynthesis of selectin ligand saccharides (**II**).
2. To synthesize novel hybrid type glycans from Globo-N-tetraose by β1-6-GlcNAc-transferase (**III**) and from chitooligosaccharide by human α1-3fucosyltransferases (**IV**).

3. Materials and methods

3.1.Key acceptor and donor saccharides.

3.1.1. Commercial saccharides.

The origin of the commercial saccharides used in part **I** is indicated in parenthesis: Lacto-N-neohexaose, (BioCarb, Lund, Sweden), GDP-[U-^{14}C]fucose, (Amersham International, Buckinghamshire, England) and unlabeled GDP-fucose (Sigma, St. Louis, MO, USA). Saccharides of work **III** include Globo-N-tetraose from Accurate Biochemicals Corp., Westbury, NY, USA GalNAcβ1-3Galα1-OMe, UDP-GlcNAc and UDP-Gal from Sigma, St.Louis, MO, USA. UDP-[6-^{3}H]GlcNAc (30 Ci/mmol) was from NEN / Du Pont, Bad Homburg, Germany. In part **IV** chitobiose was from Sigma and other chito-oligosaccharides were from Seikagaku corp. (Tokyo, Japan) and GDP-fucose was from Sigma or a kind gift from Dr B. Ernst (Universität Basel, Switzerland).

3.1.2. Preparation of acceptor saccharides.

Origins of the saccharides of part **I** have been described in the cited papers: [6-^{3}H]Galβ1-4GlcNAcβ1-3([6-^{3}H]Galβ1-4GlcNAcβ1-6)Galβ1-4Glc (Renkonen et al., 1991a); Galβ1-4GlcNAcβ1-3Galβ1-4[1-^{14}C]Glc and [^{14}C]Galβ1-4GlcNAcβ1-6Galβ1-4Glc (Renkonen et al., 1991c). The acceptor saccharides in part **II:** LNβ1-3'LNβ1-3'LN (Leppänen et al., 1997b) and LNβ1-3'Lexβ1-3'Lex (Räbinä et al., 1998) were described before. The markers SAα2-3'LNβ1-3'LNβ1-3'LN and SAα2-3'LNβ1-3'Lexβ1-3'Lex were also described in (Räbinä et al., 1998). SAα2-6'LNβ1-3'LNβ1-3'LN and SAα2-6'LNβ1-3'Lexβ1-3'Lex markers were synthesized from LNβ1-3'LNβ1-3'LN and LNβ1-3'Lexβ1-3'Lex by using α6-sialyltransferase of rat liver (Boehringer-Mannheim, Germany) and characterized by ^{1}H NMR and MALDI-TOF mass spectrometry, see part **II**.

3.2. Glycosyltransferase reactions

Glycosyltransferase preparations.
Human milk α1-3fucosyltransferase(s) was partially purified by delipidation and anion exchange chromatography according to the two first steps of the procedure described by Eppenberger-Castori et al., 1989 (part **I** and **IV**).
Human HL-60 cells (American Type Culture Collection, Rockville, MD, USA) were cultured in RPMI 1640 medium, (Life Technologies Ltd., Paisley, UK) supplemented with 10% fetal calf serum, 10 mM Hepes, 2 mM L-glutamine and 100 μg/ml gentamycin at 37°C in 5% CO_2. They were subcultured twice a week at a ratio of 1:3. The cells were lysed in the presence of a mixture of protease inhibitors (16 μg/ml benzamidine HCl, 10 μg/ml phenanthroline, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 10 μg/ml pepstatin A, 1 mM PMSF; Pharmingen, San Diego, CA) in 1% Triton X-100 on ice (experiment 1) or sonicated in ice cold water, and then lysed in 1% Triton X-100 on ice (experiment 2). Protein concentrations were determined by BCA Protein Assay Reagent Kit 23225 (Pierce, Rockford, IL) (part **II**). Hog gastric microsomes were prepared as described in (Brockhausen et al., 1983) (part **III**). Recombinant human Fuc-TV and -VI, expressed in *Spodoptera frugiperda*, were from Calbiochem (La Jolla, California, USA) (part **IV**).

Glycosyltransferase reactions.
α1-3Fucosyltransferase reactions (partially purified milk enzyme) were accomplished essentially as in Prieels *et al.*, 1981 (part **I**).
Fucosylation of chitooligosaccharides (**IV**) with human milk fucosyltransferases (EC 2.4.1.152 and EC 2.4.1.65) were carried out essentially as described in (Palcic et al., 1989) but with 2 x 360 μU of enzyme (adding the second portion after 2 days)/ 100 μl of chitosaccharides and the acceptor concentrations were 10 mM for chitobiose and 5 mM for larger saccharides, respectively, with incubation at 37°C for four days. Reactions with fucosyltransferase V (Fuc-TV, EC 2.4.1.152, recombinant, Calbiochem) were carried out under similar conditions but with 12.5 mU of the enzyme/100 μl, reaction mixtures were incubated at room temperature for five days. A vast excess of GDP-fucose was used with both of the enzymes. Fucosyltransferase VI (EC 2.4.1.152, recombinant, Calbiochem) reactions were carried out under the same reaction conditions as fot Fuc-TV except for 2 mM acceptor concentration, to which 10 mU of the enzyme/100 μl, and incubated for 3 days at 37°C. For further details see **IV**.
Sialyltransferase reactions catalyzed by HL-60 cell lysates: The unlabeled acceptors (2 - 20 nmol), the donor CMP-[^{14}C]SA [a mixture of CMP-SA, disodium salt, (Sigma, St. Louis, MO) and CMP-[^{14}C]SA, ammonium salt, Amersham International (Buckinghamshire, UK); 5 nmol, 500 000 dpm] and HL-60 cell lysate (5 μl, 44-50 μg protein) were incubated in a total volume of 10 μl in 50 mM Na-cacodylate pH 6.5 for 60 min at 37°C (part **II**).
β1-6-GlcNAc-transferase (EC 2.4.148) reaction mixtures were analogous to those described by (Piller et al., 1984), containing 55-160 nmol of GalNAcβ1-3Galα1-4Galβ1-4Glc, 400-2000 nmol of UDP-[6-^{3}H]GlcNAc, 50 mM sodium cacodylate (pH 7.0), 8.0 mM NaN_3, 2.0 mM EDTA, 2.5 μg GlcNAc/μl, 2.0 mM ATP and 45 μg/ μl of hog gastric microsome protein, in total volumes of 20-100 μl. The reaction mixtures were incubated for 6 h at 37°C (part **III**).

3.3. Glycosidase reactions

Hydrolysis with jack bean β-galactosidase (EC 3.2.1.23) and jack bean β-N-acetylhexosaminidase (EC 3.2.1.30), (Sigma), were performed as described (Renkonen et al., 1991a) (part **I**). Exhaustive digestions with the jack bean β-N-acetylhexosaminidase (EC 3.2.1.23) were performed at 37 ˚C in a similar manner but for three days, and adding 150 mU of the enzyme in 2.4 µl of 2.5 M $(NH_4)_2SO_4$ pH 7.0 after one and two days (part **III**). Degradations of chitooligosaccharides under mild and exhaustive conditions were performed as described in **IV**. Degradations with *A.ureafaciens* sialidase (EC 3.2.1.18, Boehringer-Mannheim) were performed as previously described (Seppo et al., 1996). Hydrolyses with Newcastle Disease Virus sialidase (EC 3.2.1.18, Oxford Glycosysytems, Abingdon, UK) were carried out by dissolving the dry saccharides (2-5 pmol) in 19 µl 50 mM sodium acetate pH 5.5 and adding 1mU of the enzyme in 1 µl of 10 mM phosphate buffer pH 7.0. The reaction mixtures were incubated overnight at 37°C (Corfield et al., 1986; Drzeniek and Gauche, 1970; Paulson et al., 1982) (part **II**). The oligosaccharide products were separated from the liberated [^{14}C]SA by Superdex chromatography. Defucosylations by almond meal 1 fucosidase (EC 3.2.1.111) were performed as recommended by Oxford Glycosystems (part **I**).

3.4. Chromatographic methods

Desalting was performed with 1.5 ml beds of Dowex AG-50 and Dowex AG-1 (BioRad, Richmond, CA) (parts **I** - **IV**). Descending paper chromatography was performed as described earlier (Renkonen et al., 1989) and in the articles (parts **I** and **III**). WGA-affinity chromatography was performed on columns of agarose-bound WGA as described (Renkonen et al., 1988). Two different columns were used: Column I (0.7x13.5 cm) contained 1.65 mg WGA /ml of 4% beaded agarose, while column II (0.7x15.4 cm) contained 9.6 mg WGA /ml of agarose. Column II was eluted without any addition of N-acetylglucosamine (part **I**). Gel filtration HPLC-runs (parts **I**-**IV**) were performed in columns of Superdex 75 HR 10/30 and Superdex Peptide HR 10/30 (Pharmacia, Uppsala, Sweden) at a flow rate of 1 ml/min with ultrapure water or 50 mM NH_4HCO_3, monitoring UV-absorbance at 205 or 214 nm when necessary. Anion exchange chromatography (part **III**) on a Mono Q (5/5) column (Pharmacia) was performed essentially as in (Maaheimo et al., 1995). High-pH anion exchange chromatography (HPAEC) was performed on a (4x250 mm) Dionex CarboPac PA-1 column (Dionex, Sunnyvale, CA) using a linear gradient of 25-200 mM NaOAc in 100 mM NaOH over 150 min (part **II**) as previously described (Räbinä et al., 1998), and in **IV** the samples were run isocratically with 40 or 60 mM NaOH. The saccharides were detected using pulsed amperometric detection (PAD) and by radioactivity (part **II**) (Maaheimo et al., 1995). Gel filtration chromatographies were performed in Biogel P-2 (BioRad, Richmond, CA) gel column (142 x 1.0 cm) with ultrapure water, monitoring radioactivity as well as UV-absorbance at 205 nm (part **III**) and monitoring UV-absorbance at 214 nm (part **IV**).

3.5. NMR-spectroscopy

1D ^{1}H NMR spectra (parts **I-IV**) were recorded in D_2O (Cambridge Isotope Laboratories (CIL), Woburn, MA, USA, 99.996%) with a Varian UNITY 500 spectrometer at a temperature of 23°C using a modification of the WEFT sequence (Hård et al., 1992). Chemical shifts, expressed in ppm downfield from internal sodium 4,4-dimethyl-4-silapentane-1-sulfonate, were actually measured by reference to internal acetone (δ= 2.225 ppm). In part **I**, the method was used to establish the Lewis x structure synthesized from LacNAc by comparison to published data (Ball et al., 1992; Wormald et al., 1991), details in materials and methods of **I**. Two dimensional NMR-experiments are described in parts **III** and **IV**.

3.6. Mass spectrometry

Matrix assisted laser desorption/ionization - time of flight mass spectrometry
Matrix-assisted laser desorption/ionization (MALDI) mass spectrometry of the GlcNAc-containing globopentasaccharide (**III**) was performed with the Vision 2000 reflectron time-of-flight (TOF) instrument (Finnigan MAT, Hemel Hempstead, UK). MALDI-TOF analysis of the other reaction products (from **II** and **IV**) were performed with a BIFLEX mass spectrometer (Bruker-Franzen Analytik, Bremen, Germany). See corresponding articles for experimental details.

Electrospray ionization tandem mass spectrometry.
The spectra of the novel Globo-pentasaccharide were acquired with a VG/Fisons Quattro II triple quadrupole mass spectrometer system (Micromass, Beverly, MA) fitted with an electrospray ionization source, the technical details are in article **III**. The spectra of the fucosylated chito-oligosaccharides were collected using an API265 triple quadrupole mass spectrometer (Perkin-Elmer instruments, Thornhill, Ontario, Canada) as described in article **IV**.

4. Results

4.1. Enzymatic synthesis and analysis of two Lewis x heptasaccharides (part **I**)

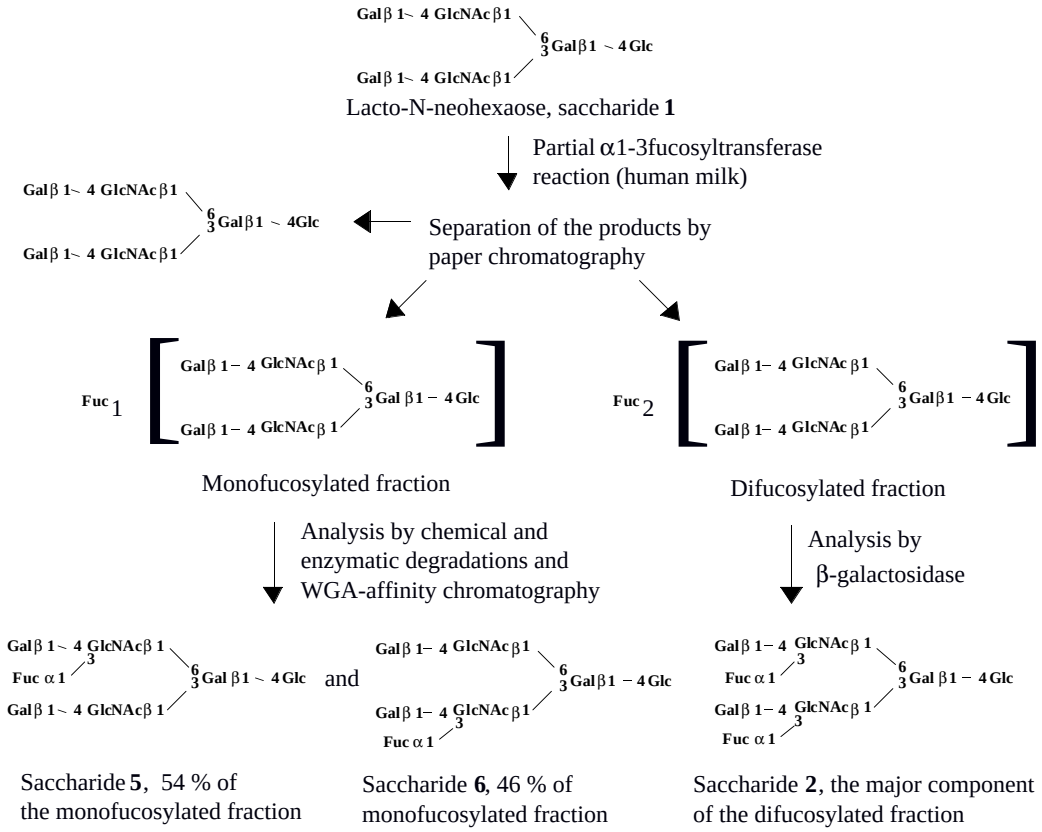
Lacto-N-neohexaose (**1**, numbering as in **I**, see Fig. 4) was partially fucosylated using an α1-3fucosyltransferase preparation from human milk. The acceptor and mono- and difucosylfractions were separated by paper chromatography. The difucosylated product had structure **2** as it resisted β-galactosidase digestion which cleaves terminal lactosamine but not the Lewis x sequence (Kobata, 1979). The fraction corresponding to monofucosylated products (**5** and **6**) was characterized by enzymatic degradation of non-fucosylated branches, defucosylation and recognition of the remaining tetrasaccharides LNβ1-3L and LNβ1-6L by wheat germ agglutinin affinity chromatography. The mixture of **5** and **6** was also first separated by WGA-affinity chromatography, and then analyzed as above. Both methods gave similar results indicating that 54% saccharide **5** and 46% of saccharide **6** were formed. The possible product monofucosyl-saccharide carrying the fucose residue in the reducing glucose **3** was not observed and the purified saccharide **2** did not incorporate fucose to the glucose residue even in a second fucosylation reaction.

4.2. Sialylation of two polylactosamines by lysates of human HL-60 cells (part **II**)

The two acceptors, LNβ1-3LNβ1-3LN and LNβ1-3Lexβ1-3Lex, were incubated with CMP-[^{14}C]SA and lysates human promyelocytic HL-60 cells. The saccharide product was purified by HPLC-chromatographies pooling saccharides with similar elution positions as the corresponding monosialylated marker saccharides. MALDI-TOF mass spectrometry of the products showed the peaks expected for monosialylated saccharides SAα2-3/6LNβ1-3LNβ1-3LN and SAα2-3/6LNβ1-3Lexβ1-3Lex. The products were treated with SAα2-3-specific Newcastle disease virus (NDV) sialidase and the [^{14}C]SA released was quantitated. HPAEC-chromatography of the NDV-sialidase resistant oligosaccharides was used to show that most of the resistant SAα2-xLNβ1-3LNβ1-3LN really had the structure SAα2-6LNβ1-3LNβ1-3LN. The experiments revealed that both of the acceptors were α2-3sialylated equally well but the α2-6sialyltransferase activity used the non-fucosylated polylactosamine more effectively than the internally fucosylated one, Table 1 (part **II**).

4.3. Transfer of GlcNAcβ1-6 to the α-galactose of globo-N-tetraose (**III**)

Globo-N-tetraose (structures and reactions see Fig. 5.) was incubated with UDP-[^{3}H]GlcNAc and hog gastric mucosal microsomes and the radiolabelled product saccharide was purified by gel filtration and paper chromatographies. The MALDI-TOF mass spectrometry showed the *m/z* of 933.5 expected for the mono-GlcNAc product, calculated $[M+Na]^+$ monoisotopic *m/z* 933.3. The saccharide was permethylated and further analyzed by electrospray ionization mass spectrometry. The doubly charged ion at *m/z* 597.2 corresponding to $[M+2Na]^{2+}$ of $(HexNAc)_2(Hex)_3$ was chosen for CID collision induced decay. The fragmentation in CID cause formation of hydroxyl groups at reducing-end side of former linkage positions in the oligosaccharide and a double bond between C1 and the ring oxygen at the non-reducing side,



No saccharides fucosylated to the glucose-residue were observed in mono- or difucosylated fractions.

Fig. 4. Partial α1-3fucosylation of lacto-N-neohexaose by the transferase(s) of human milk (part **I**).

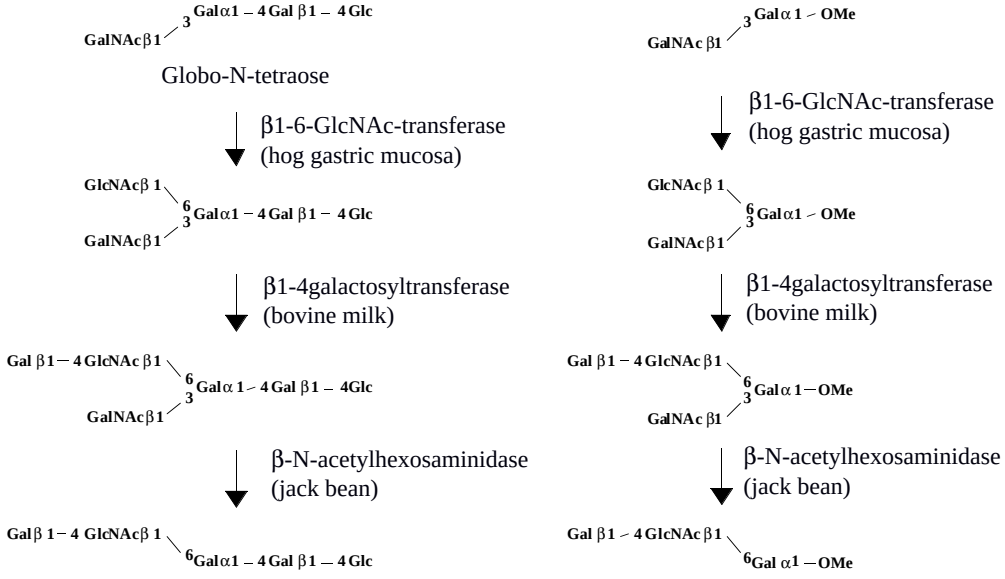


Fig. 5. Enzymatic synthesis of novel hybrid saccharides of lacto-globo-type (part **III**).

when cleavage occurs between the residues. The fragment containing the two hexoses of the reducing end had only one free hydroxyl group indicating that no branching had occurred on these residues. In contrast, the fragment from the reducing end with three hexoses contained two hydroxyls indicated a branch at the third residue. Similar fragments were obtained from the non-reducing end and no fragment containing $(HexNAc)_2$ were obtained. The data established the position of the transferred GlcNAc on the α-linked galactose.
Two-dimensional NMR-techniques, TOCSY and DQF-COSY were used to assign the chemical shifts of the acceptor and the product saccharides. Spectral overlap prevented the assignment of the C5 and C6 shifts, but fortunately the alpha galactose could be assigned in both molecules. The position of the transferred GlcNAc residue was indicated by a strong change in the chemical shift of one of protons on C6 of the α-galactose. Also an intraresidual ROESY cross peak between the signals at the positions of αGalH6 and βGlcNAcH1 indicated the site of the linkage. To get a third independent structural characterization, the terminal GlcNAc was β1-4galactosylated and the GalNAc residue was released by β-N-acetylhexosaminidase. This showed that the GlcNAc residue was not on the GalNAc residue. Similar enzymatic characterization analyzing the products by MALDI mass spectrometry was performed with the β1-6GlcNAc-transferase product of GalNAcβ1-3Galα1-OMe, reactions and structures see Fig. 5.

4.4. α1-3Fucosylation of chitooligosaccharides to chito-Lewis x glycans (**IV**)

In the final part **IV** the fucosyltransferase(s) of human milk and human recombinant fucosyltransferases, Fuc-TV and Fuc-TVI, were used for fucosylation of chitooligosaccharides, Fig. 6A. Novel chito-Lewis x-saccharide structures, GlcNAcβ1-4(Fucα1-3)GlcNAc$(\beta 1\text{-}4GlcNAc)_{0\text{-}4}$ were analyzed by NMR, mass spectrometry and enzymatic degradations. The NMR-studies indicated also that the three-dimensional structures of the glycans were similar to Lewis x: ROE-correlations were observed between the non-reducing terminal GlcNAc H2 and both H5 and H6 of the fucose. Electrospray ionization mass spectrometry was used to show that the fucose residue was linked to the non-reducing subterminal GlcNAc-residue in the novel chitooligosaccharides. Similar contacts have been observed in the known Lewis type structures Gal(NAc)β1-4(Fucα1-3)GlcNAc between the galactopyranose ring and the fucose (Bergwerff et al., 1993; Miller et al., 1992b; Wormald et al., 1991) and more recently in a lipochitin oligosaccharide from the nodulating bacteria *Mesorhizobium loti* (Olsthoorn et al., 1998). The fucosyl branch of the glycan was shown to protect the non-reducing end GlcNAc from exo-N-acetylhexosaminidase from jack beans. Using larger amounts of hexosaminidase the non-reducing GlcNAcs could be cleaved from the chito-Lex epitopes revealing the terminal sequences Fucα1-3GlcNAc-, Fig. 6B. The disaccharide Fucα1-3GlcNAc cleaved from GlcNAcβ1-4(Fucα1-3)GlcNAc was also characterized by one and two dimensional NMR-spectroscopies. Glycans with the terminal α1-3fucose-epitopes are present also in mammals, but the biosynthetic pathways to these are not known. Interestingly, commercial N-acetylhexosaminidase also contained a novel endochitinase activity, which could cleave only one GlcNAc from the reducing end of the saccharide with $GlcNAc_6$-backbone indicating that the fucose residue has a long range protective effect also against endochitinases, Fig. 6C.

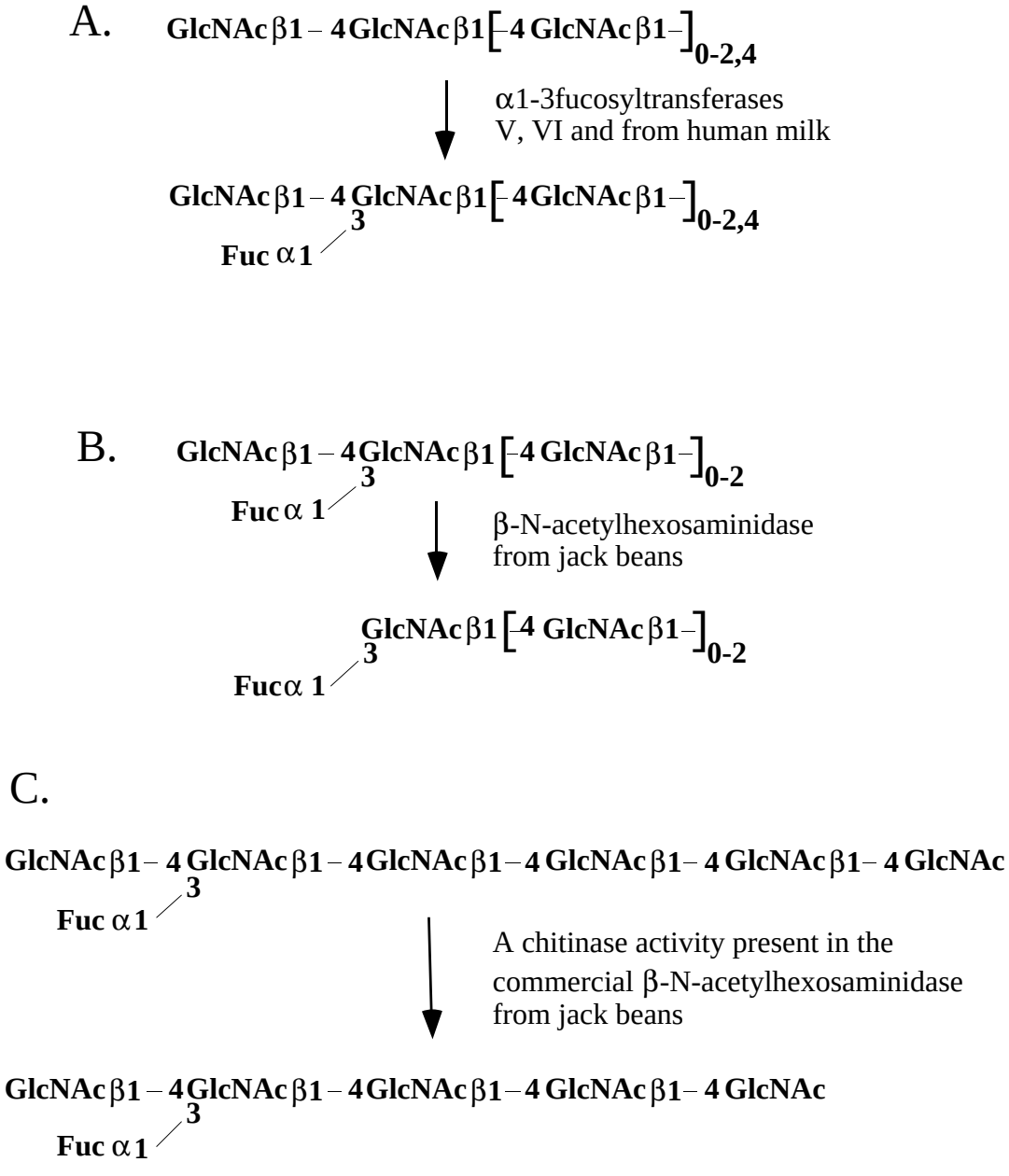


Fig. 6. The synthetic experiments of the part **IV**.

5. Discussion

5.1. Fucosylation of lacto-N-neohexaose (**I**)

We synthesized two monofucosylated Lewis x saccharides (**5** and **6**) and the difucosylated saccharide (**2**) from lacto-N-neohexaose present in human milk by the α1-3fucosyltransferases of human milk (Fuc-Thm). A similar reactivity for both branches of the saccharide LNβ1-3(LNβ1-6)LN has also been observed with Fuc-Thm (Niemelä et al., 1995) and with Fuc-TIV (Niemelä et al., 1998) and in the Fuc-TVII reactions with the α2-3sialylated form of the acceptor (Niemelä et al., 1998). Interestingly, saccharides **2** and **5** have been found in human milk (Bruntz et al., 1988; Yamashita et al., 1982; Yamashita et al., 1976) but saccharide **6** has not been described. Hence, the biosynthesis of **2** and **5** in the mammary gland may involve fucosylation of an acceptor other than lacto-N-neohexaose, perhaps GlcNAcβ1-3(LNβ1-6)L which may be formed by the branch specific action of β1-4galactosyltransferase (Augé et al., 1986; Blanken et al., 1982; Renkonen et al., 1992). Alternatively, branch specific fucosidase activity may be present, or isomer **6** has not been found although it is present in human milk.

We did not observe any fucosylation of the glucose unit in lacto-N-neohexaose which is in accordance with analytical data on human milk saccharides: none of the known fucose-containing oligosaccharides related to lacto-N-neohexaose or lacto-N-hexaose carry fucose at the reducing end glucose (Bruntz et al., 1988; Kitagawa et al., 1991; Strecker et al., 1991; Wang et al., 1992) although several milk saccharides with linear backbones contain fucose linked at the glucose unit (Egge et al., 1983; Thurl et al., 1991; Yamashita et al., 1982). Even the reducing LN in LNβ1-3(LNβ1-6)LN resisted α1-3fucosylation by the milk enzyme(s), but minor amounts the product, LNβ1-3(LNβ1-6)Lex, may be formed (Niemelä et al., 1995). The free 6'-hydroxyl group in Gal of the disaccharide at the reducing-end is most probably needed for recognition by the enzymes (de Vries et al., 1997; de Vries et al., 1995; Gosselin and Palcic, 1996; Maly et al., 1996). Human milk is suggested to contain two of the known human fucosyltransferases, Fuc-TIII and Fuc-TVI (de Vries et al., 1995). The Fuc-TIII is probably the main enzyme responsible for the fucosylation of the reducing lactose units in the linear oligosaccharides of human milk (de Vries et al., 1995; Johnson et al., 1992).

5.2. WGA-affinity chromatography of Lewis x heptasaccharides (**I**)

The present data show that the isomeric monofucosyllacto-N-neohexaoses **5** and **6** can be readily separated by WGA-agarose chromatography. The separation is based on a dramatic loss of WGA affinity associated with α1-3fucosylation of the (1->6)-linked arm of lacto-N-neohexaose. The free hydroxyl at C3 of GlcNAc (blocked by fucose in Lewis x structure) is important for WGA-affinity (Allen et al., 1973) and the oligosaccharides containing GlcNAcβ1-6-structure have especially high affinity (Renkonen et al., 1988; Renkonen et al., 1991d). The data confirm and extend our earlier observations on the separation of alditols of **5** and **6** (Renkonen et al., 1991a). Analogously the isomeric saccharides Lexβ1-3(LNβ1-6)LN and LNβ1-3(Lexβ1-6)LN have been separated under the same conditions, but in this case "the high affinity form" Lexβ1-3(LNβ1-6)LN elutes much faster, between fractions 40-80 (Niemelä et al., 1995), than Lexβ1-3(LNβ1-6)L of this study, which elutes between fractions

120-160 from the same affinity column, Fig. 3, part **I**. The difference is probably caused by interaction of the LNβ1-6-arm of Lexβ1-3(LNβ1-6)LN with the GlcNAc of the reducing end, which weakens the interaction of the high affinity GlcNAcβ1-6 with WGA. This interaction is not possible in Lexβ1-3(LNβ1-6)L which does not have GlcNAc- but Glc-residue at the reducing end. The branched saccharides LNβ1-3(LNβ1-6)L with glucose at the reducing end and LNβ1-3(LNβ1-6)LN with N-acetylglucosamine at the reducing end and their derivatives are different in the properties of the non-reducing terminal residues. This has been indicated by several enzymatic studies and a conformation where β1-6linked GlcNAc is bent to contact with the reducing GlcNAc but not with Glc in the other saccharide has been suggested (Renkonen et al., 1990; Renkonen et al., 1992; Renkonen et al., 1991b). Other examples of "branch specific" affinity of lectins (Paquet et al., 1984) and monoclonal antibodies (Kitagawa et al., 1991; Wang et al., 1992) for isomeric saccharides have been described.

5.3. Biosynthesis of sialylated and multiply fucosylated polylactosamines (**II**)

The HL-60 lysates α3-sialylated similarily both the fucosylated polylactosamine LNβ1-3'Lexβ1-3'Lex and the fucose-free analog LNβ1-3'LNβ1-3'LN, Table 3. This suggests that the α3-sialyltransferase(s) of HL-60 cells recognized mainly the distal LN units, the other parts of the two acceptors being structurally quite different (Wormald et al., 1991). In contrast, ST3Gal III from rat liver may recognize a longer epitope than the disaccharide LN because it shows 1.5-1.9 fold reaction rates for LNβ1-3'LNβ1-OR over LNβ1-3'Lexβ1-OR at the concentrations tested (Kashem et al., 1993). On the RNA-level the HL-60 cells have been shown to express more ST3Gal IV than ST3Gal III by northern blotting (Kitagawa and Paulson, 1994). A novel sialyltransferase specific for type 2 lactosamines, ST3Gal VI, is also present in the cell line (Okajima et al., 1999). Put together, these data suggest that the enzyme(s) responsible for the α3-sialylations in HL-60 cell lysates is either ST3Gal IV or ST3Gal VI or both of them. The data also suggest that α3-fucosylation of polylactosamine backbones at the peridistal LN unit, intrinsically, will inhibit α6-sialylation. α6-Sialylation of unfucosylated long chain polylactosamine ceramides in HL-60 cells *in vivo* is curiously impaired despite the expression of high amounts of α6-sialyltransferase activity capable of reacting with polylactosamines.

The present data complete two distinct sets of *in vitro* biosynthetic pathways leading from polylactosamine backbones to the selectin ligands SAα2-3'LNβ1-3'Lexβ1-3'Lex and SAα2-3'Lexβ1-3'Lexβ1-3'Lex in HL-60 cells. Successful α3-sialylation of LN-β1-3'LNβ1-3'LN, together with the previously reported data on fucosylation of SAα2-3'LN-β1-3'LNβ1-3'LN (Niemelä et al., 1998) rounds off the set of Pathways A of Fig. 3 (**II**). On the other hand, successive Fuc-TIV reactions (Niemelä et al., 1998) lead from the completed polylactosamine backbone to the internally fucosylated LNβ1-3'Lexβ1-3'Lex, and the present data on the successful α3-sialylation of this intermediate complete the set of pathways B of Fig. 7. The internal fucosylations may also take place during the elongation of the backbone as described in the generation of LNβ1-3'Lex (Kashem et al., 1993) and LNβ1-3'Lexβ1-3'Lex determinants *in vitro* (Räbinä et al., 1998). Similar reactions would be possible also with Fuc-TIV of HL-60 cell lysates.

5.4. Enzymatic synthesis of novel lacto-globo hybrid saccharides (**III**)

Novel hybrid type glycan was synthesized by transfer of β1-6-linked GlcNAc to Galα of GalNAcβ1-3Galα1-OMe and GalNAcβ1-3Galα1-4Galβ1-4Glc forming novel hybrid type saccharide sequences. Only the terminal disaccharide of globo-N-tetraose is probably required for substrate recognition by the β1-6-GlcNAc-tranferase, because the GalNAcβ1-3Galα1-OMe is also a good acceptor for the enzyme. In the hog gastric mucosa the β1-6-GlcNAc-transferase activity of broad acceptor specificity is able to glycosylate disaccharide motives GlcNAcβ1-3GalβR, Galβ1-3GalNAcαR and GlcNAcβ1-3GalNAcαR (Brockhausen et al., 1986; Piller et al., 1984). A similar broad specificity β1-6-GlcNAc-transferase has been purified to homogeneity from bovine tracheal epithelium (Ropp et al., 1991) and related enzymes are present in Novikoff ascites tumor cells (Koenderman et al., 1987), and in rat intestine (Gu et al., 1992). Members of this group of enzymes use the penultimate 3-substituted α- or β-Gal(NAc)-units close to the non-reducing end as branching sites and tolerate both the GlcNAc- and Gal-configuration at the terminal residue. The present report adds GalNAcβ1-3GalαR to the list of acceptors for the branching reaction; all structural features introduced at the C_2,C_4/C_1,C_2 of two distal monosaccharides in the novel acceptor are familiar from the acceptors identified by previous work.

Although globoside is a common glycolipid in various animal tissues, the glycolipid based on the GlcNAc-containing globo-pentasaccharide described here has not been reported. We assume that the gastric mucosal enzyme may be capable of transferring a GlcNAc-branch even to the lipid-bound globotetraose *in vitro*, because it transfers efficiently to free as well as lipid-bound trisaccharide GlcNAcβ1-3Galβ1-4Glc (Koenderman et al., 1987; Piller et al., 1984). An actual demonstration of the presence of the lacto-globosaccharides as minor components on cell surfaces may have to wait for the production of specific monoclonal antibodies against the synthetic saccharides, an approach successfully applied in several similar cases (Bouchon et al., 1992; Ding et al., 1992; Shigeta et al., 1987). The β1-4-galactosylated form of the branched globoside carries blood group I- and P-antigenic determinants and may represent an antigen recognized by the rare anti-IP-blood group antibody (Marcus et al., 1981; Ramos et al., 1994). Like the lacto-gangliohybrid (Kannagi et al., 1984), the novel lacto-globohybrids could also represent differentiation antigens, reflecting developmentally regulated shifts between glycolipid families on the cell surface (Gillard et al., 1990; Kannagi et al., 1983).

5.5. α1-3fucosylation of chitooligosaccharides (**IV**)

In the present report recombinant human fucosyltransferases, Fuc-TV and Fuc-TVI, and partially purified human milk Fuc-Ts were used to α3-fucosylate chito-oligosaccharides in a site specific way, generating products of GlcNAcβ1-4(Fucα1-3)GlcNAcβ1-OR type, Fig. 6A. The products were characterized by enzymatic degradations, MALDI-TOF-MS, ESI-MS and a variety of NMR-experiments. The NMR-data suggest that the fucose and the distal GlcNAc are stacked in a way reminiscent of the stacking of the fucose and the distal galactose in the Lewis x determinant, Galβ1-4(Fucα1-3)GlcNAc (Wormald et al., 1991), and the fucose and the distal GalNAc in the fucosylated LacdiNAc epitope (Bergwerff et al., 1993). NMR-data indicating a similar 3D structure has been reported for a bacterial Nod-factor oligosaccharide

with terminal 3'carbomoyl-GlcNAcylβ1-4(Fucα1-3)GlcNAcβ1- from *Mezorhizobium loti* (Olsthoorn et al., 1998) and for a α1-3fucosylated N-glycan core from pineapple stem bromelain (Bouwstra et al., 1990).

The reaction of chitobiose and GDP-Fuc, catalyzed by Fuc-TVI proceeded in the present experiments to near completion under conditions not too different from those required for complete fucosylation of N-acetyllactosamine in our laboratory. Twenty years ago Hoflack et al reported that chitobiose is fucosylated by a cell surface transferase of human lymphocytes, but had no activity towards endogenous acceptors on the surface (Hoflack et al., 1978); the enzyme was probably an α1-3fucosyltransferase because the known α1-6fucosyltransferases do not react with chitobiose (Voynow et al., 1991). The natural functions of the chito-Lex glycans in animals could be similar to the secreted Lex-like saccharides of the parasite *Schistosoma mansoni*, which induce B-lymphocytes to produce interleukin 10 (Velupillai and Harn, 1994), if such chito-Lex saccharides are produced by pathogens or chitosaccharides originating from pathogens can be fucosylated in animals *in vivo*. Chito-Lex- epitopes are possibly not natural non-pathogenic glycosylations of humans, at least the α1-3fucosylated N-glycan core is a very potent and cross reactive human allergen (Wilson et al., 1998). Fucosylation of some chito-type saccharides by human Fuc-TVI (Nimtz et al., 1998) and fucosylation of chitobiose 6-sulfate (product not characterized) (Tran et al., 1998) have also been reported.

Under exhaustive conditions the fucosylated chito-oligosaccharides ranging from chitobiose to chitotetraose were cleaved by jack bean β-N-acetylhexosaminidase to compounds of the type Fucα1-3GlcNAcβ1-OR, Fig. 6B. Even the fucosylated LacdiNAc sequence, GalNAcβ1-4(Fucα1-3)GlcNAc, has been cleaved by a N-acetylhexosaminidase treatment (Srivatsan et al., 1992), but the Lewis x epitope Galβ1-4(Fucα1-3)GlcNAc resists the actions of all known β-galactosidases (Arakawa et al., 1974; Kobata, 1979). Terminal Fucα1-3GlcNAc units have been reported *e.g.* in urinary N-glycopeptides from a fucosidosis patient (Michalski et al., 1991) and in the zona pellucida of porcine eggs (Mori et al., 1998). As the known α1-3FucTs do not transfer to terminal GlcNAc units, but require the 6'hydroxyl of the terminal monosaccharide for reaction (de Vries et al., 1997; de Vries et al., 1995; Maly et al., 1996; Niemelä et al., 1998), it appears possible that the terminal Fucα1-3GlcNAc groups expressed in nature may be formed by degradation from fucosylated LacdiNAc sequences or from fucosylated forms of terminal chito-oligosaccharides (Bakker et al., 1994; Haslam et al., 1999). The terminal Fucα1-glycosylations when present in oligomeric forms on cell surfaces could probably be pathologic glycosylations in human as they are recognized by mannose binding protein and the mannose receptors of macrophages (Weis et al., 1998).

In the present experiments, the commercial jack bean β-N-acetylhexosaminidase, applied under mild conditions, released from Glycan **6** only the *reducing* end GlcNAc, Fig. 6C. Plant endo-chitinases are known to belong to glycosidase families 18 and 19. One representative protein structure from both families has been resolved. The hevamine of the rubber tree belongs to family 18, having an $(\alpha/\beta)_8$ barrel structure (Terwisscha van Scheltiga et al., 1994), whereas the barley chitinase of family 19 has a completely different, lysozyme-like structure (Hart et al., 1995). Despite the large structural differencies, all known endo-chitinases of plants are

believed to bind six monosaccharides of the substrate at sites A-F, the cleavage occurring between sites D and E (Hart et al., 1995; Terwisscha van Scheltiga et al., 1994). The notion that an enzyme with an extended active site is responsible for the observed conversion of Glycan **6** to Glycan **5** is supported by the long range protective effect of the fucose unit, extending over three GlcNAc units Glycan **5**. Several examples are known among polylactosamines, where the α1-3-bonded fucose residue restricts the actions of degrading and synthesizing enzymes as well as binding of lectins in a site-specific manner (Kannagi et al., 1982; Leppänen et al., 1997a; Niemelä et al., 1999; Spooncer et al., 1984). Indeed, the fucosylated chito-oligosaccharides synthesized in the present experiments may turn out to be probes of general interest in specificity studies of endo-chitinases of plants.

Potential conservations of glycosyltransferase activities with no longer obvious functions have been observed in the effect of bovine α-lactalbumin to β1-4GalNAc-transferase of snail *Lymnaea stagnalis* (Neeleman and van den Eijden, 1996) and by fucosylation of eukaryotic N-glycan core and Lewis x antigen by chitooligosaccharide α1-6Fuc-T of nodulating bacterium (Quinto et al., 1997). The sequence homologies of the known α1-3fucosyltransferases from bacteria, invertebrates, and mammals indicate divergent evolution from common ancestor in bacteria 2 billion years ago (Oriol et al., 1999). The acceptor of the ancestor enzyme has been suggested to have been a chitobiose unit similar to that used by present N-glycan core α1-3Fuc-Ts of plants and insects (Oriol et al., 1999). The theory was further supported by the cloning of the N-glycan core α1-3Fuc-T from the plant mung bean, which revealed a suprisingly high regional homology to the other α1-3Fuc-Ts including the human Fuc-Ts (Leiter et al., 1999). Here, we observe that human N-acetyllactosamine fucosyltransferases fucosylate GlcNAcβ1-4GlcNAc-oligomers to a non-reducing subterminal residue, which may represent an evolutionary conserved glycosyltransferase activity.

5.6. Concluding remarks

Glycosyltransferases of animal cells belong to families enzymes as discussed in 1.3.2. -1.3.4. Differences of only a few amino acids can lead to quite different donor and acceptor specificities for the enzymes, giving strict specificities towards only single type of substrate and also broader specificities recognizing several acceptors. This is exemplified by the preferential recognition of type 1 lactosamines by Fuc-TIII and type 2 acceptors by Fuc-TVI while Fuc-TV has a more dual specificity (de Vries et al., 1997) or by the use of the donor UDP-GalNAc by the blood group A and UDP-Gal by the blood group B-transferases and both by the AB-form of the transferase (Seto et al., 1997). There are also common epitopes for recognition of acceptor and donor substrates or linkage structures (Zhou et al., 1999) which may be used in different families of the enzymes. The most wide variety of substrates, strictly and broadly, is recognized by the family of β1-6GlcNAc-transferases (chapter 1.3.4.). Novel enzymes of this type and their specificities have been recently described (Bierhuizen and Fukuda, 1992; Leppänen et al., 1991; Leppänen et al., 1998; Mattila et al., 1998; Sakamoto et al., 1998; Yeh et al., 1999). The presence of families of glycosyltransferases with possibly overlapping functions has allowed effective changes in glycosylations during evolution leading to tissue- and species-specific glycosylations (Kobata, 1992). One reason for the changes is

obviously the evolutionary pressure caused by the adhesions of pathogenic bacteria (Varki, 1997a).

The presence of a few hundred glycosyltransferases in animals obviously limit the otherwise huge versatility of possible animal glycosylations (Laine, 1994), though the flexibilities of the enzymatic activities, branching, poly/oligomeric structures and combinations of the epitopes increases the amount of potential structural information. This information is essential for the understanding of the biological processes in which animal glycans participate. The studies of *in vitro* biosynthesis, like those in parts **I** and **II**, are, together with direct isolation and sequencing of carbohydrates, the major tools which can be used to reveal the structures of glycans. In some cases the stuctural switches of substrate recognition by the glycosyltransferases seem to turn quite quickly during evolution. The hybrid types (see chapter 1.2.3.) and other variations in glycolipid structures in animals seem to be one modest case. The most extreme case is the exopolysaccharides of bacteria, which are at least slightly different practically in every bacterial strain, sometimes even with molecular mimicry of human glycans (Aspinall, 1998; Jennings, 1998; Jones, 1998). Another example of the structural diversity is the strikingly different species-specific saccharide sequences on the jelly layers surrounding the eggs of frogs (Morelle et al., 1998; Morelle and Strecker, 1997; Morelle and Strecker, 1998) and sea urchins (Alves et al., 1997). These saccharides are possibly required for recognition between species when the eggs are laid in the water. The examples show the potential of the glycosylation machinery in creating variable glycocodes of life. The last two parts of the study (**III** and **IV**) show that stretching the specificities of glycosyltransferases with new acceptors can give new information about the potential of the enzymes and give independent clues about glycosylations which could be found. When the work of the part **IV** was initiated neither the bacterial nodulation factor oligosaccharide (Olsthoorn et al., 1998) related to the fucosylation products nor the evolutionary relationship between the α1-3fucosyltransferases (Leiter et al., 1999; Oriol et al., 1999) were known.

6. Summary

All types of animal cells carry large amounts of oligosaccharide chains on their surfaces. The saccharides are conjugated to lipids and proteins and have numerous functions in adhesion and communication between cells. The interactions of the glycans have important roles e. g. in circulation of leukocytes and inflammation, and involving fertilization, cancer, development and, host-microbe interactions. In addition, the glycans are of importance in protein folding, targetting, and probably in organizing membranes to "raft" structures. The glycan structures are synthesized by the glycosyltransferase enzymes mainly in the Golgi apparatus of the cell. In the present study three types of glycosyltransferases were studied to reveal biosynthetic pathways to known bioactive oligosaccharides (**I** and **II**), and to find out novel glycans possibly synthesized by the glycosylation machinery (**III** and **IV**).

In part **I** the α1-3fucosylation of a human milk oligosaccharide, lacto-N-neohexaose LNβ1-3(LNβ1-6)L (LN is N-acetyllactosamine, L is lactose), was studied by partially purified fucosyltransferase(s) of human milk. The saccharide products were characterized by enzymatic degradations and affinity chromatography on the lectin wheat germ agglutinin, which can separate isomeric polylactosamines most effectively. The analysis revealed the two expected monofucosylated products Lexβ1-3(LNβ1-6)L and LNβ1-3(Lexβ1-6)L (Lex is Lewis x) and the difucosylated product Lexβ1-3(Lexβ1-6)L. The branched lactose is a non-reactive acceptor site in agreement with the known natural saccharides of human milk. Interestingly, the other major monofucosylated product, Lexβ1-3(LNβ1-6)L, has not been found among the oligosaccharides of human milk. This raises the question of how the biosynthesis of the free oligosaccharides really occur.

The second part of the thesis (**II**) deals with the biosynthesis of the sialylated and multiply fucosylated polylactosamines of human leukocytes including several important selectin counterreceptor saccharide sequences. The acceptor saccharides LNβ1-3'LNβ1-3'LN and LNβ1-3'Lexβ1-3'Lex were sialylated by radioactive CMP-NeuNAc and the lysates of the human promyelocytic leukemia cell line HL-60. The analysis of the products by specific sialidase, chromatographies, and MALDI- mass spectrometry showed that both acceptors were equally α2-3sialylated, while the midchain fucosylations were inhibitory to α2-6sialylation. This work completed the *in vitro* biosynthetic pathways to the interesting glycosylations of leukocytes.

In **III** a novel type of glycolipid glycan was synthesized. The common saccharide sequence globo-N-tetraose was branched by a broad specificity β1-6GlcNAc-transferase activity present in hog gastric mucosa and the purified product saccharide, GalNAcβ1-3(GlcNAcβ1-6)Galα1-4Galβ1-4Glc, was unambiguosly characterized by NMR, mass spectrometry and enzymatic degradations. The work revealed a novel crossing point between the biosynthetic pathways of lacto- and globoseries glycolipids and predicts at present unknown glycans which may have roles in differentiation, cancer or autoimmunity. The work also revealed a novel acceptor saccharide epitope for the β1-6GlcNAc-transferase activity: GalNAcβ1-3Galα.

In the part **IV** the fucosyltransferases of human milk and human recombinant fucosyltransferases, Fuc-TV and Fuc-TVI, were used for fucosylation of chitooligosaccharides. Novel chito-Lewis x-saccharide structures, GlcNAcβ1-4(Fucα1-3)GlcNAc(β1-4GlcNAc)$_{0-4}$ were characterized by NMR, mass spectrometry and enzymatic degradations. The NMR-studies indicated also a three-dimensional structure similar to Lewis x. The chito-Lex-type glycosylations are known from the nodulating factors of symbiotic nitrogen fixing bacteria. The fucosyl branch of the glycan was shown to protect the non-reducing end GlcNAc from exo-N-acetylhexosaminidase from jack beans. Interestingly, commercial N-acetylhexosaminidase also contained a novel endochitinase activity, which could cleave only one GlcNAc from the reducing end of the saccharide with GlcNAc$_6$-backbone indicating that the fucose residue has a long range protective effect also against endochitinases. Using larger amounts of hexosaminidase the non-reducing GlcNAcs could be cleaved from the chito-Lex epitopes revealing terminal Fucα1-3GlcNAc-sequences. Such glycans are also present in mammals, but the biosynthetic pathways to them are not known. We suggest that the terminal fucose-glycans are cleaved from chito-Lex or LexNAc, GalNAcβ1-4(Fucα1-3)GlcNAc -saccharides by N-acetylhexosaminidases.

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