Pr uction and Use of Mammalian Glycosyltransferases

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To my family

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LIST OF ORIGINAL PUBLICATIONS

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

I Mattila P., Salminen H., Hirvas L., Niittymäki J., **Salo H.**, Niemelä R., Fukuda M., Renkonen O., and Renkonen R. (1998). The centrally acting β1,6-N-acetylglucosaminyltransferase (GlcNAc to Gal): functional expression, purification, and acceptor specificity of a human enzyme involved in midchain branching of linear poly-N-acetyllactosamines. *J. Biol. Chem.* **273**: 27633-27639.

II **Salo H.**, Aitio O., Ilves K., Bencomo E., Toivonen S., Penttilä L., Niemelä R., Salminen H., Grabenhorst E., Renkonen R., and Renkonen O. (2002). Several polylactosamine-modifying glycosyltransferases also use internal GalNAcβ1-4GlcNAc units of synthetic saccharides as acceptors. *Glycobiology* **12**: 217-228.

III Sievi E., Hänninen A.-L., **Salo H.**, Kumar V., and Makarow M. (2003). Validation of the Hsp150 polypeptide carrier and *Hsp150* promoter in expression of rat α2,3-sialyltransferase in yeast. *Biotechnol. Prog.* **19**: 1368-1371.

IV **Salo H.**, Sievi E., Suntio T., Mecklin M., Mattila P., Renkonen R., and Makarow M. Team work in the yeast cell wall: mammalian glycosyltransferases synthesizing sLex. (submitted)

ABBREVIATIONS

AOX	alcohol oxidase
CHX	cycloheximide
cIGnT6	centrally acting β1,6-GlcNAc transferase
dIGnT6	distally acting β1,6-GlcNAc transferase
ER	endoplasmic reticulum
Fuc	L-fucose
FucTe	catalytic ectodomain of human α1,3-fucosyltransferase VII
FucTVII	human α1,3-fucosyltransferase VII
Gal	D-galactose
β1,4-GalT	β1,4-galactosyltransferase
GalNAc	N-acetyl-D-galactosamine
β1,4-GalNAcT	β1,4-N-acetylgalactosaminyltransferase
Glc	D-glucose
GlcNAc	N-acetyl-D-glucosamine
GST	glutathione-sulfo-transferase
Hex	hexose
HexNAc	N-acetyl-D-hexosamine
HMBC	heteronuclear multiple bond correlation
HPAEC-PAD	high-pH anion exchange chromatography with pulsed amperometric detection
HPLC	high-performance liquid chromatography
Hsp	heat shock protein
LacdiNAc, LdN	N,N-diacetyllactosdiamine, GalNAcβ1-4GlcNAc
LacNAc, LN	N-acetyllactosamine, Galβ1-4GlcNAc
Lex	Lewis x, Galβ1-4(Fucα1,3)GlcNAc
m/z	mass-to-charge ratio
MALDI-TOF MS	matrix-assisted laser desorption/ionization time-of-flight mass spectrometry
MFα	mating factor α
MOPS	4-morpholinepropanesulfonic acid
NeuNAc	N-acetyl-D-neuraminic acid, sialic acid
NMR	nuclear magnetic resonance
PLG	polylactosaminoglycan
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
Sialyl-lacNAc	sialyl-N-acetyllactosamine, NeuNAcα2,3Galβ1-4GlcNAc
sLex	sialyl Lewis x, NeuNAcα2,3Galβ1-4(Fucα1,3)GlcNAc
ST3Ne	catalytic ectodomain of rat α2,3-sialyltransferase
ST6Ne	catalytic ectodomain of rat α2,6-sialyltransferase
TCA	trichloroacetic acid
Tyv	tyvelose (3,6-dideoxy-D-arabinohexose)
WGA	wheat germ agglutinin

SUMMARY

Polylactosaminoglycans (PLGs) are composed of N-acetyllactosamine disaccharide units, and they form the sugar backbones of many glyconjugates. Their structures vary from linear to highly branched, with their degree of branching being dependent on the stage of development and differentiation of the given organism or cell type. PLGs, which are linked to cell surface proteins and lipids, carry terminal decorations responsible for their biological functions. N-acetylgalactosamine sometimes replaces the terminal galactose of PLG, resulting in N,N-diacetyllactosdiamine, which can be decorated similarly to N-acetyllactosamine. Internal N,N-diacetyllactosdiamines have not been encountered in naturally occurring PLGs. In this study, we have shown that an enzyme activity present in human serum elongates PLGs containing terminal N,N-diacetyllactosdiamine, suggesting that this disaccharide unit may occur internally in natural PLGs. These novel glycans can function as acceptors for mammalian glycosyltransferases. In addition, a method was developed to identify naturally occurring internal N,N-diacetyllactosdiamines.

Sialyl Lewis x (sLex) antigen [NeuNAcα2,3Galβ1-4(Fucα1-3)GlcNAc], a terminal decoration of PLGs, plays an important role in adhesion of leukocytes to endothelial cells. Branched PLGs carrying multiple sLex antigens can be used to inhibit this adhesion to, for example, prevent organ transplant rejection. To synthesize these for use as therapeutics, three mammalian enzymes were produced as recombinant proteins. PLG branching enzyme (IGnT6) was expressed in insect cells. It branched linear PLG at internal positions exhibiting centrally acting branching activity (cIGnT6), which could be used to synthesize multivalent inhibitors of leukocyte adhesion. Rat α2,3-sialyltransferase and human α1,3-fucosyltransferase VII were expressed in the yeasts *Saccharomyces cerevisiae* and *Pichia pastoris*. Their ectodomains were expressed as fusion proteins, which were secreted but remained bound to the yeast cell wall. This enabled the use of intact yeast cells as a source of enzymatic activity for the synthesis of sLex. Yeasts expressing the two enzymes either in separate strains or in a single strain functioned in tandem in the synthesis of sLex. The recombinant proteins were expressed under the control of the *HSP150* promoter, the strength of which was found to be comparable with strong well-known yeast promoters.

1. INTRODUCTION

1.1. Structure and biosynthesis of ordinary polylactosaminoglycans

Polylactosaminoglycans form linear or branched sugar backbones (Fig. 1) of many glycoconjugates, including N- and O-glycans of glycoproteins, some glycolipids (reviewed in Leppänen, 1997) (see Fig. 2 for common core structures), and keratan sulfate proteoglycan (Brown *et al.*, 1994a, 1994b; Greiling, 1994). The linear backbones are composed of repeating disaccharide units, either Galβ1-4GlcNAc (N-acetyllactosamine, lacNAc) (type 2 lacNAc) or Galβ1-3GlcNAc (type 1), which are joined by β1,3-linkages. The branched variations carry β1,6-linked GlcNAc units at some 3-substituted galactose residues, and these GlcNAc branches can be further elongated and branched. The linear and branched sugar backbones are often referred to as i- and I-type polylactosaminoglycans, respectively, because they represent blood group i- and I-antigens (Feizi *et al.*, 1979; Fukuda *et al.*, 1979).

The termini of the linear backbones and the branches are the carriers of the terminal structures. These terminal structures are diverse, for example, the terminal galactose can be α2,3- or α2,6-sialylated, sulfated at carbon 3 or 6, or can carry an α1,3-linked galactose. Terminal decorations also include ABO-blood group antigens and so-called Lewis antigens (Fig. 3).

The biological functions of glycoconjugates are mostly carried out by these terminal structures. They, for instance, serve as ligands for distinct lectins (carbohydrate-binding proteins) and they function as cell adhesion molecules (Rosen and Bertozzi, 1994). One important goal in enzymatic

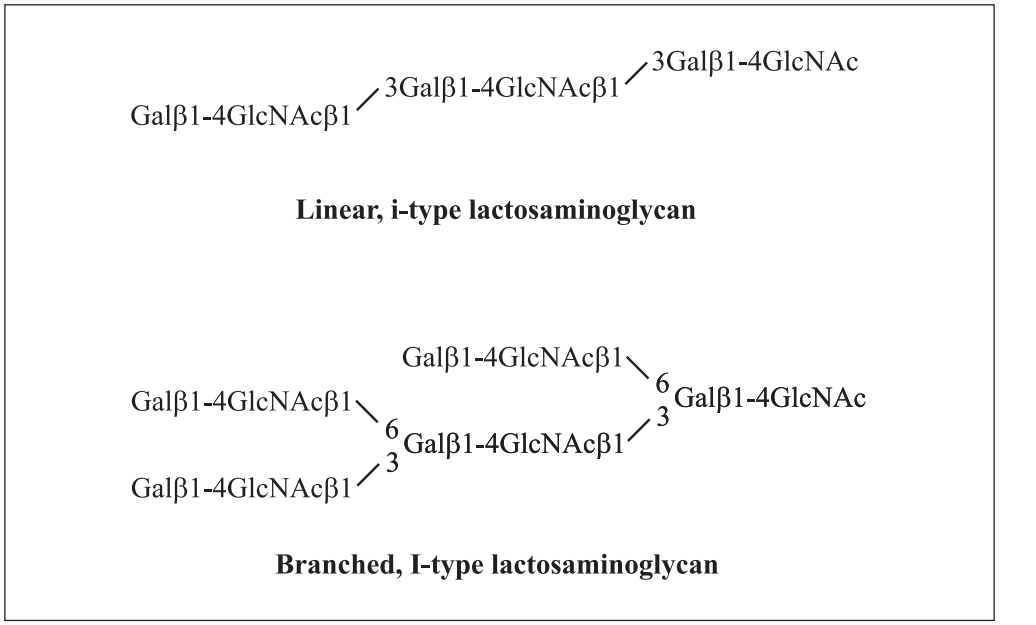


Figure 1. Linear (i-type) and branched (I-type) lactosaminoglycan.

oligosaccharide synthesis is to produce oligosaccharides carrying these binding epitopes in order to use them as counter receptors and antagonists of animal lectins to prevent certain cell adhesion events such as those involved in vascular biology and fertilization.

The linear i-type blood group antigen is synthesized by sequential action of β1,3-N-acetylglucosaminyltransferase (β1,3GlcNAcT) and β1,4-galactosyltransferase (β1,4GalT). The i-antigens are converted to I-antigens by yet another enzyme, β1,6-N-acetylglucosaminyltransferase (IGnT). Two distinct branching IGnT activities have been described *in vitro*. The "distally" acting dIGnT transfers a β1,6-GlcNAc unit to the penultimate galactose of linear polylactosaminoglycan, acting coordinately with the elongation enzymes mentioned above (Piller *et al.*, 1984; Brockhausen *et al.*, 1986; Koenderman *et al.*, 1987; Seppo *et al.*, 1990; Gu *et al.*, 1992; Helin *et al.*, 1997). This results in complex branches, which are further elongated and branched. The "centrally" acting cIGnT transfers a GlcNAc unit to the internal galactose of the preformed linear polylactosaminoglycan (Leppänen *et al.*, 1991, 1997, 1998; Gu *et al.*, 1992), resulting in multiple short branches along the polylactosaminoglycan chain.

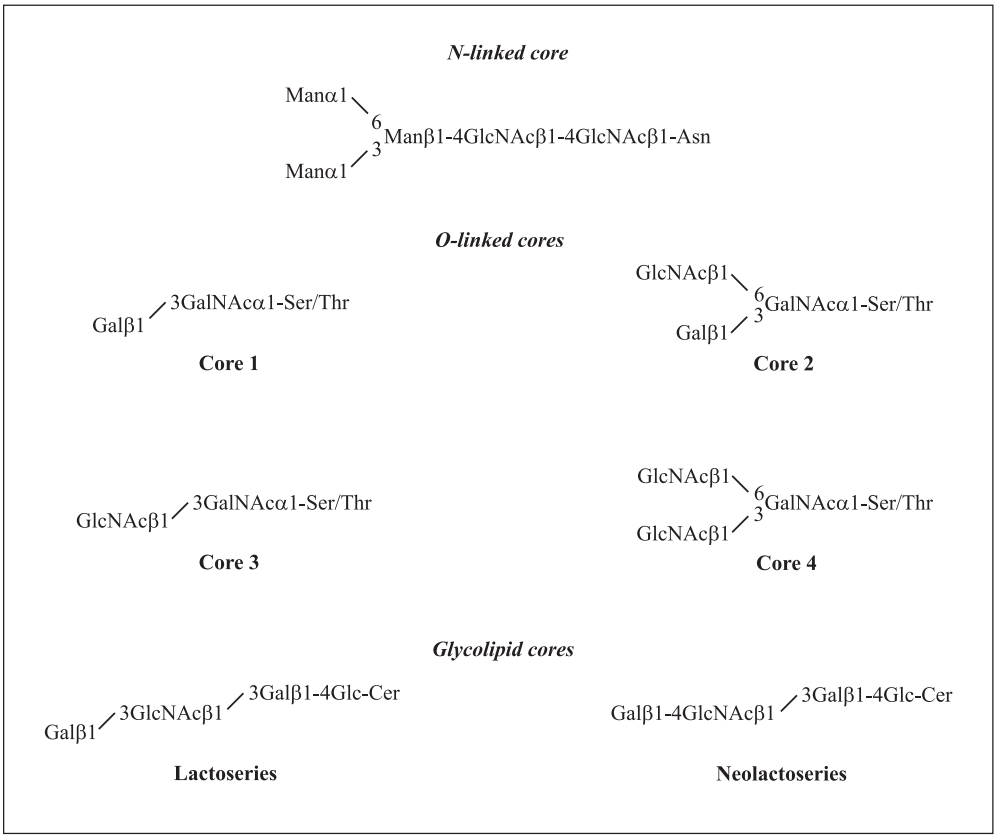


Figure 2. Examples of common core structures.

1.2. N-Acetylgalactosaminyl (GalNAc) analogs of ordinary polylactosaminoglycans

In an increasing number of cases, GalNAcβ1-4GlcNAc (N,N-diacetyllactosdiamine, lacdiNAc), an analog of lacNAc, has been found to replace the lacNAc unit in polylactosaminoglycan backbones in complex-type glycans of glycoproteins and glycolipids (reviewed in van den Eijnden *et al.*, 1997). LacdiNAc-containing sugar chains occur on vertebrate, including mammal, and invertebrate glycoconjugates (see Table 1 for structures and references). The lacdiNAc unit replaces the nonreducing lacNAc and can be terminally substituted analogously to terminal lacNAc motifs. The group of glycoconjugates carrying these chains is diverse, consisting of hormones, transport proteins, enzymes, and protective glycoproteins. Despite the wide variety of terminal structures involving the lacdiNAc determinant, truly internal lacdiNAc sequences in polylactosaminoglycans have not been reported. The GlcNAcβ1-3GalNAc bond is, however, known among glycoconjugates. O-glycan core 3 contains a GlcNAcβ1-3GalNAc linkage (Brockhausen *et al.*, 1985), and human blood fluke *Schistosoma mansoni* cercarial glycolipids are known to carry GlcNAcβ1-3GalNAc-containing glycans (Wuhrer *et al.*, 2000).

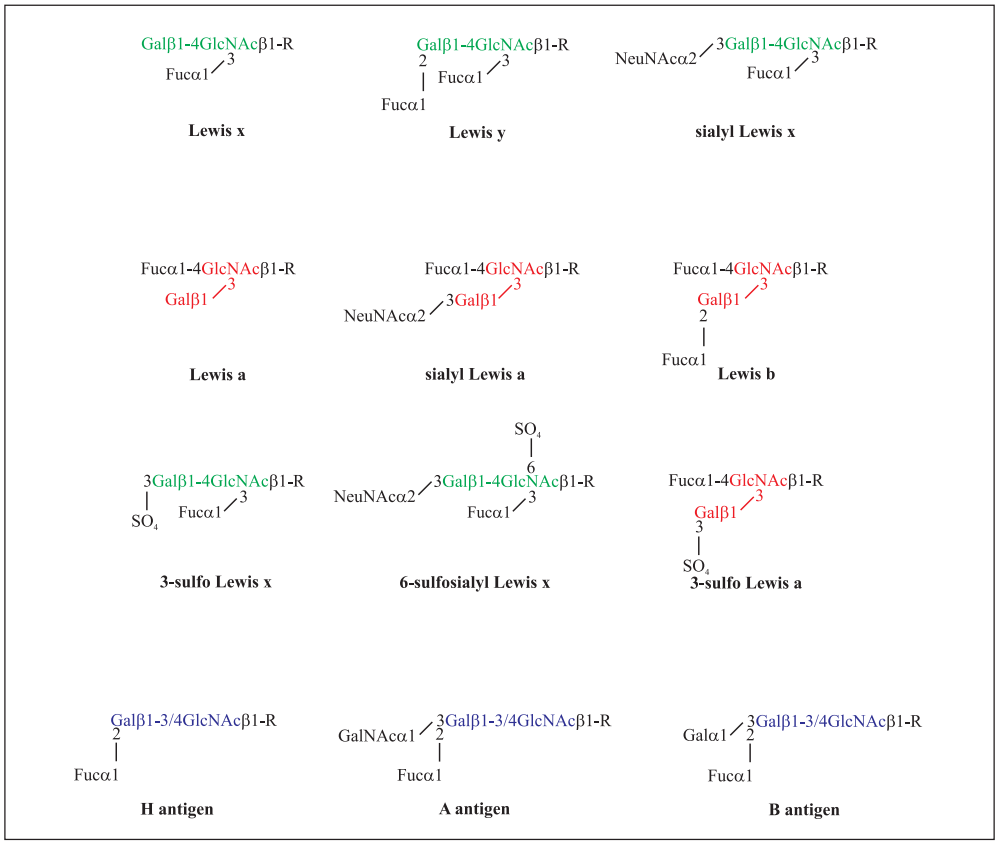


Figure 3. Structures of several terminal antigenic epitopes.

1.2.1. Structures

In the 1980s, the Asn-linked oligosaccharides of the mammalian pituitary hormone lutropin were found to contain N-acetylgalactosamine as a building block (Bahl *et al.*, 1980; Parsons and Pierce, 1980; Bedi *et al.*, 1982). Several different structures for these glycans were proposed, but due to the lack of specific methods for characterization, the exact structures were not revealed until 1985 (Green *et al.*, 1985). Since then, interest in these structures has increased, and as a result of extensive research, the list of known glycoconjugates carrying lacdiNAc-containing glycans is quite impressive. Table 1 displays the terminal structures and the occurrence of these glycans, while the structural elucidation of the corresponding glycoconjugate is presented in the reference.

1.2.2. Biosynthesis of GalNAcβ1-4GlcNAc units

There are two kinds of enzymatic β1,4-N-acetylgalactosaminyltransferase (β1,4-GalNAcT) activities known to synthesize lacdiNAc disaccharide structures, that differ from each other by the ability/disability to recognize the underlying polypeptide chain. The most studied example is the synthesis of sulfated oligosaccharides containing the lacdiNAc motif on glycoprotein hormones (see Table 1 for structures); the synthesis is highly specific and tightly regulated. Thus, the presence of sulfated oligosaccharides suggests that they play an important role in the biologic behavior of these hormones. Two of the hormones, lutropin (LH) and follitropin (FSH), are synthesized within the same cells in the pituitary. Yet LH carries N-linked oligosaccharides with terminal SO_4-4**GalNAcβ1-4GlcNAc**β1-2Manα structures, whereas N-glycans of FSH are decorated with NeuNAcα2,3**Galβ1-4GlcNAc**β1-2Manα terminal structures (Green and Baenziger, 1988a, 1988b). The oligosaccharide intermediates of these glycans are identical so the N-acetylgalactosaminyltransferase responsible for catalyzing the addition of GalNAc rather than Gal to the oligosaccharide chain has to recognize features of the underlying polypeptide chain in addition to the oligosaccharide acceptor. Smith and Baenziger (1988) discovered glycoprotein hormone-specific GalNAc transferase (β1,4-GalNAcT) in bovine interior pituitary membranes which transfers GalNAc to the terminal GlcNAc of $GlcNAc_2Man_3GlcNAc_2Asn$, and they proposed that recognition by the β1,4-GalNAcT requires the Pro-Xaa-Arg/Lys motif (where Xaa is a hydrophobic amino acid), located 6-9 residues on the amino-terminal side of an asparagine glycosylation site (Smith and Baenziger, 1992). This peptide-recognition marker is not present on the β-subunit of FSH, which explains the absence of N-linked oligosaccharides containing GalNAc on this glycoprotein hormone (Smith and Baenziger, 1992). Pro-Xaa-Arg/Lys peptide recognition marker is also present in glycoprotein hormone thyrotropin (Hiyama *et al.*, 1992), pro-opiomelanocortin (Skelton *et al.*, 1992), and tissue factor pathway inhibitor (Smith *et al.*, 1992). Each of these glycoproteins carries Asn-linked oligosaccharides with the terminal SO_4-4GalNAcβ1-4GlcNAcβ1-2Manα sequence. The presence of the peptide recognition marker on glycopeptide substrates reduces the apparent K_m of glycoprotein hormone-specific β1,4-

Table 1. Occurrence of GalNAcβ1-4GlcNAc-containing glycans

Terminal structure	Occurrence	Reference
GalNAcβ1-4GlcNAc	Bovine α-lactalbumin	Tilley *et al.* , 1991
	Bovine milk fat globule membrane proteins	Sato *et al.* , 1993
	Bovine milk butyrophilin	Sato *et al.* , 1995
	Bovine milk IgG heavy chain	Aoki *et al.* , 1995
	Bovine milk phosphoglycoprotein PP3	Girardet *et al.* , 1995
	Bovine parotid gland carbonic anhydrase VI	Hooper *et al.* , 1995
	Human urinary kallidinogenase	Tomiya *et al.* , 1993
	Human urokinase	Bergwerff *et al.* , 1995
	Human glycodelin A	Dell *et al.* , 1995
	Snake venom serine protease (batroxobin)	Lochnit and Geyer, 1995
	Schistosomal glycoproteins	Srivatsan *et al.* , 1992
	Dirofilaria immitis (dog heartworm)	Kang *et al.* , 1993
	Rnase 1 from Capan-1 tumor cells	Peracaula *et al.* , 2003
	Honeybee royal jelly glycoprotein	Kimura *et al.* , 2002
	Matrix metalloproteinase-1 (HT-1080 fibrosarcoma cells)	Saarinen *et al.* , 1999
NeuAcα2-3GalNAcβ1-4GlcNAc	Snake venom serine protease (ancrod)	Pfeiffer *et al.* , 1992
	Snake venom serine protease (batroxobin)	Tanaka *et al.* , 1992
NeuAcα2-6GalNAcβ1-4GlcNAc	Pituitary hormones	Weisshaar *et al.* , 1991
	Bovine lactotransferrin	Coddeville *et al.* , 1992
	Bovine mammary epithelial CD36	Nakata *et al.* , 1993
	Bovine milk phosphoglycoprotein PP3	Girardet *et al.* , 1995
	Human urokinase	Bergwerff *et al.* , 1995
	Human glycodelin A	Dell *et al.* , 1995
	Recombinant human protein C	Yan *et al.* , 1993
	Recombinant tissue plasminogen activator	Chan *et al.* , 1991
	Rat placental prolactin family	Manzella *et al.* , 1997
SO_4-4GalNAcβ1-4GlcNAc	Pituitary hormones	Baenziger and Green, 1988; Hiyama et al., 1992
	Bovine pro-opiomelanocortin	Siciliano *et al.* , 1994
	Bovine submaxillary gland carbonic anhydrase VI	Hooper *et al.* , 1995
	Human Tamm-Horsfall glycoprotein	Hård *et al.* , 1992
	Human urokinase	Bergwerff *et al.* , 1995
	Recombinant tissue factor pathway inhibitor	Smith *et al.* , 1992
	Murine pro-opiomelanocortin	Skelton *et al.* , 1992
	Cellular adhesion molecule Tenascin-R	Woodworth *et al.* , 2002
GalNAcβ1-4(Fucα1-3)GlcNAc	Bovine pro-opiomelanocotin	Siciliano *et al.* , 1993
	Human urokinase	Bergwerff *et al.* , 1995
	Human glycodelin A	Dell *et al.* , 1995
	Recombinant human protein C	Yan *et al.* , 1993
	Snake venom serine protease (batroxobin)	Lochnit and Geyer, 1995
	Honeybee venom phospholipase A_2	Kubelka *et al.* , 1993
	Honeybee venom hyaluronidase	Kubelka *et al.* , 1995
	Schistosomal glycoproteins	Srivatsan *et al.* , 1992
	Dirofilaria immitis (dog heartworm)	Kang *et al.* , 1993
	Matrix metalloproteinase-1 (HT-1080 fibrosarcoma cells)	Saarinen *et al.* , 1999
Tyv1-3GalNAcβ1-4(Fucα1-3)GlcNAc	*Trichinella spiralis* (intracellular parasite)	Reason *et al.* , 1994
Galα1-3GalNAcβ1-4GlcNAc	*Ascaris suum* (pig parasitic nematode)	Lochnit *et al.* , 1997
Galβ1-3GalNAcβ1-4GlcNAc	*Lymnaea stagnalis* (hemocyanin)	van Kuik *et al.* , 1987

GalNAcT for the oligosaccharide acceptor from 1-2 mM to 5-10 μM (Smith and Baenziger, 1988, 1992). Due to these differences in K_m-values, this enzyme is referred to as PXR/K-specific β1,4-GalNAcT. The sulphotransferase, which acts after the GalNAc has been added to the oligosaccharide chain, requires only the GalNAcβ1-4GlcNAcβ1-2Manα acceptor structure for addition of sulfate to the 4-hydroxyl of the terminal GalNAc (Skelton *et al.*, 1991; Xia *et al.*, 2000).

Bovine milk glycoproteins are surprisingly rich in lacdiNAc-containing complex-type glycans (see Table 1 for references). β1,4-GalNAcT activity has been identified and characterized in lactating bovine mammary gland membranes (van den Nieuwenhof *et al.*, 1999). This enzyme uses acceptor substrates carrying terminal GlcNAc in β-configuration and even free GlcNAc (van den Nieuwenhof *et al.*, 1999), differing from the bovine pituitary gland β1,4-GalNAcT activity by being independent of a specific peptide motif in the underlying protein (Smith and Baenziger, 1992). β1,4-GalT activity, which is also present in the bovine mammary gland, relates very promiscuously to donor substrates, thus being able to use UDP-GalNAc instead of UDP-Gal (Palcic and Hindsgaul, 1991). However, β1,4-GalNAcT activity seems not to be due to the flexible behavior of β1,4-GalT activity, as demonstrated by competition experiments between these two enzymes and by studying responsiveness against an inhibiting antibody raised against bovine β1,4-GalT (van den Nieuwenhof *et al.*, 1999). Furthermore, in the presence of α-lactalbumin, a modifier protein present in lactating mammary gland directing the β1,4-GalT to synthesize lactose (Galβ1-4Glc) instead of lacNAc (Galβ1-4GlcNAc) (Brew *et al.*, 1968; Schanbacher and Ebner, 1970), the β1,4-GalNAcT acts on Glc to form GalNAcβ1-4Glc (van den Nieuwenhof *et al.*, 1999). This observation also distinguishes β1,4-GalNAcT from β1,4-GalT; the latter enzyme is incapable of transferring GalNAc to Glc despite the presence of α-lactalbumin (Do *et al.*, 1995).

However, regardless of the presence of terminal lacdiNAc determinant on human glycans, the enzyme responsible for the synthesis of this structure was not cloned until 2003. Takashi Sato *et al*. (2003) cloned a novel human β1,4-GalNAcT that catalyzes the formation of lacdiNAc structure, and the transcript of the enzyme was shown to be highly expressed in the stomach, colon, and testis by quantitative real-time PCR (Sato *et al.*, 2003). The truncated form of the enzyme was expressed in HEK293T cells, and it showed catalytic activity towards GlcNAc in β-configuration *in vitro*, forming GalNAcβ1-4GlcNAc both with N- and O-glycan-derived acceptor glycans. An experiment with asialo/agalacto fetal calf fetuin as an acceptor glycoprotein, which carries both N- and O-glycans, implies that the cloned β1,4GalNAcT recognizes both of these glycans (Sato *et al.*, 2003).

A peptide motif-independent β1,4-GalNAcT activity has been also described in schistosomes (Neeleman *et al.*, 1994; Srivatsan *et al.*, 1994), snails (Mulder *et al.*, 1995; Neeleman and van den Eijnden, 1996), Lepidopteran insect cells (van Die *et al.*, 1996), human 293 kidney cells (Do *et al.*, 1997), and nematode *C. elegans* (Do *et al.*, 1997; Kawar *et al.*, 2002).

1.2.3. Terminal decoration of the GalNAcβ1-4GlcNAc unit *in vitro*

Enzymatic *in vitro* synthesis of the terminally decorated lacdiNAc oligosaccharides has offered the possibility to study the biosynthesis of these structures. The terminal sulfation of lacdiNAc determinant *in vitro* has been carried out using bovine pituitary

membrane extracts to sulfate the chemically synthesized trisaccharide GalNAcβ1-4GlcNAcβ1-2Man (Skelton *et al.*, 1991; Dharmesh *et al.*, 1993). Different kinds of acceptor saccharides containing the lacdiNAc determinant have been fucosylated *in vitro* by, for example, using human milk α3/4-fucosyltransferase (Bergwerff *et al.*, 1993), recombinant human fucosyltransferase V (Pykäri *et al.*, 2000), recombinant human fucosyltransferase IV (Study **II**), and recombinant human fucosyltransferase VI (Nyame *et al.*, 1999). Partially purified bovine colostrum α2,6-sialyltransferase (Nemansky and van den Eijnden, 1992) and α2,6-sialyltransferase from different bovine tissues expressed in COS-7 cells (Mercier *et al.*, 1999) could catalyze the *in vitro* synthesis of terminal NeuNAcα2-6GalNAcβ1-4GlcNAc.

1.2.4. Biological significance of lacdiNAc-based oligosaccharide chains

As a result of intensive research during the 1990s, numerous glycoconjugates carrying lacdiNAc-based oligosaccharide chains have been reported (for references, see Table 1). Despite the wide variety of structures encountered, their biological significance remains obscure. The functions of these unique structures are difficult to study due to their presence in negligible amounts in biological materials, but as the sensitivity of the research methods develops, more functions will be revealed. The proposed biological functions of pituitary hormones and glycodelin A are presented here as examples.

1.2.4.1. Pituitary hormones

The hormones lutropin (LH), follitropin (FSH), thyrotropin (TSH), and chorionic gonadotropin (CG) are a family of closely related glycoproteins. Each hormone is a dimer consisting of a common α-subunit and a hormone-specific β-subunit (Pierce and Parsons, 1981). Lutropin from three different animal species bears N-linked oligosaccharides terminating with SO_4-4GalNAcβ1-4GlcNAcβ1-2Manα (Green *et al.*, 1985; Green and Baenziger, 1988a, 1988b). Native bovine lutropin (SO_4-4GalNAcβ1-4GlcNAcβ1-2Manα) and desulfated lutropin (GalNAcβ1-4GlcNAcβ1-2Manα) were studied for their capability to bind to the lutropin/chorionic gonadotropin receptor and to produce cAMP and progesterone. No differences in cAMP and progesterone production were present between these two glycoforms of the same protein, indicating that the interaction of glycoprotein hormones with the lutropin/chorionic gonadotrophin receptor is not modulated by sulfation of bovine lutropin oligosaccharides (Baenziger *et al.*, 1992).

Recombinant bovine lutropin produced in Chinese hamster ovary (CHO) cells carrying N-linked oligosaccharides with terminal structures NeuNAcα2-3Galβ1-4GlcNAcβ1-2Man and native bovine lutropin had a surprisingly large difference in their metabolic clearance rates, at 1.7%/min and 7.3%/min, respectively. This implies that the presence of sulfated rather than sialylated oligosaccharides on bovine lutropin is responsible for a shorter circulatory half-life, having a significant impact on *in vivo* bioactivity of this hormone (Baenziger *et al.*, 1992). A receptor present on hepatic

reticuloendothelial and Kupffer cells is specific for SO_4-4GalNAcβ1-4GlcNAcβ1-2Man structure (Fiete *et al.*, 1991). It binds to lutropin with an apparent K_d of 1.63 x 10^{-7}M, and the bound ligand is rapidly internalized, transported to lysosomes, and degraded, resulting in a rapid clearance of lutropin from circulation (Fiete *et al.*, 1991). For example, the regulation of the circulatory half-life of lutropin by this receptor is critical for embryo implantation *in vivo* (Mi *et al.*, 2002). The rat liver SO_4-4GalNAcβ1-4GlcNAcβ1-2Man-specific receptor is closely related both antigenically and structurally to the macrophage mannose receptor isolated from the rat lung, but they differ in their ability to bind ligands bearing terminal SO_4-4GalNAc or Man (Fiete and Baenziger, 1997). The cDNA isolated from mouse lung encodes the macrophage/endothelial cell mannose receptor that binds SO_4-4GalNAcβ1-4GlcNAcβ1-2Man- or Man-terminating oligosaccharides at independent sites (Fiete *et al.*, 1997). The cysteine-rich domain at the N-terminus of this receptor binds to oligosaccharides with terminal SO_4-4GalNAc, while calcium-dependent carbohydrate recognition domains (CRD) account for binding of Man-terminating ligands (Fiete *et al.*, 1998; Roseman and Baenziger, 2000).

1.2.4.2. Glycodelin A

Glycodelin is a major glycoprotein that is synthesized in the endometrium in response to the hormones progesterone and relaxin. In addition to the endometrium, glycodelin is synthesized in the fallopian tube, bone marrow, breast, seminal vesicles, and various glands in the body, and these isoforms are differentially glycosylated depending on the tissue from which they are derived (reviewed in Seppälä *et al.*, 2001). Glycodelin A, the amniotic fluid isoform has three consensus sites for N-glycosylation (Asn28, Asn63, Asn85) (Julkunen *et al.*, 1988), the first two of which are glycosylated (Dell *et al.*, 1995). The major nonreducing epitopes in the complex-type glycans are Galβ1-4GlcNAc (lacNAc), GalNAcβ1-4GlcNAc (lacdiNAc), NeuNAcα2-6Galβ1-4GlcNAc (sialylated lacNAc), NeuNAcα2-6GalNAcβ1-4GlcNAc (sialylated lacdiNAc), Galβ1-4(Fucα1-3)GlcNAc (Lewis x), and GalNAcβ1-4(Fucα1-3)GlcNAc (fucosylated lacdiNAc) (Dell *et al.*, 1995). Glycodelin A has been shown in the hemizona assay to inhibit the binding of human sperm to the zona pellucida, the binding which is essential for fertilization (Oehninger *et al.*, 1995). This binding is proposed to require a selectin-like interaction between human sperm and the zona pellucida (Patankar *et al.*, 1993). A recombinant form of the human protein C expressed in human kidney 293 cells carries biantennary N-linked oligosaccharides terminating with a GalNAcβ1-4(Fucα1-3)GlcNAc sequence (Yan *et al.*, 1993). This sequence is a potent inhibitor of E-selectin-mediated adhesion (Grinnell *et al.*, 1994), a prerequisite binding for anti-inflammatory properties of this anti-coagulant factor. By blocking the selectin-mediated adhesion event by this same carbohydrate epitope, glycodelin A performs as a contraceptive. Human kidney 293 cells can be used to produce recombinant glycodelin, which carries the same type of glycan structures as contraceptive glycodelin A (van den Nieuwenhof *et al.*, 2000), thus providing

a means of developing a naturally occurring contraceptive agent.

In addition to the contraceptive activities of glycodelin A, it is known to have immunosuppressive properties (Pockley *et al.*, 1988; Pockley and Bolton, 1989, 1990; Okamoto *et al.*, 1991). These properties have been proposed to be mediated by the recognition of α2,6-sialylated lacdiNAc termini on glycodelin A by CD22, a B cell-associated receptor (Dell *et al.*, 1995).

1.3. Glycosyltransferases

Glycosyltransferases that act by adding monosaccharides one at a time from high-energy donors to specific positions on the acceptor saccharide are dealt with in this section. Transferases involved in the synthesis of dolicol-linked oligosaccharide precursor in N-glycan biosynthesis, those involved in initiation of O-linked glycosylation by adding monosaccharides to either serine or threonine, and those involved at the beginning of glycolipid biosynthesis by utilizing ceramide as an acceptor molecule are not considered here.

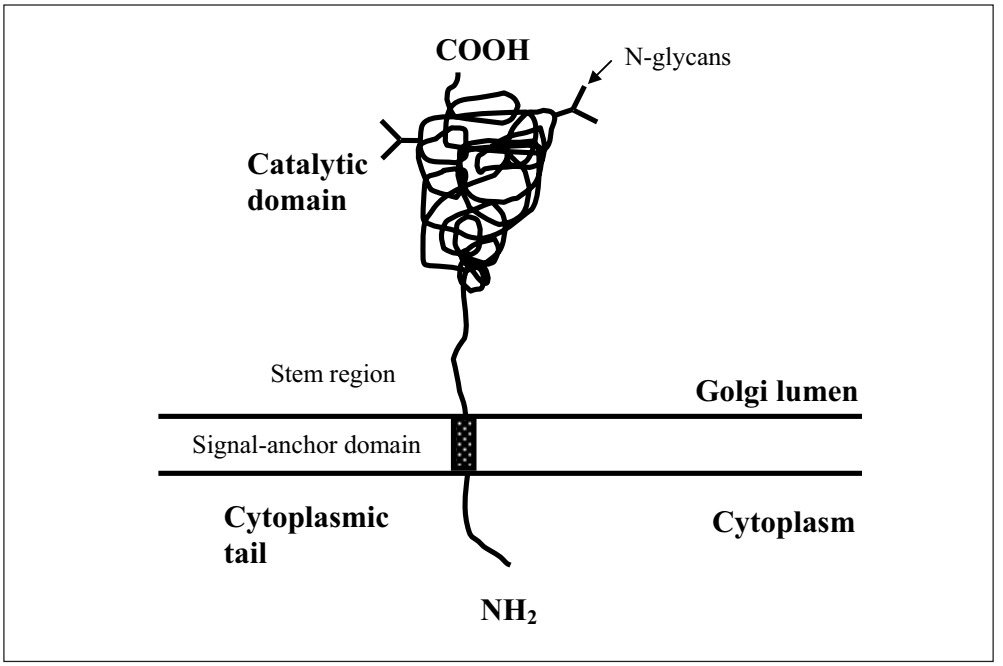


Figure 4. General structure of Golgi glycosyltransferases.

1.3.1. General features of glycosyltransferases

1.3.1.1. Structure

Glycosyltransferases are enzymes functioning in the biosynthesis of oligo- and polysaccharides, which are attached to proteins, lipids, or proteoglycans. All Golgi glycosyltransferases are type II transmembrane proteins. They consist of a short amino-terminal cytoplasmic domain, a transmembrane domain, and a stem region followed by a carboxy-terminal catalytic domain facing the lumen of the Golgi apparatus (Fig. 4). Glycosyltransferases themselves are often posttranslationally modified by glycosylation, especially by N-glycosylation. Although these enzymes share a common topology, sequence similarity between glycosyltransferases belonging to different families is rare. Instead, glycosyltransferases within functionally related families share amino acid sequences. For example, the members of the human α1,3-fucosyltransferase family (FucT-III, FucT-V, FucT-VI) share 85% of amino acids in their primary sequences (reviewed in Niemelä, 1999). Soluble forms of the glycosyltransferases are present in biological fluids, and they are formed via proteolytic cleavage, which occurs at the stem region of the transferase.

1.3.1.2. Subcellular localization

Proteins destined for the secretory pathway travel from the endoplasmic reticulum (ER) to the plasma membrane via the Golgi apparatus. Protein glycosylation begins in the ER and continues in the Golgi apparatus while the proteins move along the secretory pathway. The glycosyltransferases are located in distinct but overlapping areas in the Golgi, forming concentration gradients across the stack. This subcompartmentalization of glycosyltransferases is consistent with their mode of action in protein glycosylation. Glycosyltransferases act sequentially such that the oligosaccharide product of one enzyme functions as the acceptor saccharide for another enzyme. There has been a lively debate concerning the mechanisms by which the glycosyltransferases are segregated from the secretory traffic and retained in appropriate subcompartments of the Golgi apparatus. The lack of sequence homology among glycosyltransferases suggests the absence of a common Golgi retention signal.

The following two mechanisms have been proposed to explain how resident Golgi glycosyltransferases are retained in this organelle and how they are organized within Golgi membranes: a) the lipid bilayer thickness model (Bretscher and Munro, 1993; Masibay *et al.*, 1993) and b) the oligomerization or kin recognition hypothesis (Machamer, 1991; Nilsson *et al.*, 1993). The first mechanism is based on the theory that the retention of the enzyme depends on the length of the transmembrane domain of the protein and the thickness of the membrane in the Golgi complex. A shorter transmembrane domain prevents the Golgi proteins from entering cholesterol-rich transport vesicles, thus resulting in Golgi retention. This hypothesis was supported by the observation that increasing the length of the ST6Gal I transmembrane domain from 17 amino acids to 23 leucines resulted in the localization of the enzyme into the plasma membrane (Munro, 1991). On the other hand, proteins targeted to the plasma membrane were accu-

mulated in the Golgi apparatus when their transmembrane domains were shortened from 23 amino acids to 17 residues (Munro, 1995). The second mechanism relies on the assumption that the glycosyltransferases are induced to form insoluble homo- or hetero-oligomers when they arrive at the correct Golgi compartment. These large protein aggregates are excluded from the transport vesicles destined for later secretory compartments. Two medial-Golgi enzymes, GlcNAcT I and Mann II (mannosidase II), were relocated into the ER when either one was supplied with an ER retention signal, implying an interaction between these two enzymes (Nilsson *et al.*, 1994). The trans-Golgi enzyme GalT I, by contrast, was not retained by either ER-retained medial-Golgi transferase (Nilsson *et al.*, 1994), further supporting the idea that the enzymes are oligomerized. But neither the bilayer thickness model nor the oligomerization or kin recognition hypothesis alone explains the localization of glycosyltransferases in the Golgi complex. Opat *et al.* (2001) have suggested yet another model for the localization of medial- and late-Golgi resident transferases. This model is based on the dual transport system across the Golgi stack, where vesicular transport and cisternal progression occur simultaneously. In this model, medial-Golgi enzymes are thought to form large oligomers that are excluded from anterograde vesicular transport. Instead they move by cisternal progression through the Golgi stack (for detailed information about anterograde vesicular transport and cisternal progression, see Storrie and Nilsson, 2002). Upon arrival in the trans-Golgi network (TGN), these large oligomers are packed into retrograde transport vesicles and returned to the medial-Golgi. By contrast, late-Golgi enzymes could exploit the anterograde vesicular traffic and arrive quickly at the TGN. The low pH of the TGN may result in complex formation between late-Golgi enzymes, which has been demonstrated for one of the late-Golgi enzymes, namely ST6Gal I (Chen *et al.*, 2000). Complex formation restricts the forward movement of late-Golgi enzymes in the secretory pathway.

1.3.1.3. Function

Glycosyltransferases act by adding monosaccharide units from high-energy donors to saccharide chains. They catalyze trans-glycosylation reactions and are extremely regioselective and stereo-specific (Ichikawa *et al.*, 1992), distinguishing between different carbon atoms in a ring and the α/β-anomericity of the monosaccharide units. Their catalytic activity is usually dependent on divalent cations (the most common ones being Mg^{2+} and Mn^{2+}), and their pH optimum from 5.0 to 7.0 reflects the pH found in the ER-Golgi pathway. There are several factors affecting the final structure of the glycans on a certain glycoconjugate traveling through the Golgi. These factors include the expression of certain glycosyltransferases in a given time and cell type. The availability of nucleotide sugar donors in the Golgi lumen is also essential. The donors are synthesized in the cytoplasm (or in the nucleus in the case of CMP-sialic acid), after which they are transported across the Golgi membrane by specific nucleotide sugar transporters (reviewed in Gerardy-Schahn *et al.*, 2001) to the lumen, where the catalytic ectodomains of the glycosyltransferases are located. In

addition to the availability of nucleotide sugar donors, the concentration of the acceptors in the lumen of the Golgi influences the glycosylation of a given glycoconjugate. The presence of nucleotide sugars, or oligosaccharide-hydrolyzing enzymes, combined with the competition between glycosyltransferases for the same substrate molecules also affect the final structures of glycans being synthesized.

1.3.2. Need for expression of recombinant glycosyltransferases

Glycans expressed on cell surfaces perform many biologically important functions. They are involved in adhesion and recognition events, including selectin-mediated cell adhesion, host-pathogen recognition, and carbohydrate-mediated immune recognition. Prevention of these binding events is desirable in, for example, acute xenograft rejection and pathogen invasion. This could be accomplished by soluble oligosaccharides, which compete for binding to natural ligands, thus inhibiting the first critical steps of adhesion or recognition. The biological activities are mostly carried out by the complex end structures of glycans, which protrude from cell surfaces to be recognized by their counter receptors. Examples of these terminal epitopes are presented in Figure 3. Oligosaccharides to be used as binding inhibitors are difficult, if not impossible, to purify in sufficient quantities from tissues or cell cultures. Nevertheless, these oligosaccharides can be synthesized *in vitro* either chemically, enzymatically, or chemo-enzymatically (Ichikawa *et al.*, 1992). So far, the chemical synthesis of complex oligosaccharide structures lies beyond any reasonable cost-benefit ratio, so enzymatic and chemo-enzymatic syntheses are the methods of choice. *In vivo,* these terminal glycan structures are assembled on the common core structures present on N- and O-glycans and glycolipids, and their formation is catalyzed by specific glycosyltransferases located in the Golgi apparatus as described above. The glycosyltransferases are very difficult to obtain from tissue extracts or biological fluids using traditional biochemical methods because of their presence in scarce amounts and the presence of other disturbing enzymatic activities.

In 1989 John B. Lowe and his co-workers (Larsen *et al.*, 1989) introduced expression cloning to the field of glycobiology. This technique enables cloning of a cDNA that encodes a glycosyltransferase without the need to purify the enzyme protein. Because most glycosyltransferases are glycosylated themselves, they have to be correctly glycosylated and properly folded to be biologically active. All of these posttranslational modifications take place in the organelles of the secretory pathway in eukaryotic cells while the proteins are moving from the site of translation to their final destinations through the ER-Golgi pathway. The expression system to be used, whether it is mammalian, eukaryotic, or prokaryotic, must be carefully evaluated as a whole. The factors that should be considered are cell growth characteristics, expression levels, intra- and extracellular expression, posttranslational modifications, and biological activity of the protein as well as its intended use.

1.4. Production of recombinant proteins in yeasts

The yeast *Saccharomyces cerevisiae* has been used for almost three decades as a host organism for the expression of recombinant proteins of eukaryotic origin for research, industrial, and medical purposes (reviewed in Romanos *et al.*, 1992). *S. cerevisiae* has also been used for thousands of years by mankind in brewing and baking, and this is why it has been granted the GRAS (generally recognized as safe) status. It is easy and simple to cultivate on inexpensive growth media, and many well-known techniques are available for genetic and molecular manipulation of foreign genes. It is one of the best-characterized eukaryotes, with its entire genome being sequenced in 1996. The secretory pathway of *S. cerevisiae* contains the same cellular compartments as mammalian cells. Thus, it has the means to perform posttranslational modifications and

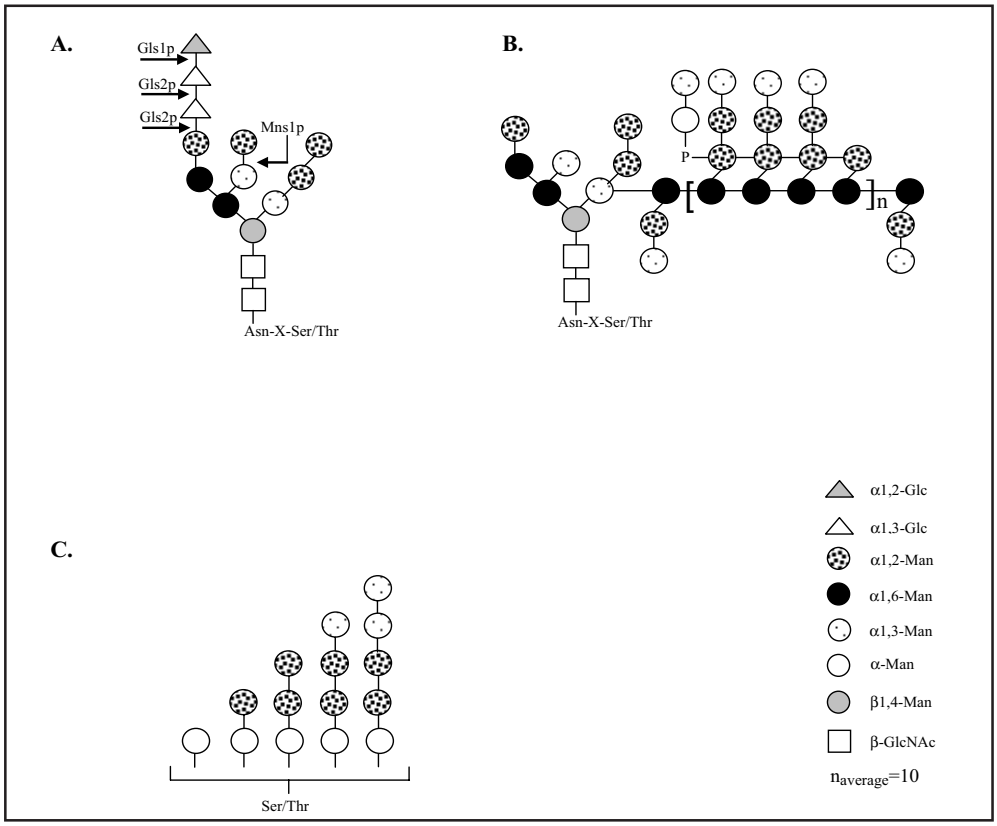


Figure 5. N- and O-glycosylation in *S. cerevisiae*. **A.** Structure of the core N-glycan attached to asparagine residues on proteins within the sequence Asn-x-Ser/Thr (x≠Pro). Before the newly glycosylated protein is transported from the ER to the Golgi, the glycan is trimmed by glycosidases at sites designated with arrows. **B.** The Golgi-elongated polymannose type N-glycan. **C.** O-glycosylation starts in the yeast ER by the attachment of the first mannose to Ser/Thr residues on the protein, whereas elongation takes place in the Golgi. Yeast O-glycans contain 1-5 mannose residues. The symbols on the right indicate the glycosidic linkages created between the monosaccharides.

secretion, resulting in protein products that are, if not identical, more similar to the native protein than those expressed in prokaryotic cells.

Glycosylation is the most common and the most complex form of posttranslational modification. It affects the final structure of the glycosylated protein, and thus, its biological activity *in vivo*, and the majority of therapeutic recombinant proteins are glycosylated in their native form. Yeasts are capable of performing both O- and N-glycosylation of secretory proteins. The N-glycosylation begins with the transfer of a preformed oligosaccharide core from dolicyl pyrophosphate to the amide group of asparagine within the tripeptide, Asn-X-Thr(Ser), where X may be any amino acid except proline. However, the N-glycans on glycoproteins produced by *S. cerevisiae* consist primarily of mannose residues, and the average mannose chain length can be as large as 50-100 units. The O-glycans consist solely of mannose chains attached to serine or threonine residues (Fig. 5) (Herscovics and Orlean, 1993).

Hyperglycosylation reduces the benefits of *S. cerevisiae* as an expression system. Many yeasts, such as methylotrophic *Pichia pastoris* and *Hansenula polymorpha,* tend not to glycosylate proteins to this extent (Faber *et al.*, 1995; Bretthauer and Castellino, 1999; Gellissen, 2000; Lin Cereghino and Cregg, 2000), although the glycans are also composed of mannose. High-mannose-type glycans are recognized by mannose receptors on mammalian cells, and the glycoproteins are removed from the circulation by endocytosis. In addition, high-mannose-type glycans are immunogenic. This dramatically reduces the effect of heterologous therapeutic proteins produced in yeast. As a consequence, extensive work has been carried out to produce secretory glycoproteins devoid of high-mannose-type glycans in yeast (Melnick *et al.*, 1990; Yip *et al.*, 1994; Lehle *et al.*, 1995). An ambitious cell engineering project aimed at constructing the complete mammalian-type glycosylation system in *S. cerevisiae* using glycosylation mutants (*Δoch1, Δmnn1, Δmnn4*), which are unable to polymannosylate the glycans (Chiba *et al.*, 1998; Malissard *et al.*, 1999). The N-glycosylation pathways of mammalian cells and yeast cells diverge in the Golgi apparatus (Herscovics and Orlean, 1993). The α1,2-mannosidase from *Aspergillus satoi* was successfully expressed in the yeast ER. This enzyme converted the $Man_8GlcNAc_2$ structure produced by the glycosylation mutants into $Man_5GlcNAc_2$ structure (Chiba *et al.*, 1998), which is the intermediate form for mammalian hybrid- and complex-type sugar chains. But to achieve elongation and termination of complex mammalian glycans, additional enzymes and nucleotide sugar transporters must be introduced into yeast. Mammalian cells therefore remain the primary expression system for generating glycosylated therapeutic recombinant proteins (Eckart and Bussineau, 1996; Grabenhorst *et al.*, 1999).

1.4.1. *Saccharomyces cerevisiae*

Efficient expression of recombinant protein in yeast requires the use of expression vectors that contain a promoter and terminator, selection marker, and DNA sequences encoding for a signal peptide and polypeptide carrier.

1.4.1.1. Expression vectors

Yeast expression vectors work as shuttle vectors, i.e. they possess bacterial sequences which aid in the vectors being selected and propagated in *E. coli* (Romanos *et al.*, 1992; Malissard *et al.*, 1999). Both integrative and episomally replicating plasmids are used to transform yeast cells. Episomal autonomously replicating plasmids can be divided into two subgroups: YEp and YCp. The YEp plasmids use the partitioning system of the endogenous yeast plasmid known as 2μ circle, giving rise to a high copy number (10-40 copies per cell). Characteristic to YCp is the chromosomal centromere, which connects the plasmid with the mitotic spindle apparatus. As a result, the plasmid copy number is limited (1-2 copies per cell) (Malissard *et al.*, 1999). Expression of episomal plasmids requires continuous selection on a minimal growth medium, resulting in lower cell densities than in a rich medium. Chromosomal integration of integrating vectors (YIp) offers a more stable alternative to episomal maintenance of foreign DNA (Romanos *et al.*, 1992). YIp vectors integrate normally by homologous recombination, and they contain yeast chromosomal DNA to target integration, the selectable marker, and the bacterial replicon (Romanos *et al.*, 1992). After the desired DNA sequence has been integrated into the yeast chromosome, the cells can be grown on a rich medium to much higher cell densities than in a minimal medium without risking the loss of the desired gene (Malissard *et al.*, 1999).

1.4.1.2. Promoters

Efficient transcription of foreign genes in yeast is dependent on promoters of yeast origin (Hitzeman *et al.*, 1981). Both regulated and constitutively active promoters are used. The advantage of the use of the former is that the growth stage can be separated from the expression stage; production of proteins toxic to the host organism, for intance, requires the use of a regulated promoter. The cells can thus be grown to high cell densities before they begin to express the toxic protein. The use of poorly regulated promoters is unsuitable in large-scale cultures, in which the risk for selection of nonexpressing cells is high. Glycolytic promoters (*ADH1*, *PGK*, *GAP*) are the most powerful ones of *S. cerevisiae*, but they are poorly regulated (Romanos *et al.*, 1992; Malissard *et al.*, 1999). Galactose-regulated promoters (*GAL1*, *GAL7*, *GAL10*) are the most powerful tightly regulated promoters of *S. cerevisiae*. They are strongly repressed by glucose, but many strains have mutations in the galactose permease gene (*GAL2*), and thus, are not inducible by galactose (Romanos *et al.*, 1992). Another class of regulated promoters is glucose-repressible promoters of *ADH2*, *SUC2,* and *CYC1* genes, which encode alcohol dehydrogenase II, invertase, and iso-1-cytochrome c, respectively (Romanos *et al.*, 1992; Malissard *et al.*, 1999). Genes involved in galactose or sucrose metabolism are repressed by glucose because yeasts prefer glucose that directly enters the glycolytic pathway. In addition to these metabolically expressed promoters, inorganic salts can be used to activate certain promoters. These include the promoter of acid phosphatase gene (*PHO5*), which is regulated by inorganic

phosphate, and the promoter of copper metallothionein (*CUP1*), which is tightly regulated by Cu^{2+} ions (Romanos *et al.*, 1992).

Yet another class of yeast promoters is of interest because the induction is independent of culture conditions. They can be regulated without interfering with the culture just by shifting the cells to a higher growth temperature. A gene encoding secretory heat shock protein hsp150 is regulated by heat shock and nitrogen starvation. Shifting the cells from their physiological temperature of 24°C to 37°C increases the steady-state level of the *HSP150* mRNA and the amount of synthesized hsp150 protein (Russo *et al.*, 1992, 1993). This promoter has been used to induce the expression of several heterologous proteins in *S. cerevisiae* (Simonen *et al.*, 1994, 1996; Mattila *et al.*, 1996).

1.4.1.3. Terminators

Yeast transcription terminators are needed for the proper formation of mRNA 3' ends and for obtaining maximal expression levels. Often the yeast terminator corresponding to the promoter is used just to simplify the vector construction, and for the same reason, a terminator from the 2μ circle (either *FLP* or *D* gene terminator) can be exploited (Romanos *et al.*, 1992; Malissard *et al.*, 1999).

1.4.1.4. Selectable markers

The yeast transformants must be screened for successful transformation. This is done with the aid of selectable markers, either auxotrophic or dominant, introduced into the cloning vectors. The most commonly used markers for selection of yeast transformants are *LEU2*, *TRP1*, *URA3,* and *HIS3*. Thus, mutant strains, auxotrophic for leucine, tryptophane, uracil, and histidine, are used. Dominant selectable markers usually encode resistance to some antibiotic. Antibiotic resistance markers, commonly used in yeast transformation, include aminoglycoside G418 resistance marker, hygromycin B resistance marker, and chloramphenicol resistance marker (Romanos *et al.*, 1992).

1.4.1.5. Signal peptides

S. cerevisiae secretes only about 0.5% of its own proteins. Thus, the secreted heterologous protein is easy to purify from the growth medium, and this eliminates the need for costly and low-yielding cell-disruption and refolding steps. Although intracellular expression of proteins requires extra purification steps, it is the system of choice for heterologous proteins that are normally expressed in the cytoplasm (Eckart and Bussineau, 1996). As in higher eukaryotes, targeting of proteins to the secretory pathway is directed by an N-terminal signal peptide required for translocation of the protein into the ER lumen (Blobel and Dobberstein, 1975). A classical signal peptide is composed of a charged N-terminus, followed by a hydrophobic core and a consensus sequence for cleavage in the ER by signal peptidase. Despite signal peptides being recognized with low specificity in yeast, foreign signal peptides do not usually work (Romanos *et al.*, 1992). Therefore, the most widely used signal peptides are of yeast origin, like those of acid phosphatase, invertase, and mating factor α (Brake *et al.*, 1984; Malissard *et al.*, 1999).

1.4.1.6. Carrier polypeptides

Once in the ER, a default pathway is thought to direct a protein either to the plasma membrane, unless it contains a specific signal that retains it in the ER or Golgi, or to the vacuole. However, there are many stages along the yeast secretory pathway where the journey of the protein being secreted may be hampered, and usually heterologous proteins are incorrectly folded and fail to be secreted (Romanos *et al.*, 1992). Many of the retained proteins can be rescued by fusing them to a carrier polypeptide, which helps them to achieve their proper conformation and guides them out of the ER and through the secretory pathway. The most commonly used carrier polypeptide is the prepro-fragment (amino acids 1-83) of the *S. cerevisiae* α-factor precursor (Brake *et al.*, 1984; Singh *et al.*, 1984; Brake, 1990). α-Factor, a 13-amino acid mating pheromone required for mating, is secreted by *S. cerevisiae* α-cells (Thorner, 1981). The pheromone molecules are initially synthesized as larger precursor polypeptides, which are then processed to yield the mature species (Fig. 6) (Julius *et al.*, 1983). The proposed prepro-α-factor precursor (165 amino acids in total) of *S. cerevisiae* consists of a 19-amino acid hydrophobic signal peptide, an additional leader region of 66 amino acids, and four identical repeats of the mature 13-amino acid pheromone separated by similar spacer peptides (6-8 residues) (Kurjan and Herskowitz, 1982; Brake *et al.*, 1983). Processing of prepro-α-factor precursor requires four different proteolytic activities (Fig. 6). Signal peptidase cleaves between amino acids 19 and 20, releasing the signal peptide (pre) in the ER (Waters *et al.*, 1988). The resulting pro-α-factor is cleaved by Kex2 endoproteinase, which cleaves on the carboxyl side of the Lys-Arg sequence in the spacer peptide of each repeat (Leibowitz and Wickner, 1976; Julius *et al.*, 1984). Kex1 carboxypeptidase removes the arginyl and lysyl residues at the C-terminus of each of the first three repeats (Dmochowska *et al.*, 1987). Finally, before the mature pheromone is released to the extracellular environment, the membrane-bound dipeptidylaminopeptidase

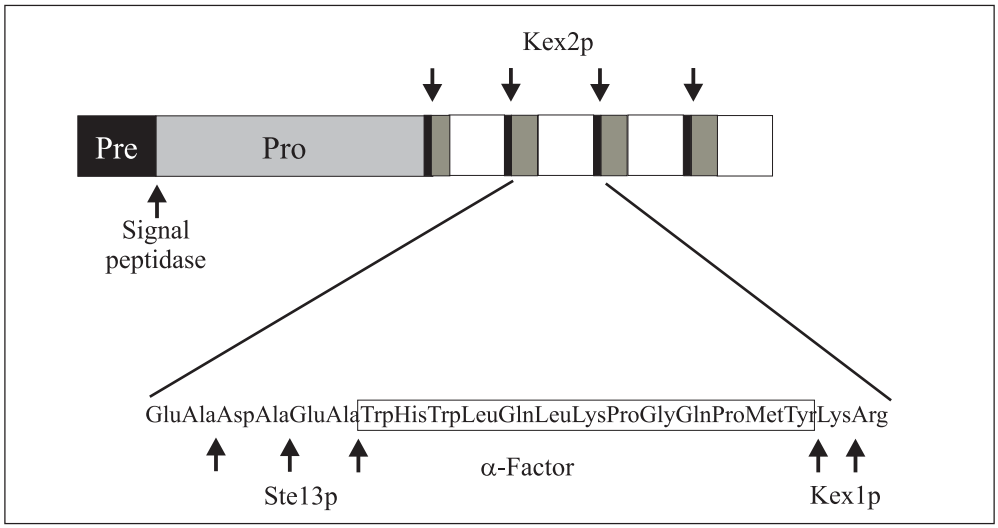


Figure 6. Structure and processing of the prepro-α-factor. For detailed explanation of the processing steps, see Section 1.4.1.6.

Ste13 removes the Glu-Ala and Asp-Ala dipeptides from the N-terminus of each repeat (Julius *et al.*, 1983). The prepro-region of the mating factor α-precursor contains enough information to target the heterologous proteins fused to its C-terminus for processing and secretion in *S. cerevisiae* (Brake *et al.*, 1984; Brake, 1990).

Another carrier polypeptide is derived from the secretory heat shock protein Hsp150 of *S. cerevisiae*, which has been cloned and characterized in our laboratory (Fig. 8A) (Russo *et al.*, 1992). Hsp150 is constitutively expressed, highly O-glycosylated, and secreted efficiently to the culture medium (Russo *et al.*, 1992). Indeed, it is one of the few proteins secreted to the culture medium of *S. cerevisiae* (Tanner and Lehle, 1987). *HSP150* gene expression is regulated by heat shock and nitrogen starvation (Russo *et al.*, 1993). The primary translation product of *HSP150* gene (413 amino acids) consists of an 18-amino acid signal peptide, followed by subunits I (amino acids 19-72) and II (amino acids 73-413). Subunit II is divided into 11 tandem repeats of 19 amino acids (amino acids 73-299) and C-terminal amino acids (300-413) (Russo *et al.*, 1992). The signal peptide is cleaved off in the ER by signal peptidase, and Kex2p in the Golgi apparatus removes subunit I, yielding peptide fragments of 53 and 341 amino acids (Russo *et al.*, 1992). The Hsp150Δ carrier is an N-terminal fragment of the authentic Hsp150 secretory protein containing the first 321 amino acids (see Fig. 8D and 8H) (Simonen *et al.*, 1994, 1996; Jämsä *et al.*, 1995). The Hsp150Δ carrier promotes proper folding in the yeast ER of heterologous proteins fused to its C-terminus, which results in efficient secretion of the fusion proteins (Simonen *et al.*, 1994, 1996; Jämsä *et al.*, 1995; Mattila *et al.*, 1996).

1.4.2. *Pichia pastoris*

Despite the successes of *S. cerevisiae* as a host organism for the expression of recombinant proteins, low yields often limit its use. Methylotrophic yeasts, such as *Pichia pastoris*, provide much higher yields than *S. cerevisiae*. Moreover, they do not extend the N-glycans, as encountered with *S. cerevisiae* (Faber *et al.*, 1995; Gellissen, 2000; Lin Cereghino and Cregg, 2000). The increasing popularity of the *P. pastoris* expression system arises from the simplicity and similarity of the techniques to those of *S. cerevisiae*, as well as from the ability of *P. pastoris* to produce foreign proteins at high levels either intra- or extracellularly. The capability of *P. pastoris* to carry out posttranslational modifications and the commercial availability of the expression system as a kit also make it an attractive choice as a host organism (Lin Cereghino and Cregg, 2000). The heterologous gene expression in *P. pastoris* is based on a strong and regulatable promoter derived from genes of the methanol utilization pathway (Gellissen, 2000). When grown on methanol, this yeast produces 30% of total soluble protein as an alcohol oxidase (Ellis *et al.*, 1985; Cregg *et al.*, 1993), one of the key components of the methanol utilization pathway. The initial reactions of this pathway (Fig. 7) (Egli *et al.*, 1980; Gleeson and Sudbery, 1988; Gellissen, 2000; Lin Cereghino and Cregg, 2000) take place in specialized organelles, peroxisomes, with subsequent steps occurring in the cytoplasm.

In peroxisomes, methanol is oxidized to formaldehyde (HCHO) and hydrogen peroxide (H_2O_2). *P. pastoris* has two

alcohol oxidase genes, *AOX1* and *AOX2*. Studies of strains with disrupted *AOX* genes have revealed that Aox1p is the dominant alcohol oxidase and that its expression is controlled at the transcription level (Cregg *et al.*, 1989). Regulation of the *AOX1* gene involves a repression/derepression mechanism and an induction mechanism. The repression/derepression mechanism is based on the presence and then absence of a repressing carbon source, such as glucose or glycerol, but the presence of methanol is essential for high-level production of Aox1p (Tschopp *et al.*, 1987). Formaldehyde formed in the oxidization reaction enters both the cytosolic dissimilatory pathway to yield energy and the assimilatory pathway to generate biomass so *P. pastoris* can utilize methanol as its sole carbon source (Egli *et al.*, 1980; Ellis *et al.*, 1985; Gleeson and Sudbery, 1988; Lin Cereghino and Cregg, 2000). In addition, methanol is

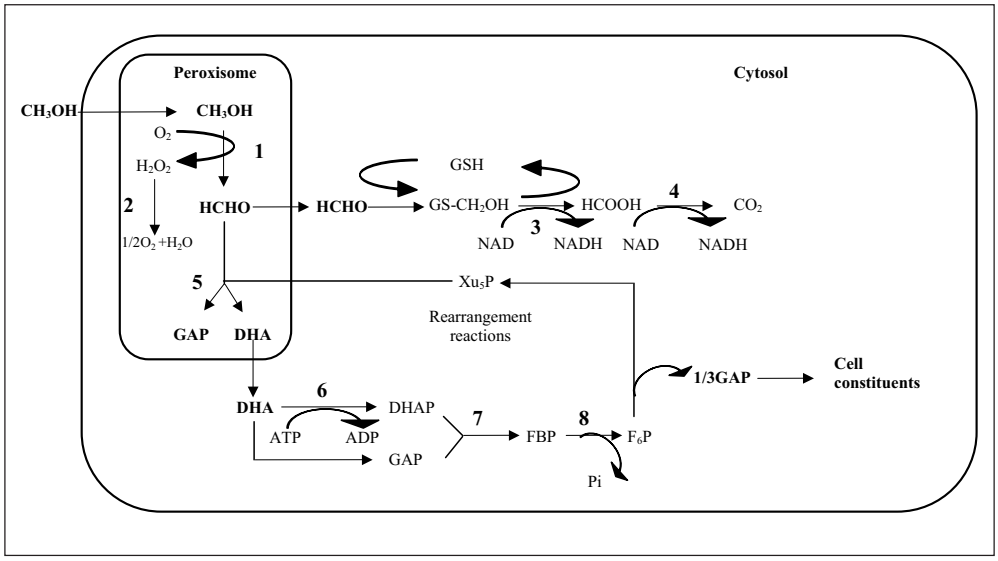


Figure 7. Methanol utilization pathway and its compartmentalization in *Pichia pastoris*. Methanol (CH_3OH) is oxidized by alcohol oxidase (**1**) within the peroxisome to produce formaldehyde (HCHO) and hydrogen peroxidase (H_2O_2). Hydrogen peroxidase is reduced to water (H_2O) and molecular oxygen (O_2) by catalase (**2**). Formaldehyde may diffuse to the cytosol, where it spontaneously reacts with reduced glutathione (GS). This complex is subsequently oxidized to formate (HCOOH) and then carbon dioxide (CO_2) by the actions of formaldehyde dehydrogenase (**3**) and formate dehydrogenase (**4**), respectively. Alternatively, formaldehyde may react with xylulose 5-phosphate (Xu_5P) by the action of dihydroxyacetone synthase (**5**) within the peroxisome to produce glyceraldehyde 3-phosphate (GAP) and dihydroxyacetone (DHA). Phosphorylation of DHA to dihydroxyacetone phosphate (DHAP) by the action of dihydroxyacetone kinase (**6**) in the cytosol facilitates the production of fructose 1,6-bisphosphate (FBP) by fructose 1,6-bisphosphate aldolase (**7**) and its dephosphorylation to fructose 6-phosphate by fructose 1,6-bisphosphatase (**8**). Subsequent rearrangement reactions generate Xu_5P, which is recycled to react with formaldehyde. The net product of this pathway is generation of one-third of the GAP molecules for biomass (adapted from Gleeson and Sudbery, 1988).

toxic or at least growth-inhibitory to several other organisms, and thus, it reduces contamination of *P. pastoris* cultures. However, in some cases, the use of the methanol-inducible *AOX1* promoter is not suitable, when, for example, producing food products or in large-scale fermentations, where large quantities of methanol become dangerous. Alternative promoters are *Pichia pastoris GAP*, *FLD1*, *PEX8*, and *YPT1* (Liu *et al.*, 1995; Waterham *et al.*, 1997; Sears *et al.*, 1998; Shen *et al.*, 1998;).

One disadvantage of *P. pastoris* as a host organism is the shortage of selectable markers. They have been limited to the biosynthetic pathway genes *HIS4* from *P. pastoris* or *S. cerevisiae*, *ARG4* from *S. cerevisiae*, and *Sh ble* from *Streptoalloteichus hindustanus*, which codes for resistance to the bleomycin-related drug zeocin. However, a new set of biosynthetic markers has been isolated and characterized (Lin Cereghino *et al.*, 2001), thus providing more alternatives for selecting *P. pastoris* transformants.

P. pastoris has an additional advantage over *S. cerevisiae* as a host organism for production of heterologous proteins: it is a poor fermenter. When grown to high cell densities, *S. cerevisiae* produces ethanol as the fermentation product. Ethanol rapidly reaches the toxic level, which limits further growth and production of recombinant proteins. *P. pastoris* prefers aerobic growth and can therefore reach extremely high cell densities (OD_{600} ~ 500). This phenomenon is particularly important when the concentration of the secreted protein is proportional to the concentration of the cells in the culture (Lin Cereghino *et al.*, 2002).

2 AIMS OF THE STUDY

I To evaluate the acceptor specificity of β1,6-N-acetylglucosaminyltransferase derived from human PA1 cells.

II To explore the capability of mammalian glycosyltransferases to catalyze the formation of polylactosaminoglycans containing an internal lacdiNAc unit.

III To determine the acceptor and substrate specificities of glycosyltransferases and glycosidases, respectively, with lacdiNAc-containing saccharides.

IV To compare the folding and secretion of the catalytic ectodomain of rat α2,3-sialyltransferase fused to the Hsp150Δ and MFα carriers in the yeasts *Saccharomyces cerevisiae* and *Pichia pastoris*, and to compare the strength of the *HSP150* promoter with that of the *GAL1*, *ADH1,* and *PGK1* promoters.

V To express rat α2,3-sialyltransferase and human α1,3-fucosyltransferase VII in *Saccharomyces cerevisiae* and *Pichia pastoris*, and to use the recombinant yeast strains in the synthesis of sLex antigen, a determinant of selectin ligands.

3. MATERIALS AND METHODS

Experimental methods used in this study are listed in Table 2. Detailed descriptions of the methods are given in the original publications or references therein. The glycosyltransferases and glycosidases used are listed in Tables 3 and 4, respectively, and the detailed reaction conditions are described in the original publications. Table 5 presents all of the acceptor and control glycans used, with detailed information on their synthesis given in the references. Table 6 lists the applied yeast strains, and a schematic representation of the carrier and fusion proteins is presented in Figure 8.

Table 2. Methods

Method	Study
Chromatography	
Size exclusion chromatography	**II**
Ion exchange chromatography	**II-IV**
Affinity chromatography	**I, II**
Paper chromatography	**I**
Mass spectrometry and NMR spectroscopy	
Positive-ion MALDI-TOF MS	**I, II**
One-dimensional ^{1}H-NMR spectroscopy	**I, II**
Two-dimensional NMR spectroscopy	**II**
Molecular biology	
Bacterial transformation	**I, III, IV**
Baculovirus transfer vector construction/plasmid contruction	**I, III, IV**
Cell wall isolation	**III, IV**
Immunoprecipitation	**III, IV**
Isolation and treatment of cell walls	**III, IV**
β-Lactamase activity assay	**III**
Metabolic labeling	**III, IV**
Northern blotting	**I**
Nucleotide sequencing	**III, IV**
Proteinase K digestion	**IV**
SDS-PAGE	**I, III, IV**
Sf9 insect cell transfection	**I**
Western analysis	**I, III, IV**
Yeast transformation (*P. pastoris*)	**III, IV**
Yeast transformation (*S. cerevisiae*)	**III, IV**

Figure 8.

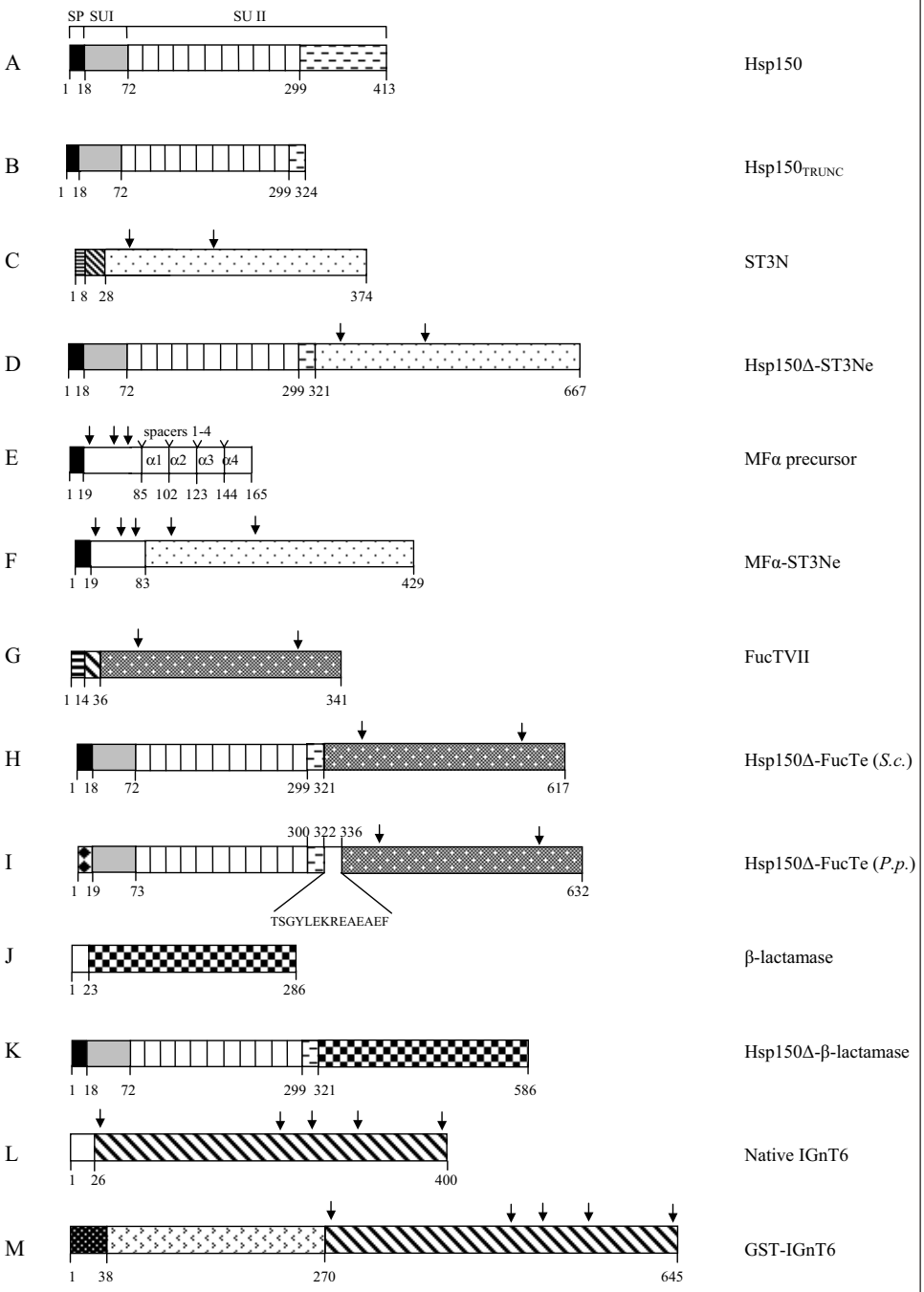

SP SUI
SU II
A
1 18 72 299 413
Hsp150
B
1 18 72 299 324
Hsp150TRUNC
C
1 8 28 374
ST3N
D
1 18 72 299 321 667
Hsp150Δ-ST3Ne
spacers 1-4
α1 α2 α3 α4
E
1 19 85 102 123 144 165
MFα precursor
F
1 19 83 429
MFα-ST3Ne
G
1 14 36 341
FucTVII
H
1 18 72 299 321 617
Hsp150Δ-FucTe (S.c.)
300 322 336
I
1 19 73 632
TSGYLEKREAEAEF
Hsp150Δ-FucTe (P.p.)
J
1 23 286
β-lactamase
K
1 18 72 299 321 586
Hsp150Δ-β-lactamase
L
1 26 400
Native IGnT6
M
1 38 270 645
GST-IGnT6

Table 3. Glycosyltransferase reactions

Glycosyltransferase	Source of enzyme	EC number	Study
β1,4-Galactosyltransferase	Bovine milk, Sigma	EC 2.4.1.90	**II**
β1,3-Galactosyltransferase	Colo 205 cell lysate		**II**
β1,4-N-Acetylgalactosaminyltransferase	Bovine milk, Sigma	EC 2.4.1.90	**II**
β1,3-N-Acetylglucosaminyltransferase	Human serum		**II**
Distally acting β1,6-N-Acetylglucosaminyltransferase	Hog gastric mucosal microsomes		**II**
Centrally acting β1,6-N-Acetylglucosaminyltransferase (a)	Human, produced in *S. frugiperda*	EC 2.4.1.150	**I, II**
Centrally acting β1,6-N-Acetylglucosaminyltransferase (b)	Rat serum		**II**
α1,3-Fucosyltransferase IV	Human, produced in BHK-21 cells	EC 2.4.1.152	**II**
α1,3-Fucosyltransferase VII	Human, produced in yeast	EC 2.4.1.152	**IV**
α2,3-Sialyltransferase	Rat, produced in yeast	EC 2.4.99.5	**III, IV**

Table 4. Glycosidase reactions

Glycosidase	Source of enzyme	EC number	Study
Endo-β-galactosidase	*Bacteroides fragilis* , Boehringer	EC 3.2.1.103	**I, II**
β-Galactosidase	Jack bean, Seikagaku	EC 3.2.1.23	**I, II**
β-N-Acetylhexosaminidase	Jack bean, Sigma	EC 3.2.1.52	**II**
β2,3,4,6,-N-Acetylglucosaminidase	*Streptococcus pneumoniae,* produced in *E. coli,* Calbiochem	EC 3.2.1.30	**II**
β1,4-Galactosidase (*Diplococcus pneumoniae*)	*Diplococcus pneumoniae,* Boehringer	EC 3.2.1.23	**II**

Figure 8. Carrier polypeptides and recombinant proteins used in this study. **(A)** Hsp150 consists of a signal peptide (SP) and subunits (SU) I and II. SUII is composed of 11 repeats of homologous 19 amino acid peptides and a unique C-terminal fragment. **(B)** Truncated Hsp150 ($Hsp150_{Trunc}$) consists of the 319 N-terminal amino acids of Hsp150, and 5 additional amino acids (NLINC) in the C-terminus. **(C)** Rat liver ST3N consists of an N-terminal cytoplasmic tail of 8 amino acids, a transmembrane domain (amino acids 9-28), and a catalytic ectodomain of 346 amino acids. **(D)** ST3Ne joined to the C-terminus of Hsp150Δ carrier. **(E)** The mating factor α (MFα) precursor is composed of the 85-amino acid leader sequence and four 13-amino acid MFα repeats (α1-α4), which are separated from each other by a peptide spacer of 5-8 amino acids that contains the Kex2p cleavage site. **(F)** ST3Ne fused to the MFα carrier without the Kex2p processing site. **(G)** Human α1,3-fucosyltransferase VII consists of 14-amino acid N-terminal cytoplasmic tail, a transmembrane domain (amino acids 15-36), and a catalytic ectodomain of 295 amino acids. **(H)** Catalytic ectodomain of human α1,3-fucosyltransferase VII (FucTe) fused to the C-terminus of the Hsp150Δ carrier. **(I)** FucTe fused to the Hsp150Δ carrier. The original signal peptide is replaced by the MFα signal peptide. FucTe and the Hsp150Δ carrier are separated by a Kex2 protease cleavage site. **(J)** β-lactamase of *E. coli* contains a 23-amino acid signal sequence, and the enzyme itself is composed of 263 amino acids. **(K)** β-lactamase joined to the C-terminus of Hsp150Δ carrier. **(L)** Native IGnT6. **(M)** GST-IGnT6 consists of an N-terminal gp67 signal peptide (amino acids 1-38), a glutathione-S-transferase (GST) coding region (amino acids 39-270), and the sequence coding for the stem and the Golgi lumenal regions of native IGnT6.
The numbers indicate the last amino acid of each domain. N-glycosylation sites are designated with arrows.

Table 5. Acceptor and control oligosaccharides

NO	Oligosaccharide		Reference	Study
1	GlcNAcβ1-3Galβ1-4GlcNAc*		Seppo *et al*., 1990	I
2	Galβ1-4GlcNAcβ1-3Galβ1-4GlcNAc*		Renkonen *et al*., 1991	I
3	Galβ1-4GlcNAcβ1-3Galβ1-4GlcNAcβ1-3Galβ1-4GlcNAcβ1-3Galβ1-4GlcNAc		Salminen *et al*., 1997	I
4	Galβ1-4GlcNAcβ1-3Galβ1-4GlcNAc* (Fucα1-3)		Räbinä *et al*., 1997	I
5	GalNAcβ1-4GlcNAcβ1-3Galβ1-OMe	(glycan **1** in Study **II**)	Palcic and Hindsgaul, 1991	II
6	GalNAcβ1-4GlcNAcβ1-OMe	(glycan **9** in Study **II**)	Palcic and Hindsgaul, 1991	II
7	Galβ1-4GlcNAcβ1-3Galβ1-4Glc	(glycan **14** in Study **II**)	Nyame *et al*, 1999	II
8	Galβ1-4GlcNAcβ1-3Galβ1-3GlcNAc		Sigma	III
9	Galβ1-4GlcNAc		Sigma	IV
10	NeuNAcα2-3Galβ1-4GlcNAc		Sigma	IV
11	Galβ1-4GlcNAcβ1-3Galβ1-4GlcNAcβ1-3Galβ1-OMe		Niemelä *et al*., 1998	II
12	Galβ1-4GlcNAcβ1-3Galβ1-4GlcNAcβ1-3Galβ1-4GlcNAc (Fucα1-3)		Niemelä *et al*., 1999	II

* Otherwise identical but radiolabeled oligosaccharides were synthesized as described in given reference but using either ^{14}C- or ^{3}H-labeled nucleotide sugar donors.

Table 6. Yeast strains

S. cerevisiae **strain**	**Relevant mutation**	**Recombinant protein**	**Study**	**Source/Reference**
H1	none		III, IV	R. Schekman
H23	*Δhsp150*		III, IV	Russo *et al.* , 1992
H335	none	(P_{HSP150})Hsp150Δ-β-lactamase	III	Simonen *et al.,* 1994
H430	*Δhsp150*	$Hsp150_{TRUNC}$	III	Fatal *et al.* , 2002
H626	*Δhsp150*	Hsp150Δ-ST3Ne	III, IV	Mattila *et al* , 1996
H649	*Δhsp150*	Hsp150Δ-α1,3FucTe	IV	This study
H970	*Δhsp150*	Hsp150Δ-ST3Ne, Hsp150Δ-α1,3FucTe	IV	This study
H1120	*Δhsp150*	MFα-ST3Ne	III	This study
H1208	*Δhsp150*	(P_{PGK1})Hsp150Δ-β-lactamase	III	This study
H1209	*Δhsp150*	(P_{GAL1})Hsp150Δ-β-lactamase	III	This study
H1210	*Δhsp150*	(P_{ADH1})Hsp150Δ-β-lactamase	III	This study
P. pastoris **strain**	**Relevant mutation**	**Recombinant protein**	**Study**	**Source/Reference**
P714	none		III, IV	Invitrogen
P1402	none	Hsp150Δ-ST3Ne	III, IV	This study
P1403	none	MFα-ST3Ne	III	This study
P1755	none	Hsp150Δ-α1,3FucTVII	IV	This study

4. RESULTS

4.1. Centrally acting β1,6-N-acetylglucosaminyltransferases

Multiply branched polylactosaminoglycans with distal lacNAc units decorated with α2,3-N-acetylneuraminic acid and α1,3-fucose (sLex) (Fig. 9) have been shown in *in vitro* experiments to be nanomolar L-selectin antagonists (Renkonen *et al.*, 1997). To obtain multivalent sLex polylactosaminoglycans in reasonable amounts to study their capability of inhibiting adhesion of lymphocytes to endothelium, or bacteria and viruses to host tissues *in vivo*, their enzymatic *in vitro* synthesis should be optimized. With the aid of recombinant DNA technology, enzymes can be produced in higher amounts than with traditional purification methods from tissues.

There are two kinds of β1,6-GlcNAc transferases involved in *in vitro* branching of polylactosaminoglycans: distally acting β1,6-GlcNAc transferases (dIGnT6), which catalyze the β1,6-GlcNAc bond formation to the peridistal galactose residue, and centrally acting β1,6-GlcNAc transferases (cIGnT6), which catalyze the bond formation to the internal galactose residues in linear polylactosaminoglycans (for references, see Introduction section 1.1., and Discussion section 5.4.) (Fig. 10). The *in vitro* synthesis of multiply branched polylactosaminoglycan chains (reviewed in Renkonen, 2000) using mammalian blood serum (Leppänen *et*

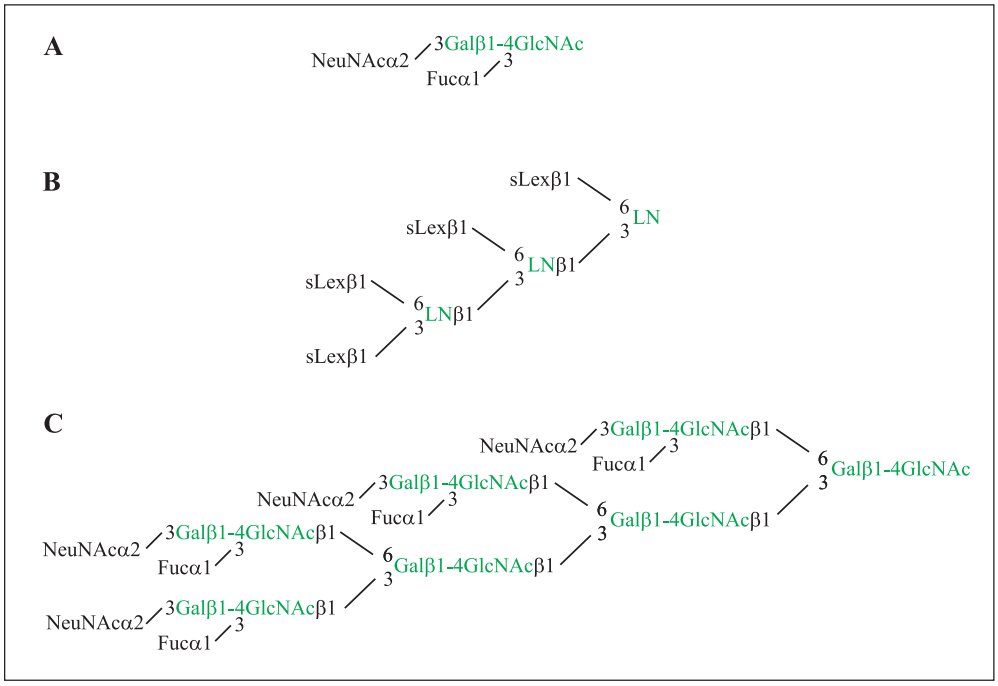


Figure 9. (A) Sialyl Lewis x (sLex) tetrasaccharide. **(B)** and **(C)** Linear tetravalent sLex glycan presented in two different ways.

al., 1991, 1997) and human (PA1) embryonal carcinoma cell lysates (Leppänen *et al.*, 1998), both of which contain centrally acting β1,6-GlcNAc activities, suggests that cIGnT6 rather than dIGnT6 activity is responsible for naturally occurring multiply branched polylactosaminoglycans. *IGnT* cloned from human PA1 cells (Bierhuizen *et al.*, 1993) was expressed in insect cells, and the acceptor specificity of the recombinant enzyme was evaluated in Study **I**.

4.1.1. Expression of human embryonal carcinoma cell β1,6-N-acetylglucosaminyltransferase in insect cells (**I**)

The cDNA coding for the β1,6-GlcNAc transferase (IGnT6) responsible for branching of polylactosaminoglycans in human PA1 cells has been cloned and sequenced (Bierhuizen *et al.*, 1993), but the acceptor specificity of the enzyme had not been studied. To produce pure enzyme for *in vitro* specificity studies, the cDNA encoding the soluble ectodomain of human IGnT6 (Fig. 8L) (amino acids 26-400) was inserted into the pAcSecG2T baculovirus vector to form the transfer vector pAcSecG2T-IGnT6, which was used to transfect Sf9 insect cells. Northern blot analysis revealed a new RNA transcript (2.3 kb) in infected cells (**I**, Fig. 2A). The fusion protein (GST-IGnT6) was detected by Western blot analysis using a monoclonal antibody against GST (Glutathione-S-Transferase), which recognized bands of 67 and 74 kDa in cell lysates at 48-96 hours after infection (**I**, Fig. 2B). An effective one-step purification was carried out using affinity chromatography with glutathione-agarose beads, which bind to the GST moiety of the fusion protein. Cell lysate samples were run through the column and the protein was eluted with glutathione. Coomassie Blue staining after SDS-PAGE of the purified fusion protein revealed a major band at 67 kDa (**I**, Fig. 4). The yield of the recombinant fusion protein was 750 µg/ 10^9 infected Sf9 cells in one liter of the suspension culture.

4.1.2. Determination of the acceptor specificity of the recombinant GST-IGnT6 (**I**)

The polylactosaminoglycans used as acceptors are presented in Table 5. The enzymatic activity and acceptor specificity of the recombinant GST-IGnT6 were first studied by using Sf9 cell lysates as the enzyme source. The radiolabeled acceptor saccharides (Table 5, glycans **1** and **2**) were incubated with the sugar nucleotide donor UDP-GlcNAc and the cell lysate in appropriate buffer conditions, and the reaction products were analyzed with paper chromatography. The acceptor trisaccharide GlcNAcβ1-3[^{14}C]Galβ1-4GlcNAc (glycan **1**) migrated alone, suggesting the absence of product saccharides (**I**, Fig. 5A). In contrast, the

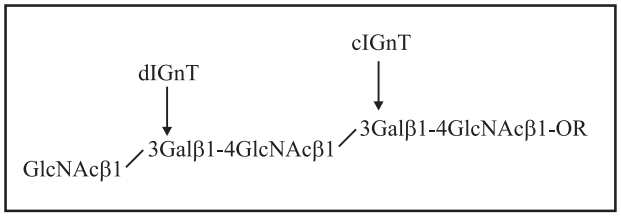


Figure 10. Site-specificity of distally and centrally acting β1,6-N-acetylglucosaminyltransferases.

acceptor tetrasaccharide [^{14}C]Galβ1-4GlcNAcβ1-3Galβ1-4GlcNAc (glycan **2**) was converted in significant amounts into a product that migrated like a pentasaccharide (data not shown).

The acceptor specificity of the affinity-purified recombinant GST-IGnT6 was studied by incubating the fusion protein with UDP-GlcNAc and [^{3}H]Galβ1-4GlcNAcβ1-3Galβ1-4GlcNAc (Table 5, glycan **2**). According to its radioactivity, the reaction mixture contained 28% of a pentasaccharide product, which comigrated with the control pentasaccharide [^{3}H]Galβ1-4GlcNAcβ1-3(GlcNAcβ1-6)Galβ1-4GlcNAc in paper chromatography (**I**, Fig. 5B, peak 1), leaving 72% of the acceptor tetrasaccharide (**I**, Fig. 5B, peak 2) unreacted.

Another reaction mixture containing the purified GST-IGnT6 in a higher concentration to improve the product yield was analyzed with MALDI-TOF mass spectrometry. The spectrum revealed two major peaks, indicating the presence of the pentasaccharide product and acceptor tetrasaccharide. About half of the tetrasaccharide was converted into the product pentasaccharide, as calculated from the peak intensities (**I**, Fig. 6A).

Jack bean (exo)-β-galactosidase and *Bacteroides fragilis* endo-β-galactosidase digestions were performed to study the position of the newly formed β1,6-GlcNAc branch in the pentasaccharide product, and the reaction products were analyzed with paper chromatography. Jack bean (exo)-β-galactosidase treatment removed the tritium-labeled galactose from the reducing end of the pentasaccharide (**I**, Fig. 7A). This suggested that the GlcNAc was not transformed to the distal galactose with β1,3- or β1,6-linkage. Endo-β-galactosidase should cleave the internal β-galactoside bond in a linear tetrasaccharide substrate (Scudder *et al.*, 1984), but should not hydrolyze the branched Galβ1-4GlcNAcβ1-3(GlcNAcβ1-6)Galβ1-4GlcNAc (Leppänen *et al.*, 1997). The incubation of the pentasaccharide with endo-β-galactosidase did not yield any breakdown products (**I**, Fig. 7B), indicating that the GlcNAc was transferred to the internal galactose in the linear acceptor tetrasaccharide to form pentasaccharide product [^{3}H]Galβ1-4GlcNAcβ1-3(GlcNAcβ1-6)Galβ1-4GlcNAc.

The ^{1}H-NMR spectrum of the product pentasaccharide (**I**, Fig. 8, Table II) showed identical structural reporter group resonances with authentic Galβ1-4GlcNAcβ1-3(GlcNAcβ1-6)Galβ1-4GlcNAc (Maaheimo *et al.*, 1997).

In conclusion, the β1,6-GlcNAc transferase activity responsible for branching of polylactosaminoglycans in human PA1 cells possesses centrally acting β1,6-GlcNAc transferase activity rather than distally acting activity.

4.1.3. Production of multiply branched polylactosaminoglycan using recombinant fusion protein (**I**)

Tetravalent sLex glycan (Fig. 9) has been shown to inhibit L-selectin-dependent lymphocyte adhesion to cardiac endothelium during acute heart transplant rejection (Renkonen *et al.*, 1997). In the Stamper-Woodruff binding assay (Stamper and Woodruff, 1976), where the lymphocytes were preincubated with different concentrations of tetravalent sLex glycan, the IC_{50} (50% inhibitory concentration) value was 1 nM

(Renkonen *et al.*, 1997). To test these observations *in vivo*, larger amounts of the multivalent sLex glycan are needed. The capability of the recombinant fusion protein GST-IGnT6 to branch linear octasaccharide acceptor (Table 5, glycan **3**) was studied. The incubation of the purified recombinant fusion protein and acceptor glycan with sugar nucleotide donor UDP-GlcNAc resulted in a mixture of deca- and nonasaccharide products, and intact acceptor octasaccharide, as shown by MALDI-TOF mass spectrometry (**I**, Fig. 6B). This indicates that the recombinant β1,6-GlcNAc transferase is able to form multiple branches on linear acceptor saccharide, although the efficiency should be improved before increasing the amount of synthesis.

4.2. Reactivity of GalNAcβ1-4GlcNAc determinant with glycosyltransferases and glycosidases (II)

The GalNAcβ1-4GlcNAc (lacdiNAc) determinant replaces the nonreducing Galβ1-4GlcNAc (lacNAc) unit in some human and bovine glycoconjugate glycans, and it is also common in lower vertebrates and invertebrates (for more detailed information, see Table 1). These terminal lacdiNAc determinants can be further modified similarly to their lacNAc analogs. They can be sialylated (Weisshaar *et al.*, 1991; Bergwerff *et al.*, 1995; Dell *et al.*, 1995), fucosylated (Srivatsan *et al.*, 1992; Siciliano *et al.*, 1993; Bergwerff *et al.*, 1995; Dell *et al.*, 1995; Lochnit and Geyer, 1995), and sulfated (Green *et al.*, 1985; Hiyama *et al.*, 1992, and reviewed in Baenziger and Green, 1988). Terminal lacdiNAc units also serve some specific biological functions (Smith and Baenziger, 1988; Fiete *et al.*, 1991; Grinnell *et al.*, 1994; Dell *et al.*, 1995; Seppälä *et al.*, 2001), and some of them are known to be immunogenic (Nyame *et al.*, 1999, 2000; van Remoortere *et al.*, 2000, 2001). A polylactosamine-type elongation reaction of the lacdiNAc determinant generating GlcNAcβ1-3GalNAcβ1-4GlcNAc-OR has not been reported, nor has the existence of truly internal lacdiNAc determinants in natural polylactosaminoglycans.

4.2.1. Elongation of GalNAcβ1-4GlcNAcβ1-OR to a novel GlcNAcβ1-3GalNAcβ1-4GlcNAcβ1-OR determinant by enzyme activity present in human serum (**II**)

Human serum contains β1,3-GlcNAc transferase activity capable of elongating the i-type polylactosaminoglycans (=repeating N-acetyllactosamine units linked together via β1,3-bonds that form linear sugar backbone, Fig. 1) (Piller *et al*., 1983; Yates and Watkins, 1983; Hosomi *et al.*, 1984; Seppo *et al.*, 1990). To study whether this enzyme activity catalyzes the GlcNAcβ1-3GalNAc bond formation to the distal GalNAcβ1-4GlcNAc determinant, GalNAcβ1-4GlcNAcβ1-OR acceptor saccharides [Table 7, glycans **1**, **9**, and **14** (numbering as in Study **II**, Table I)] were incubated with UDP-GlcNAc in human serum or human serum concentrate (Yates and Watkins, 1983). Of glycans **1** and **9**, 12-13 mol% were converted into product saccharides **2** and **10**, respectively. In the

case of glycan **14**, where $(NH_4)_2SO_4$-concentrate of human serum was used as an enzyme source, the product yield was 27 mol%. The MALDI-TOF mass spectra (**II**, Fig. 1) of product saccharides gave major signals that were assigned to the molecular ions of product glycans **2**, **10**, and **15** (Table 7). Full assignment of the ^{1}H and ^{13}C signals from various two-dimensional spectra (**II**, Table III) and the clear interglycosidic correlation in the heteronuclear multiple bond correlation (HMBC) spectrum (**II**, Fig. 3) confirmed the positions of glycosidic linkages in glycan **2**. The correlation between the distal *e*-GlcNAc H1 and the *d*-GalNAc C3 (**II**, Fig. 3) (for monosaccharide denotations, see Table 7) shows that the novel β-glycosidic linkage was GlcNAcβ1-3GalNAc. Identical two-dimensional NMR experiments to those of glycan **2** were performed with glycan **10**, and ^{1}H and ^{13}C signals were assigned (**II**, Table III). The data showed great similarity between the analogous resonances of glycans **2** and **10**. The HMBC spectrum of glycan **10** identified the novel linkage as GlcNAcβ1-3GalNAc (data not shown). One-dimensional ^{1}H-NMR spectrum of glycan **15** (**II**, Table IV) revealed the structural reporter group resonances of acceptor glycan **14** plus one equivalent resonances at 4.574 and 4.174 ppm. Three equivalents resonance was seen at 2.022 ppm. These resonances arose from the distal *e*-GlcNAc H1, the peridistal *d*-GalNAc H4, and the NAc protons (methyl protons of N-acetyl group) of *e*-GlcNAc, respectively. The reporter group signals being identical to those of glycans **2** and **10** suggests that a distal GlcNAcβ1-3GalNAc determinant was also formed here.

These data imply that β1,3-GlcNAc transferase activity present in human serum is able to catalyze GlcNAcβ1-3GalNAc bond formation to lacdiNAc structure. Human serum converted only about 12 mol% of the GalNAcβ1-4GlcNAcβ1-OR into GlcNAcβ1-3GalNAcβ1-4GlcNAcβ1-OR. Under similar reaction conditions, 40-70% of i-type polylactosaminoglycans Galβ1-4GlcNAcβ1-OR are converted into GlcNAcβ1-3Galβ1-4GlcNAcβ1-OR (Leppänen *et al.*, 1997), which suggests that i-type polylactosaminoglycans are better acceptors than GalNAcβ1-4GlcNAcβ1-OR for elongation enzymes present in human serum. In these *in vitro* studies, only one acceptor saccharide was offered to the enzyme present in human serum. If several acceptor saccharides had to compete against each other to be elongated, the elongation of GalNAcβ1-4GlcNAcβ1-OR acceptors would be less than 12%.

4.2.2. Reactions catalyzed by mammalian glycosyltransferases with novel GlcNAcβ1-3GalNAcβ1-4GlcNAcβ1-OR acceptors (**II**)

An overview of the following enzymatic reactions catalyzed by different mammalian glycosyltransferases is presented in Figure 11. Table I of Study **II** with the numbered key oligosaccharides is reproduced here as Table 7 to facilitate the discussion. Glycans **2**, **10**, and **15** were subjected to enzymatic reactions that converted the novel GlcNAcβ1-3GalNAcβ1-4GlcNAcβ1-OR glycans into several types of polylactosaminoglycan analogs carrying internal lacdiNAc

determinants. The results from these experiments indicate that this trisaccharide motif functions as an acceptor for several mammalian glycosyltransferases, which are known to modify ordinary polylactosaminoglycans.

4.2.2.1. β1,4-Galactosyltransferase

Bovine β1,4-galactosyltransferase has been shown to behave flexibly with different donor (UDP-Gal/UDP-GalNAc) and acceptor saccharides (Palcic and Hindsgaul, 1991). The GlcNAcβ1-3GalNAcβ1-4GlcNAcβ1-OR glycans (**2**, **10**, and **15**) were incubated with β1,4-galactosyltransferase and UDP-Gal. The product saccharides (glycans **3**, **11**, and **17**) were formed in high yields (over 90 mol%). The MALDI-TOF mass spectra of glycans **3**, **11**, and **17** revealed major signals at *m/z* 988.3, 826.2, and 1136.3, respectively, which were assigned to the acceptor glycans together with one hexose unit. One-dimensional ^{1}H-NMR spectra of product glycans **3** (**II**, Table II), **11**, and **17** (**II**, Table IV) contained the reporter group resonances of the acceptor glycans and additional H1 doublets at 4.475 ppm (in the case of glycan **3** at 4.474 ppm) arising from the novel β1,4-linked galactose. The reactions also caused the downfield shifts of the H1 of the *e*-GlcNAc residues, seen also with distal β1,4-galactosylation of ordinary i-type polylactosaminoglycans (Leppänen *et al.*, 1997).

Glycan **2** was incubated with β1,4-galactosyltransferase and UDP-GalNAc. The reaction gave a product pentasaccharide, glycan **7**, in a yield exceeding 95 mol%. The MALDI-TOF mass spectrum of the purified glycan **7** gave a major signal, which represented the molecular ion of $Hex_1HexNAc_4OMe$. The one-dimensional ^{1}H-NMR spectrum confirmed the glycan **7** to contain two adjacent lacdiNAc units (**II**, Fig. 2F, Table II).

4.2.2.2. β1,3-Galactosyltransferase

Colon carcinoma cells (Colo 205) are known to contain β1,4-galactosyltransferase and β1,3-galactosyltransferase activities (Holmes, 1989). Isshiki *et al*. (1999) cloned, expressed, and characterized the β1,3-GalT activity from Colo 205 cells and named it β1,3Gal-T5. Glycan **2** was incubated with Colo 205 cell lysate and UDP-Gal in the presence of α-lactalbumin, which inhibits β1,4-galactosyltransferase activity of the cell lysate against GlcNAc (Brew *et al.*, 1968; Schanbacher and Ebner, 1970). The minor β1,4-galactosylated product (glycan **3**) was degraded by linkage-specific β1,4-galactosidase from *Diplococcus pneumoniae* (Hughes and Jeanloz, 1964; Renkonen *et al.*, 1989), leaving the β1,3-galactosylated product (glycan **8**) intact. The resulting glycan **2** was hydrolyzed with β2,3,4,6-N-acetylglucosaminidase from *Streptococcus pneumoniae*. The MALDI-TOF mass spectrum confirmed the product saccharide composition to be $Hex_2HexNAc_3OMe$. High-pH anion exchange chromatography (HPAEC) on a PA1 column separates slowly eluting Galβ1-3GlcNAc isomers from faster eluting Galβ1-4GlcNAc isomers (Townsend *et al.*, 1988). Glycan **8** emerged from the column at 4.77 min in 40 mM NaOH. Glycan **3** (representing the Galβ1-4GlcNAc isomer of glycan **8**) in an identical run gave a major peak at 2.56 min. The MALDI-TOF MS and

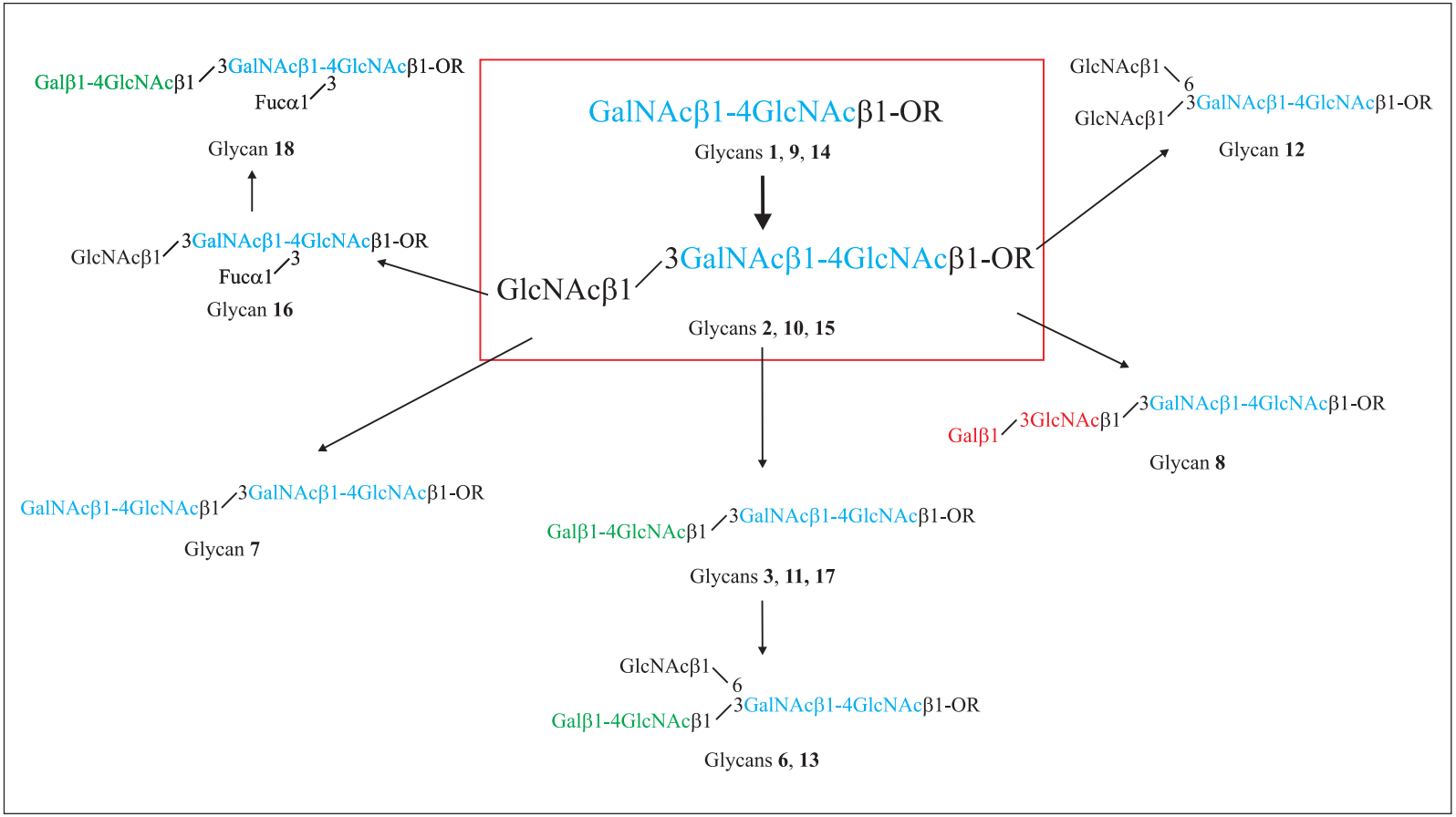
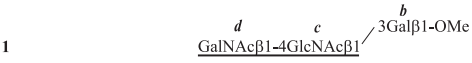
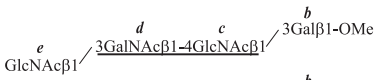
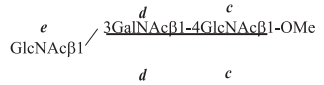
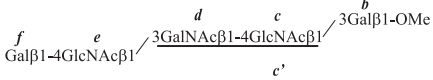
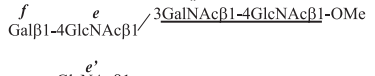
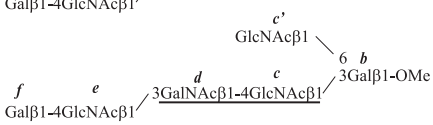
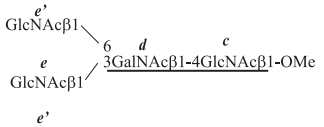
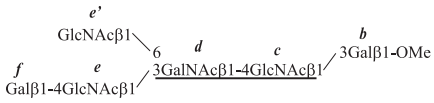
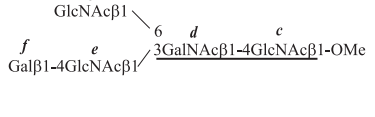
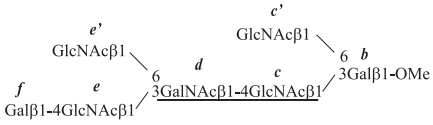
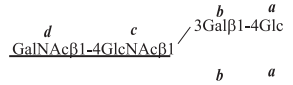
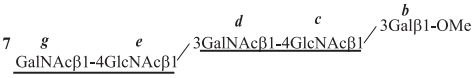
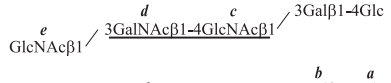
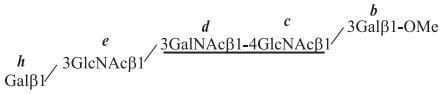
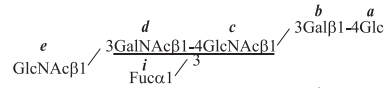
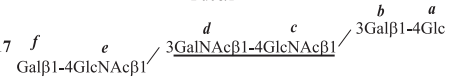
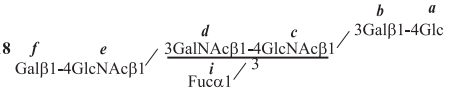


Figure 11. An overview of the *in vitro* reactions catalyzed by mammalian glycosyltransferases with acceptor saccharides containing internal lacdiNAc determinant of Study **II**.

Table 7. Structures of the key oligosaccharides and denotations of the monosaccharide residues used in Study II (Table 1 of Study II).

NO	Saccharide	NO	Saccharide
1		9	
2		10	
3		11	
4		12	
5		13	
6		14	
7		15	
8		16	
		17	
		18	

HPAEC data together confirmed the glycan **8** to represent a pentasaccharide with a distal Galβ1-3GlcNAc determinant.

4.2.2.3. β1,6-N-Acetylglucosaminyltransferases

Glycan **3** was incubated with UDP-GlcNAc in a rat serum concentrate (Leppänen *et al.*, 1997) containing β1,6-N-acetylglucosaminyltransferase activity (cIGnT6) capable of branching ordinary i-type polylactosaminoglycans (Gu *et al.*, 1992; Leppänen *et al.*, 1997). The MALDI-TOF mass spectra proved the reaction mixture to consist of doubly branched glycan **6**, glycans **4** and **5** carrying a single β1,6-GlcNAc branch, and acceptor glycan **3**. The structural analysis of glycans **4**, **5**, and **6** was carried out studying the ^{1}H-NMR spectra of glycan **6** (**II**, Fig. 2E, Table II), the mixture of glycans **4** and **5** (**II**, Fig. 2D), and acceptor glycan **3** (**II**, Fig. 2C, Table II). Comparing these data together and to

the published ^{1}H-NMR data (Leppänen *et al.*, 1997; Maaheimo, 1998), the structure of glycan **6** was interpreted to consist of acceptor glycan **3** carrying β1,6-GlcNAc branches at the ***b***-Gal and at the internal GalNAc. The mixture of glycans **4** and **5** consisted of acceptor glycan **3** carrying a β1,6-GlcNAc branch either at the ***b***-Gal or at the internal GalNAc.

The purified, recombinant form of the centrally acting β1,6-N-acetylglucosaminyltransferase from human PA1 cells (Study **I**) was used to branch glycan **11**. The MALDI-TOF mass spectrum (**II**, Fig. 5) consisted of two major peaks representing the product glycan **13** and the acceptor glycan **11**. The product yield calculated from the peak intensities was 20 mol%. When glycan **11** and control saccharide Galβ1-4GlcNAcβ1-3Galβ1-4GlcNAc were incubated in similar reaction conditions, the yields were 7 mol% and 97 mol%, respectively (data not shown), indicating that the lacdiNAc-containing glycan was a poor acceptor for the recombinant IGnT6 as compared with the control saccharide.

Hog gastric mucosal microsomes contain distally acting β1,6-N-acetylglucosaminyl-transferase activity (Piller *et al.*, 1984; Seppo *et al.*, 1990; Helin *et al.*, 1997). Glycan **10** was incubated with UDP-GlcNAc in a hog gastric microsome suspension. The MALDI-TOF mass spectrum confirmed the composition of the product to be $HexNAc_4OMe$. The one-dimensional ^{1}H-NMR spectrum of glycan **12** (**II**, Table IV) revealed all of the structural reporter group resonances of the acceptor glycan **10** and an additional H1 doublet at 4.566/4.578 ppm that emerged from the β1,6-linked *e'*-GlcNAc-H1. In addition to the two NAc proton singlets arising from the two GlcNAc residues in glycan **10**, the new NAc proton resonance at a lower field arising from β1,6-linked GlcNAc was detected at 2.061 ppm. An almost identical NAc proton resonance is seen in GlcNAcβ1-3(GlcNAcβ1-6)Galβ1-4GlcNAcβ1-OMe, which was synthesized from GlcNAcβ1-3Galβ1-4GlcNAcβ1-OMe in the manner described previously (Maaheimo, 1998). To conclude, the dIGnT activity present in hog gastric mucosal microsomes is able to catalyze the β1,6-branch formation to peridistal GalNAc in addition to peridistal Gal.

4.2.2.4. α1,3-Fucosyltransferase IV

Glycan **15** was incubated with GDP-Fuc and partially purified recombinant human α1,3-fucosyltransferase IV (FucTIV) expressed in BHK-21 cells (Grabenhorst *et al.*, 1998). The MALDI-TOF mass spectrum (data not shown) revealed a major peak (88% calculated from the peak intensities) representing a monofucosylated reaction product (glycan **16**). A minor peak (12% from the peak intensities) represented the unreacted acceptor glycan **15**, which was separated from the fucosylated glycan **16** using wheat germ agglutinin (WGA) affinity chromatography (Niemelä *et al.*, 1999). Glycan **16** was β1,4-galactosylated as described for glycans **2**, **10**, and **15**, and product glycan **18** was subjected to further characterization. The one-dimensional ^{1}H-NMR spectrum of glycan **18** (**II**, Table IV) revealed the structural reporter group resonances of glycan **17** and a fucosylated internal lacdiNAc determinant (Bergwerff *et al.*, 1993). FucTIV had operated site-specifically on the internal lacdiNAc

unit, as indicated by the absence of the reporter group resonances of Glc-bonded fucose (de Vries *et al.*, 1995), proving that FucTIV is able to fucosylate lacdiNAc units.

To conclude, the results from the experiments with mammalian glycosyltransferases revealed the first successful enzymatic synthesis of a GlcNAcβ1-3GalNAcβ1-4GlcNAc motif, and that this trisaccharide motif can be β1,4-galactosylated, β1,3-galactosylated, β1,4-N-acetylgalactosaminylated, β1,6-N-acetylglucosaminylated, and α1,3-fucosylated with glycosyltransferases known to modify ordinary mammalian polylactosaminoglycans.

4.2.3. Enzymatic degradation of polylactosaminoglycans containing internal GalNAcβ1-4GlcNAc unit (**II**)

Three different hydrolases were studied to determine their capability to degrade lacdiNAc-containing substrate saccharides and to find a suitable method for detection and isolation of putative lacdiNAc analogs among naturally occurring polylactosaminoglycans.

4.2.3.1. Endo-β-galactosidase

Glycan **11** and control oligosaccharide Galβ1-4GlcNAcβ1-3Galβ1-4GlcNAcβ1-3Galβ1-OMe were digested with endo-β-galactosidase (EβG) from *B. fragilis*. The MALDI-TOF mass spectrum of the digest of glycan **11** (**II**, Fig. 6A) revealed a minor peak, which represented the breakdown product Galβ1-4GlcNAcβ1-3GalNAc of the substrate glycan **11**. The major peak represented the intact substrate, approximately two-thirds of which had survived the degradative action of the enzyme. The control saccharide was cleaved completely in similar reaction conditions as seen in the MALDI-TOF mass spectrum (**II**, Fig. 6B). In conclusion, the lacdiNAc-containing glycan **11** was cleaved slowly, being a poor substrate for EβG.

4.2.3.2. β-Galactosidase and β-N-acetylhexosaminidase

When glycan **17** was incubated with jack bean exohydrolases β-galactosidase and β-N-acetylhexosaminidase, it was completely degalactosylated, as seen in MALDI-TOF mass spectrum (**II**, Fig. 7A), but only a very small fraction of it (~6% of peak intensities) had lost GlcNAc. Glycan **18** was digested similarly, resulting in glycan **16** and leaving the GlcNAcβ1-3GalNAc bond uncleaved. The MALDI-TOF mass spectrum of a parallel control reaction with the heptasaccharide Galβ1-4GlcNAcβ1-3Galβ1-4(Fucα1-3)GlcNAcβ1-3Galβ1-4GlcNAc (**II**, Fig. 7B) revealed a complete loss of the distal galactose and an almost complete loss of the subterminal β1,3-linked GlcNAc.

The results from this "double degradation" experiment provide a method for producing naturally occurring lacdiNAc-containing polylactosaminoglycans simply by degrading ordinary ones sequentially with jack bean exohydrolases, leaving lacdiNAc analogs intact at the GlcNAcβ1-3GalNAc stage.

4.3. Expression of recombinant proteins in yeasts (III, IV)

Yeasts have many advantages over cultured animal cells as hosts for the expression of recombinant proteins. Yeasts are easy to cultivate on inexpensive growth media, and a wide array of techniques for genetic manipulation of genes to be expressed is available. Importantly, foreign proteins can be directed by yeast-derived signal peptides to the secretory pathway, where posttranslational modifications take place. If the protein being expressed is a secretory protein, it has to be transported through the secretory compartments to be correctly modified. In addition, the secretion of heterologous proteins from yeast is desirable to simplify the purification of the recombinant protein since the level of endogenous secreted proteins is quite low. And finally, many yeasts are safe organisms, which have been awarded the GRAS (generally recognized as safe) status.

4.3.1. Expression of rat liver α2,3-sialyltransferase in yeasts *Saccharomyces cerevisiae* and *Pichia pastoris* (**III**)

The catalytic ectodomain of α2,3-sialyltransferase of rat liver (ST3Ne) has been shown to acquire an active and secretion-competent form in *Saccharomyces cerevisiae*, but only when fused to the Hsp150Δ carrier (Mattila *et al.*, 1996; Sievi *et al.*, 1998). The Hsp150Δ carrier is an N-terminal fragment of a natural secretory protein of yeast (Fig. 8A). It promotes proper folding in the ER of heterologous proteins fused to its C-terminus and efficient secretion of the fusion protein (Simonen *et al.*, 1994, 1996; Holkeri *et al.*, 1996). To compare the catalytic activity and secretion competence of the Hsp150Δ carrier to those of the commercially available MFα carrier, ST3Ne was fused to the C-terminus of both carriers (Fig. 8), and the fusion proteins were expressed in *S. cerevisiae* and *P. pastoris* under the control of *HSP150* and *AOX1* promoters, respectively. *HSP150* is upregulated by shift of the cells from 24°C to 37°C and by nitrogen starvation (Russo *et al.*, 1993). *AOX1* codes for the alcohol oxidase, a key enzyme of the methanol utilization pathway in methylotrophic yeast *P. pastoris*, and its strong and highly regulated promoter is induced by methanol (Cregg *et al.*, 1989). The fusion proteins did not contain the Kex2p cleavage site, resulting in the secretion of the full-length fusion proteins. The activity of ST3Ne was measured from the cell lysates, the cell walls, and the culture media after overnight cultivation. In *S. cerevisiae*, 60% of the MFα-ST3Ne activity was in the medium, 40% in the cell wall, and virtually none inside the cells. In the case of Hsp150Δ-ST3Ne, more of the activity remained inside the cells, and most of the externalized protein stayed in the cell wall (**III**, Fig. 1C, a and b). In *P. pastoris* (**III**, Fig. 1C, c and d), no significant differences were obsereved between the carriers in the distribution of ST3Ne activity within the cell and the cell exterior. With both carriers, a small amount of ST3Ne activity remained intracellular, and most of the secreted ST3Ne activity was cell-wall-associated. In both yeast species, more total activity was achieved with the Hsp150Δ-ST3Ne fusion protein than with MFα-ST3Ne. To conclude, both

carriers promoted proper folding of the catalytically active ST3Ne portion of the fusion protein in the ER of both yeast species.

4.3.2. Binding of fusion proteins to the *S. cerevisiae* cell wall (**III**)

Yeast cells are surrounded by a porous cell wall, a rigid but dynamic structure that is essential for their viability, providing physical and osmotic support. The cell wall is composed of mannoproteins (40%), β1,3-glucan (50%), β1,6-glucan (10%), and chitin (1-3%). The β1,3-glucan-chitin complex is the major constituent of the inner cell wall (Lipke and Ovalle, 1998), while the mannoproteins reside on the outer surface of the wall. Highly branched

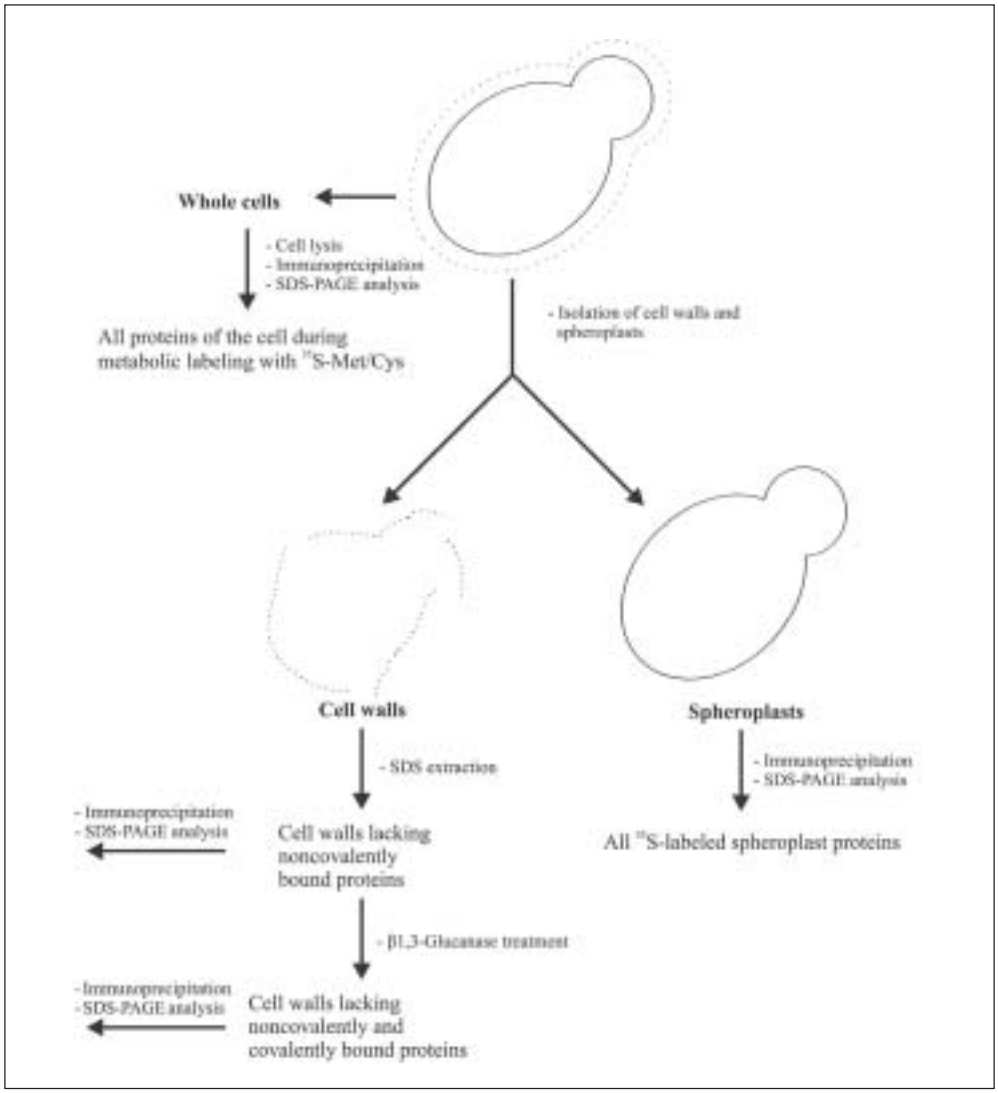


Figure 12. Experimental setup to study the binding mechanism of the fusion proteins to *S. cerevisiae* cell walls.

β1,6-glucan interconnects the cell wall components, mannoproteins, β1,3-glucan, and chitin (Kollar *et al.*, 1997).

Hsp150Δ-ST3Ne is secreted in active, N-glycosylated, and disulfide-bonded form to the *S. cerevisiae* cell wall, to which the *de novo* synthesized molecules remain tightly bound (Mattila *et al.*, 1996; Sievi *et al.*, 1998). The binding mechanism of Hsp150Δ-ST3Ne and MFα-ST3Ne to the *S. cerevisiae* cell wall was studied next. *S. cerevisiae* cells were metabolically labeled, and the cell walls were isolated. Samples consisting of whole-cell lysates, spheroplasts, SDS extracts of cell walls, or SDS-extracted cell walls digested with β1,3-glucanase (Fig. 12) were immunoprecipitated with Hsp150 antiserum or MFα antiserum, and the precipitates were analyzed with SDS-PAGE (**III**, Fig. 2A). Both fusion proteins were released from the cell walls with SDS, indicating that their binding was noncovalent.

The following reporter proteins served as controls. Truncated Hsp150 consisting of 319 N-terminal amino acids (Fig. 8B) is expressed mostly in secreted form (Fatal *et al.*, 2002), and the small fraction remaining cell wall bound was also released with SDS (**III**, Fig. 2B). The authentic Hsp150 (Fig. 8A) was not released with SDS, but was with β1,3-glucanase treatment, indicating that its binding was covalent. To conclude from these results, the ST3Ne component was responsible for the noncovalent binding of the fusion proteins to the cell wall, whereas the C-terminal domain bound authentic Hsp150 covalently to the cell wall.

4.3.3. Determination of the strength of the *HSP150* promoter (**III**)

The *HSP150* promoter was studied in *S. cerevisiae* by comparing its strength with three well-known strong promoters, namely *GAL1*, *ADH1*, and *PGK1*. The *HSP150* promoter provides a basal level of expression at a physiological temperature of 24°C and is upregulated upon shift of the cells to a heat shock temperature of 37°C (Russo *et al.*, 1993). *E. coli* β-lactamase fused to the Hsp150Δ carrier (Hsp150Δ-β-lactamase), which is rapidly secreted to the culture medium (Simonen *et al.*, 1994), was used as a reporter protein. *S. cerevisiae* strains expressing Hsp150Δ-β-lactamase under the control of promoters *HSP150*, *GAL1*, *ADH1*, and *PGK1* were grown overnight at 24°C to early logarithmic growth phase. Cells were pelleted, resuspended in fresh medium, and the cultivation was continued at 24°C, except for the *HSP150* promoter strain, which was cultivated at 37°C. After 8 hours, in the case of the *HSP150* promoter, 1.5 mU/ml of β-lactamase activity was measured in the culture medium. For the *GAL1*, *ADH1*, and *PGK1* promoters, 2.1, 3.15, and 5.5. mU/ml of β-lactamase activity, respectively, was in the medium (**III**, Fig. 3). In all cases, insignificant amounts of β-lactamase activity were present in cell lysate samples. Thus, the strength of the *HSP150* promoter at 37°C was comparable with that of the *GAL1* promoter at 24°C.

4.3.4. Expression of Hsp150Δ-FucTe in *Saccharomyces cerevisiae* and *Pichia pastoris* (**IV**)

The cDNA fragment encoding the Hsp150Δ carrier was fused to that encoding the catalytic ectodomain of human α1,3-fucosyltransferase VII (FucTe) to express Hsp150Δ-FucTe (Fig. 8H). The recombinant gene was placed under the control of the *HSP150* promoter (Russo *et al.*, 1993) and was integrated into the genome of a *S. cerevisiae* strain from which the *HSP150* gene had been deleted. To study whether Hsp150Δ-FucTe was expressed, the cells were pulse-labeled and chased. After the pulse, a protein of 102 kDa was immunoprecipitated with Hsp150 antiserum from the cell lysate (**IV**, Fig. 3, lane 2). After a chase of 30 minutes, the 102-kDa protein had disappeared, but two proteins of about 150 kDa and 120 kDa were detected from the cell lysate (**IV**, Fig. 3, lane 4). No proteins could be immunoprecipitated from the culture medium (**IV**, Fig. 3, lanes 1 and 3). The 150-kDa protein must have been the mature, extensively O-glycosylated fusion protein, which remained cell wall bound. We assume that the 102-kDa protein was the primary O-glycosylated form located in the ER, whose glycans were elongated during the chase, resulting in the 120-kDa protein.

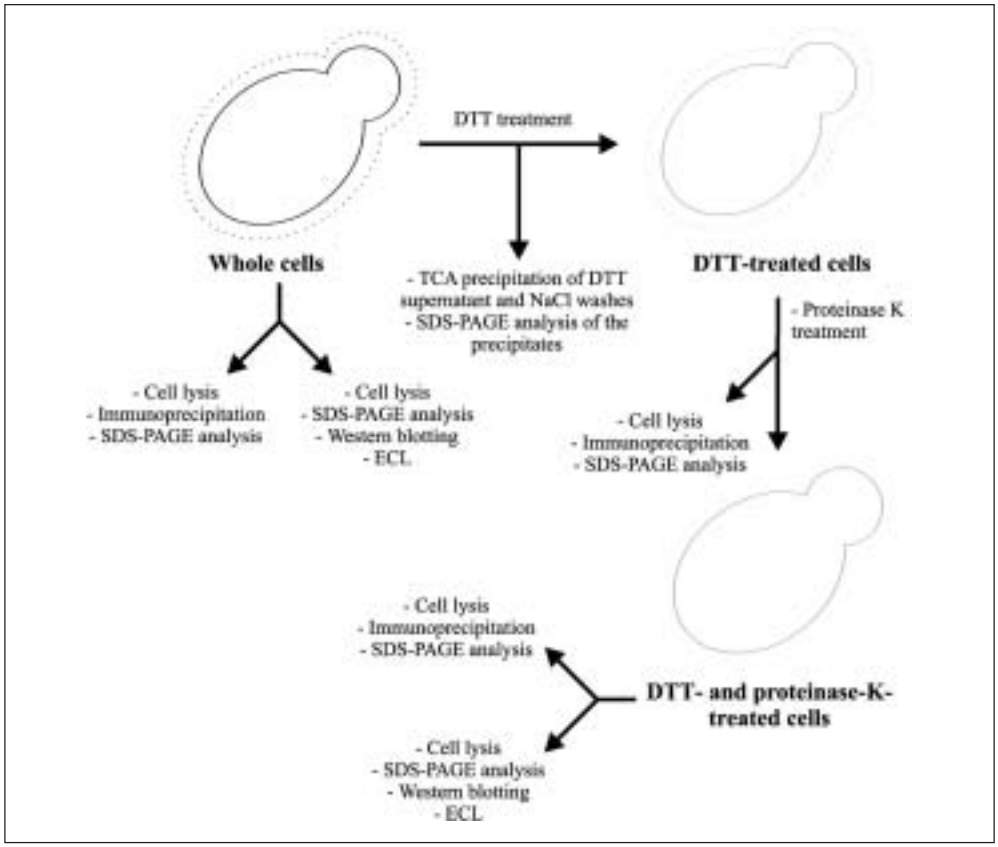


Figure 13. Experimental setup to study the location of the fusion protein Hsp150Δ-FucTe in metabolically labeled *S. cerevisiae* cells.

A *P. pastoris* strain was transformed with an expression vector constructed to produce Hsp150Δ-FucTe. In this case, a recognition site for the Golgi Kex2 protease was constructed between the Hsp150Δ carrier and FucTe (Fig. 8I). The chimeric gene was expressed under the methanol-inducible *AOX1* promoter in the genome of the host strain, and the transformants were grown for 12 days on methanol. The cells and medium samples were screened for expression of Hsp150Δ-FucTe by Western blotting using Hsp150 antiserum, and the transformant giving the strongest signal was chosen and named P1755 (Table 6) (data not shown).

4.3.5. Binding of Hsp150Δ-FucTe to the *S. cerevisiae* cell wall (**IV**)

To see whether Hsp150Δ-FucTe remained intracellular or was transported to the cell wall like Hsp150Δ-ST3Ne, the *S. cerevisiae* strain expressing Hsp150Δ-FucTe (Table 6) was pulse-labeled and chased for an hour. After the chase, a cell lysate sample was immunoprecipitated with Hsp150 antiserum directly, and a parallel sample after proteinase K digestion in the presence of DTT (Fig. 13). Mature Hsp150Δ-FucTe of 150 kDa was detected in the absence of proteinase K digestion, whereas it had disappeared after the digestion (not shown). This implies that Hsp150Δ-FucTe is located in the cell wall and not inside the cells. Proteinase K had destroyed the cell wall but left the cells otherwise intact according to the following control experiment. Cell samples before and after proteinase K treatment were analyzed by Western blotting with antiserum against cytosolic GAPDH. Similar signals were detected, suggesting that proteinase K had not attacked cytosolic components. When the control experiment was repeated using antiserum against the cell wall protein Bgl2p, only the untreated cell sample gave a signal, implying that proteinase K had destroyed cell wall-bound Bgl2p (data not shown).

Cell wall proteins of *S. cerevisiae* are either covalently bound to the complex glucose polymer β-glucan or they are intercolocalized in the glucan layer without covalent bonds. To study whether the binding of Hsp150Δ-FucTe to the *S. cerevisiae* cell wall was covalent or noncovalent (Fig. 12), a set of parallel samples of *S. cerevisiae* cells expressing Hsp150Δ-FucTe was metabolically labeled and chased. Immunoprecipitation of a whole-cell sample revealed a 150-kDa protein representing mature Hsp150Δ-FucTe (**IV**, Fig. 4, lane 1). Another cell sample was subjected to the release of the cell walls. The resulting spheroplasts did not contain the 150-kDa protein (**IV**, Fig. 4, lane 2), indicating that the fusion protein was bound to the cell wall. SDS extraction of the isolated cell walls of yet another cell sample released the 150-kDa protein (**IV**, Fig. 4, lane 3). Another cell wall sample was, after SDS extraction, digested with β1,3-glucanase (**IV**, Fig. 4, lane 4), but no protein was released. This confirms that Hsp150Δ-FucTe was bound noncovalently to the cell wall.

4.3.6. FucTVII activity in *S. cerevisiae* and *P. pastoris* (**IV**)

Next, we studied whether Hsp150Δ-FucTe expressed in *S. cerevisiae* and *P. pastoris* was catalytically active. The intact *S. cerevisiae* cells expressing Hsp150Δ-FucTe were incubated after overnight cultivation with sialyl-lacNAc

and GDP-[^{14}C]Fuc. Of 200 000 cpm of [^{14}C]Fuc, 32 000 cpm had been transferred to sialyl-lacNAc (data not shown), yielding NeuNAcα2,3-Galβ1,4([^{14}C]Fucα1,3)GlcNAc (radioactive sLex). In the case of the parental strain, only 970 cpm was transferred.

The *P. pastoris* strain expressing Hsp150Δ-FucTe was grown in methanol. The FucTVII activity of the culture medium (**IV**, Fig. 5, columns A), spheroplast lysates (columns B), and intact cells (columns C) was determined on days 3, 6, and 9. The majority of the FucTVII activity remained in the cell walls throughout the cultivation, peaking at day 9 (55 000 cpm of 200 000 cpm) (**IV**, Fig. 5). Thus, Hsp150Δ-FucTe was expressed in catalytically active form in the cell walls of S. *cerevisiae* and *P. pastoris*.

4.3.7. Teamwork of FucTVII and ST3N in the yeast cell wall (**IV**)

We next studied whether Hsp150Δ-ST3Ne and Hsp150Δ-FucTe could work *in tandem* to synthesize sLex from lacNAc, CMP-NeuNAc, and GDP-[^{14}C]Fuc. Human fucosyltransferase VII can transfer fucose only to α2,3-sialylated lacNAc (Niemelä *et al.*, 1998), thus [^{14}C] fucosylation implies that sialylation has occurred first. The *S. cerevisiae* strains expressing Hsp150Δ-ST3Ne and Hsp150Δ-FucTe (H626 and H649, respectively) were cultivated overnight separately and thereafter combined in the same test tube containing lacNAc, CMP-NeuNAc, and GDP-[^{14}C]Fuc. More than 11 000 cpm (total 200 000 cpm) of radioactive glycan was produced (**IV**, Fig. 6A, column a).

The *S. cerevisiae* strain coexpressing Hsp150Δ-ST3Ne and Hsp150Δ-FucTe was then constructed and cultivated overnight. When the cells were provided with lacNAc, CMP-NeuNAc, and GDP-[^{14}C]Fuc, about 8500 cpm had been transferred to lacNAc (column b). The parental *S. cerevisiae* strain incubated as above served as a negative control (column c). The *S. cerevisiae* strain expressing Hsp150Δ-FucTe alone was incubated with lacNAc and GDP-[^{14}C]Fuc. This experiment confirmed that FucTVII could not fucosylate unsialylated lacNAc (column d).

Finally, the *P. pastoris* strains expressing Hsp150Δ-ST3Ne and Hsp150Δ-FucTe were cultivated separately for 7 days in methanol, combined in the same test tube, and incubated with lacNAc, CMP-NeuNAc, and GDP-[^{14}C]Fuc. Approximately, 32 000 cpm (total 200 000 cpm) of radioactive sLex was obtained (**IV**, Fig. 6B, column a). Because the culture medium of the *P. pastoris* strain expressing Hsp150Δ-FucTe contained some FucTVII activity (IV, Fig. 5, column a), *P. pastoris* cells expressing Hsp150Δ-ST3Ne were incubated in that medium in the presence of lacNAc, CMP-NeuNAc, and GDP-[^{14}C]Fuc. Much less radioactive sLex was produced (column b) than above, consistent with the notion that most of the fusion proteins remained in the cell wall. The negative control experiments were carried out as described above for *S. cerevisiae* (columns c and d).

To conclude, ectodomains of human fucosyltrasferase VII and rat α2,3-sialyltransferase functioned *in tandem* in the cell wall of intact yeast cells, synthesizing sLex from CMP-NeuNAc, GDP-[^{14}C]Fuc, and lacNAc (**IV**, Fig. 7).

5. DISCUSSION

5.1. Use of mammalian glycosyltransferases

5.1.1. Substrate specificity of recombinant β1,6-N-acetylglucosaminyltransferase

The i- and I-blood group antigens are glycan structures that are characterized by linear and branched repeats of N-acetyllactosamine, respectively, and they are present on various glycolipids and glycoproteins (Fig. 1) (Feizi *et al.*, 1979; Fukuda *et al.*, 1979; Koscielak *et al.*, 1979; Watanabe *et al.*, 1979). The expression of i- and I-antigens is developmentally regulated, and structural changes in these carbohydrates are also seen in cell differentiation and malignancy (reviewed in Leppänen, 1997). Because of the building block, N-acetyllactosamine, they are also referred to as lactosaminoglycans. The linear i-type blood group antigen is synthesized by sequential action of β1,3-N-acetylglucosaminyltransferase and β1,4-galactosyltransferase. It has long been suspected that a precursor-product relationship exists between i- and I-antigens and that the synthesis of branched I-type glycan would require yet another enzymatic activity, that of I-branching β1,6-N-acetylglucosaminyltransferase (IGnT) (Fukuda *et al.*, 1979; Koscielak *et al.*, 1979; Piller *et al.*, 1984). β1,6-N-acetylglucosaminyltransferase has been identified in microsomal preparations from hog gastric mucosal microsomes, and this enzyme activity catalyzes the reaction GlcNAcβ1-3Galβ1-4GlcNAc + UDP-GlcNAc → GlcNAcβ1-3(GlcNAcβ1-6)Galβ1-4GlcNAc (Piller *et al.*, 1984), thus representing distally acting IGnT6 transferase (dIGnT) (Fig. 10) (Piller *et al.*, 1984; Brockhausen *et al.*, 1986; Koenderman *et al.*, 1987; Seppo *et al.*, 1990; Gu *et al.*, 1992; Helin *et al.*, 1997). β1,6-N-acetylglucosaminyltransferase activity, which functions as a centrally acting branching enzyme (cIGnT) (Fig. 10), was described in 1991 (Leppänen *et al.*, 1991). Human (Leppänen *et al.*, 1991), as well as bovine, equine, rat, and ovine (Leppänen *et al.*, 1997), sera contain β1,6-GlcNAc-transferase activities, which catalyze multiple branching to internal galactose residues of a linear polylactosaminoglycan backbone, as confirmed with degradative experiments, MALDI-TOF mass spectrometry, and NMR spectroscopy (Leppänen *et al.*, 1997).

The cDNA encoding IGnT was first cloned from human embryonal carcinoma cells (PA1 cells) (Bierhuizen *et al.*, 1993), which are known to contain large amounts of polylactosaminoglycans covalently bound to proteins (Rasilo *et al.*, 1980; Rasilo and Renkonen, 1982; Fukuda *et al.*, 1985), as well as cIGnT rather than dIGnT activity (Leppänen *et al.*, 1998). The cDNA codes for a type II transmembrane protein, and when expressed in Chinese hamster ovary cells (CHO), which normally express only linear i-antigens (Sasaki *et al.*, 1987; Smith *et al.*, 1990), the cells acquired I-branched polylactosaminoglycan structures (Bierhuizen *et al.*, 1993). In Study **I,** the catalytic ectodomain of this enzyme was fused to GST, the recombinant fusion protein was expressed in Sf9 insect cells, and the acceptor specificity of the fusion

protein was examined. The recombinant protein catalyzed the transfer of GlcNAc from UDP-GlcNAc to the linear tetrasaccharide Galβ1-4GlcNAcβ1-3Galβ1-4GlcNAc (Table 5, glycan **2**) to position 6 of the underlined Gal residue, as confirmed by mass spectrometry, degradative experiments, and ^{1}H-NMR spectroscopy. The recombinant protein did not, however, react with UDP-GlcNAc and trisaccharide acceptor GlcNAcβ1-3Galβ1-4GlcNAc (Table 5, glycan **1**), indicating the need for the presence of at least one complete LacNAc unit bonded to position 3 of the reacting galactose residue. When the recombinant IGnT6 was incubated with the octasaccharide $(Galβ1\text{-}4GlcNAc)_4$ (Table 5, glycan **3**), the MALDI-TOF mass spectrum of the product glycan confirmed that two additional HexNAc units had been added. Thus, the recombinant enzyme could form multiple branches on a preformed i-type polylactosaminoglycan chain. Taken together, Sf9 insect cells as an expression system provides the means to produce functional glycosyl-transferases, and the cDNA isolated from human embryonal carcinoma cells of line PA1 (Bierhuizen *et al.*, 1993) codes for centrally acting β1,6-GlcNAc transferase activity.

To date, three centrally acting human IGnT activities have been cloned and characterized, namely IGnT1, IGnT2, and IGnT3. IGnT1 stands for the enzyme described above, and it was the first one to be cloned and characterized (Bierhuizen *et al.*, 1993 and Study **I**). All three isoforms consist of 3 exons and share the second and third exons (Inaba *et al.*, 2003; Yu *et al.*, 2003). They are considered to be generated by alternative splicing of a single gene. Transcripts of *IGnT1* and *IGnT2* show similar tissue distribution. The expression pattern of *IGnT3*, by contrast, is clearly different. Bone marrow cells preferentially express the *IGnT3* transcript. This transcript is present in reticulocytes, but not in fetal liver (Inaba *et al.*, 2003; Yu *et al.*, 2003), suggesting that the biosynthesis of I-blood group antigens in terminally differentiated erythrocytes results from IGnT3 activity.

The naturally occurring polylactos-aminoglycans usually consist of a long main chain with several short branches (Fukuda, 1985). cIGnT activities have been suggested to be responsible for the multiple short branches in I-antigens (Leppänen *et al.*, 1997). If dIGnT activity was responsible for the multiple branching, acting coordinately with β1,4-GalT and β1,3-GlcNAcT, branched branches would predominate over short branches. The recombinant IGnT described in Study **I** helps to synthesize multiply branched glycans, which can be modified further to carry multivalent sLex sequences. These structures have been shown to be nanomolar inhibitors of L-selectin-mediated adhesion of lymphocytes to activated endothelium (Renkonen *et al.*, 1997), and their synthesis is described in detail elsewhere (Renkonen *et al.*, 1997; Salminen *et al.*, 1997).

5.1.2. Elongation of GalNAcβ1-4GlcNAcβ1-OR to a novel GlcNAcβ1-3GalNAcβ1-4GlcNAcβ1-OR

Polylactosaminoglycans consist of repeating lacNAc units joined by β1,3-linkages. They can be β1,6-branched, and the linear backbones as well as the

branches are the carriers of the terminal structures, which participate in biological activities of glycoconjugates. However, in a number of glycoconjugates studied so far, the lacdiNAc determinant (GalNAcβ1-4GlcNAc) replaces the terminal lacNAc unit (Table 1). This terminal lacdiNAc unit can be decorated with different capping monosaccharides similar to their ordinary lacNAc-containing analogs (reviewed in van den Eijnden *et al.*, 1997). The differently modified lacdiNAc units can have immunogenic properties (Nyame *et al.*, 1999, 2000; van Remoortere *et al.*, 2000, 2001), and they are suspected of modifying the biological activities of the glycoconjugates carrying them (Smith and Baenziger, 1988; Fiete *et al.*, 1991; Grinnell *et al.*, 1994; Dell *et al.*, 1995). However, truly internal lacdiNAc units in naturally occurring polylactosaminoglycans have not been reported, although GlcNAcβ1-3GalNAc linkage is encountered in the core 3 structure of O-glycans (Fig. 2) (Brockhausen *et al.*, 1985) and in *Schistosoma mansoni* glycolipids (Wuhrer *et al.*, 2000). Here, β1,3-N-acetylglucosaminyltransferase activity present in human serum (Piller and Cartron, 1983; Yates and Watkins, 1983; Hosomi *et al.*, 1984; Seppo *et al.*, 1990) catalyzed the formation of GlcNAcβ1-3GalNAc linkage between the incoming GlcNAc monosaccharide and the terminal lacdiNAc determinant in acceptor saccharides of type GalNAcβ1-4GlcNAcβ1-OR. This novel, internal lacdiNAc determinant was identified by NMR data, which included long-range correlations in heteronuclear multiple bond correlation spectra. The yields were, however, considerably lower (12.5 mol%) than those obtained before using acceptor saccharides presenting solely lacNAc units in their backbones (40-70 mol%) (Leppänen *et al.*, 1997). Here, the reaction conditions were optimized to favor bond formation. The conditions were not competitive since only lacdiNAc-containing acceptor saccharides were present. As some purified glycosyltransferases are truly multifunctional, such as sialyl-α2,3-transferase, also known as ST3Gal II, which transfers sialic acid efficiently to Gal and GalNAc (Toivonen *et al.*, 2001), we thought that the same enzyme activity present in human serum is in our *in vitro* experiments responsible for elongating both lacNAc- and lacdiNAc-terminating acceptors. Furthermore, to favor product formation, the acceptor and donor sugar concentrations were kept high to force the reaction to proceed forward (0.24 mM acceptor saccharide, 4.8 mM UDP-GlcNAc).

As the reaction conditions *in vivo* differ dramatically from those of our *in vitro* experiments described above, internal lacdiNAc units may not be as abundant in human polylactosaminoglycans. One of our findings could assist in enriching and characterizing these structures in naturally occurring polylactosaminoglycans. The sequential action of two degradative enzymes, namely jack bean β-galactosidase and β-N-acetylhexosaminidase, hydrolyzes ordinary, desialylated, and defucosylated polylactosaminoglycans monosaccharide by monosaccharide (Niemelä *et al.*, 1998). In polylactosaminoglycans containing internal lacdiNAc units, the action of degradative enzymes stops at the GlcNAcβ1-3GalNAcβ1-4GlcNAcβ1-OR stage, as we have

shown in Study **II**. Thus, this degradative treatment identifies internal lacdiNAc unit-containing polylactosaminoglycans among ordinary ones, providing a means to study their occurrence.

As shown in Figure 11, the synthetic GlcNAcβ1-3GalNAcβ1-4GlcNAcβ1-OR saccharides proved to be functional acceptors for enzyme-assisted β1,3-galactosylation, β1,4-galactosylation, β1,4-N-acetylgalactosaminylation, α1,3-fucosylation, and several β1,6-N-acetylglucosaminylation reactions catalyzed by mammalian glycosyltransferases known to function with ordinary polylactosaminoglycans. The reactions catalyzed by β1,3-galactosyltransferase (Colo 205 cell lysate) (Holmes, 1989), distally acting β1,6-N-acetylglucosaminyltransferase (hog gastric mucosal microsomes) (Piller *et al.*, 1984; Seppo *et al.*, 1990; Helin *et al.*, 1997), and centrally acting β1,6-N-acetylglucosaminyltransferase (rat serum) (Gu *et al.*, 1992; Leppänen *et al.*, 1997) were carried out with crude enzyme preparations. Hence, determining whether the same enzyme activity or two different activities are responsible for catalyzing the reactions with lacNAc- and lacdiNAc-containing acceptor glycans is not possible. Data from our experiments, nevertheless, imply that this trisaccharide motif functions as an acceptor for several pure glycosyltransferases or crude glycosyltransferase preparations that modify natural mammalian polylactosaminoglycans.

To summarize, polylactosaminoglycanbranching enzyme (IGnT6) cloned from human embryonal carcinoma cells (PA1) was expressed in insect cells, and its acceptor specificity was studied. It showed catalytic activity towards internal Gal residues along the linear polylactosaminoglycan, thus exhibiting centrally acting branching activity (cIGnT6). The recombinant enzyme could catalyze the formation of multiple branches on a linear, preformed acceptor glycan, which is the first step in the synthesis of multivalent binding inhibitors of leukocyte adhesion.

We also showed that enzyme activity present in human serum could elongate the acceptor glycans containing terminal lacdiNAc. A novel GlcNAcβ1-3lacdiNAc linkage was formed that generated an internal lacdiNAc determinant, a determinant that has not been encountered in naturally occurring polylactosaminoglycans. These novel glycans functioned as acceptors for mammalian glycosyltransferases, and in addition, a method was developed for searching for naturally occurring internal lacdiNAc determinants.

5.2. Production of mammalian glycosyltransferases in yeast

5.2.1. Hsp150Δ carrier functions in expression of rat α2,3-sialyltransferase in *Saccharomyces cerevisiae* and *Pichia pastoris*

The Hsp150Δ carrier polypeptide, which is an N-terminal fragment of a natural secretory glycoprotein of *Saccharomyces cerevisiae* (Russo *et al.*, 1992), functions in production of heterologous proteins in *S. cerevisiae* by conferring proper folding and secretion competence (Simonen *et al.*, 1994, 1996; Jämsä *et al.*, 1995; Holkeri *et al.*, 1996; Mattila *et al.*, 1996; Sievi *et al.*, 1998). The catalytic ectodomain of rat α2,3-sialyltransferase (ST3Ne) has previously been expressed in yeast *S. cerevisiae* fused to the Hsp150Δ carrier polypeptide, and the fusion protein was externalized but remained bound to the cell wall. This made it possible to use whole yeast cells as an enzyme source for sialylation of glycans and asialo-glycoproteins (Mattila *et al.*, 1996; Sievi *et al.*, 1998).

The cell wall association of the fusion protein Hsp150Δ-ST3Ne was noncovalent, as it could be released with SDS, not needing β1,3-glucanase treatment (**III**). The ST3Ne portion was thought to be responsible for the attachment of the fusion protein to the cell wall because the authentic Hsp150 binds covalently (also shown by Kapteyn *et al.*, 1999) and Hsp150Δ as such is secreted to the culture medium (Fatal *et al.*, 2002).

The performance of the Hsp150Δ carrier in *Pichia pastoris* was studied next and compared with that of the MFα carrier in both *S. cerevisiae* and *P. pastoris*. The MFα carrier is commercially available and widely used. It is the prepro-region of the yeast *S. cerevisiae* mating factor α (Fig. 6) (Kurjan and Herskowitz, 1982; Brake *et al.*, 1983; Julius *et al.*, 1983). The use of the methylotrophic yeast *P. pastoris* as an expression system is attractive when high product levels are desired. The expression system is based on strong, regulated promoters derived from methanol metabolism pathway genes (Fig. 7). This system is commercially available as a kit and has a number of biotechnological applications (Gellissen, 2000; Lin Cereghino and Cregg, 2000).

The efficiency of the Hsp150Δ carrier in *S. cerevisiae* and *P. pastoris* in expression of ST3Ne in catalytically active form was at least as high as that of the MFα carrier (**III**, Fig. 1C). The Hsp150Δ carrier was also functional under the control of the alcohol oxidase promoter (*AOX*) in *P. pastoris*. Thus, this carrier appears to be as effective as the MFα carrier in promoting the proper folding and transport of catalytically active ST3Ne in the ER of *S. cerevisiae* and *P. pastoris*.

The catalytic ectodomain of rat α2,6-sialyltransferase (ST6Ne) has also been expressed as a Hsp150Δ fusion protein (Hsp150Δ-ST6Ne). The ST6Ne portion of Hsp150Δ-ST6Ne acquired a catalytically active conformation in the ER and was transported to the Golgi apparatus. But instead of being externalized, the fusion protein was targeted to the vacuole and degraded there. The signal guiding the fusion protein to destruction was speculated to reside in the ST6Ne portion because the Hsp150Δ fusion proteins have been

considered to be stable (Sievi *et al.*, 2001).

5.2.2 Strenght of the *HSP150* promoter is comparable with that of *GAL1*

Efficient transcription of foreign genes is achieved with promoters of yeast origin, while the use of foreign promoters does not result in accurate initiation of transcription (Romanos *et al.*, 1992). We compared the strength of the *HSP150* promoter with that of three well-known strong promoters of *S. cerevisiae*, namely the *GAL1*, *ADH1*, and *PGK1* promoters. The *HSP150* promoter, derived from the *HSP150* gene of *S. cerevisiae*, was cloned and characterized in our laboratory (Russo *et al.*, 1992). It is upregulated by heat and nutrient stress (Russo *et al.*, 1993). The *HSP150* 5'-flanking region contains three heat shock elements (HSE) (Russo *et al.*, 1992). Site-directed mutagenesis of one of these elements located between the TATA box and the transcription initiation site abolished heat activation of transcription (Russo *et al.*, 1993). Here, the strength of the *HSP150* promoter was found to be comparable with that of the *GAL1* promoter. The *ADH1* and *PGK1* promoters, which belong to the glycolytic promoters of *S. cerevisiae*, were shown to be considerably stronger (3.15 U/ml and 5.5 U/ml, respectively) than *HSP150* and *GAL1* promoters (1.5 U/ml and 2.1 U/ml, respectively), but their disadvantage lies in their nonregulated nature (Romanos *et al.*, 1992). This makes them a poor promoter choice when the growth stage needs to be separated from the expression stage (e.g. with toxic products) or when dealing with large-scale cultures. The *HSP150* promoter is simply activated by shifting the cells from a physiological temperature of 24-30°C to 37°C. To activate the *GAL1* promoter, one needs to change the carbon source of the culture medium from glucose to galactose.

Comparing the strengths of the *GAL1* and *HSP150* promoters, we paid attention to cell densities. The optical density (OD_{600}) of the *HSP150* strain was twice as high as that of the *GAL1* promoter strain (**III**, Fig. 3). If the production of the reporter protein were dependent on the concentration of the cells in the culture, the *GAL1* promoter would have been two times stronger than the *HSP150* promoter.

5.2.3. Expression of the catalytic ectodomain of human α1,3-fucosyltransferase VII in *S. cerevisiae* and *P. pastoris*

Lymphocyte extravasation into the inflamed tissue is initiated by interactions between L-selectin and their counter receptors on endothelial cells, which carry sLex tetrasaccharide epitopes [NeuNAcα2-3Galβ1-4(Fucα1-3)GlcNAc] in response to inflammatory stimuli (Turunen *et al.*, 1994, 1995). In an organ transplant, this infiltration of lymphocytes to the graft is not desirable. The tetravalent sLex glycan (Fig. 9) has been shown to efficiently inhibit lymphocyte adhesion to the endothelium *in vitro* (Renkonen *et al.*, 1997), suggesting that this glycan can be used as an anti-inflammatory drug. To produce recombinant human α1,3-fucosyl-transferase VII for sLex synthesis, we transformed *S. cerevisiae* and *P. pastoris* with chimeric genes that encoded the fusion protein Hsp150Δ-FucTe (Fig. 8H and 8I) under the control of the *HSP150* and *AOX1* promoters, respectively. Yeast was chosen as the host organism because

it lacks endogenous sLex-synthesizing transferases and is capable of modifying proteins with, for example, a disulfide bond. In addition, it is able to secrete proteins and can be grown to high cell densities in inexpensive media. In both yeast species, the Hsp150Δ carrier succeeded in guiding the fusion proteins through the secretory pathway. Most of the FucTe activity remained bound to the cell wall, which enabled whole yeast cells to be used as an enzyme source. The recombinant *S. cerevisiae* and *P. pastoris* strains were able to synthesize sLex from sialyl-α2,3-N-acetyllactosamine and GDP-Fuc.

5.2.4. Teamwork of FucTVII and ST3N in the yeast cell wall

We also used yeast strains expressing the catalytic ectodomain of rat α2,3-sialyltransferase (ST3Ne) and FucTe combined to synthesize sLex from lacNAc, CMP-NeuNAc, and GDP-Fuc. The yeast strains were cultivated separately overnight and then added to the same test tube. The product yields were too low (picomole scale) for determination of the linkage position or the α/β-anomericity. The cDNA coding for the secretory FucTVII has also been expressed in a B-cell lymphoma cell line (Shinoda *et al.*, 1997) and in baculovirus-infected insect cells (Shinkai *et al.*, 1997). In both cases, the recombinant proteins showed α1,3-fucosyltransferase activity toward terminally α2,3-sialylated lacNAc. Hsp150Δ-ST3Ne expressed in *S. cerevisiae* is known, according to degradative experiments and ^{1}H-NMR, to catalyze the transfer of sialic acid in α2,3-linkage to lacNAc (Mattila *et al.*, 1996). Human fucosyltransferase VII efficiently fucosylates only sialylated type 2 oligosaccharides (Natsuka *et al.*, 1994; Sasaki *et al.*, 1994; Shinoda *et al.*, 1997; Niemelä *et al.*, 1998), forming tetrasaccharide sLex but not trisaccharide Lex. Our control experiments with lacNAc and GDP-Fuc confirmed that Hsp150Δ-FucTe utilized only sialylated lacNAc as an acceptor. In conclusion, the catalytic ectodomain of human fucosyltransferase VII expressed in yeast transferred fucose to α2,3-sialylated lacNAc, probably with an α1,3-linkage.

Both *S. cerevisiae* and *P. pastoris* have previously been used to produce glycosyltransferases. The cDNA from HeLa cells coding for the full-length membrane-bound form of β1,4-galactosyltransferase (β1,4-GalT) has been expressed in *S. cerevisiae* (Krezdorn *et al.*, 1993). The enzyme activity of the recombinant β1,4-GalT could be detected in crude cell lysates. Using N-acetylglucosamine or glucose as acceptors in combination with α-lactalbumin, the enzyme catalyzed the formation of the β1,4-bond between galactose and N-acetylglucosamine or glucose, as estimated with ^{1}H-NMR analysis (Krezdorn *et al.*, 1993). Full-length human α2,6-sialyltransferase expressed in *S. cerevisiae* (Krezdorn *et al.*, 1994) catalyzed the expected α2,6-linkage between sialic acid and acceptors asialofetuin and N-acetyllactosamine, as confirmed with ^{1}H-NMR. However, due to the absence of hyperglycosylation and the Man-α1,6-Man epitope, two posttranslational modifications normally carried out in the Golgi apparatus, both β1,4-galactosyltransferase and α2,6-sialyltransferase were speculated to have stayed in the ER in enzymatically active form (Krezdorn *et al.*, 1994). Sievi *et al.*

(2001) reported the expression in *S. cerevisiae* of the catalytic ectodomain of rat α2,6-sialyltransferase (ST6Ne) fused to the Hsp150Δ carrier. Hsp150Δ-ST6Ne was catalytically active and reached the Golgi compartment. But instead of being externalized, the fusion protein was transported from the Golgi to the vacuole for destruction. The loss of enzymatic activity of the newly synthesized protein occurred within one hour (Sievi *et al.*, 2001). Because Hsp150, the Hsp150Δ carrier, and Hsp150Δ-ST3Ne are externalized (Russo *et al.*, 1992; Jämsä *et al.*, 1995; Mattila *et al.*, 1996), the signal targeting Hsp150Δ-ST6Ne to the vacuole was thought to reside in the ST6Ne portion (Sievi *et al.*, 2001).

As the purification of membrane-bound proteins is difficult and time-consuming, the sequences coding for the N-terminal cytoplasmic and transmembrane domains of human β1,4-GalT have been replaced by the yeast invertase signal peptide (Kleene *et al.*, 1994). The catalytic ectodomain of human β1,4-GalT acquired a catalytically active form in *S. cerevisiae* in the absence of a carrier polypeptide. The enzymatic activity could be recovered from cell lysates with ultracentrifugation, indicating the presence of a cell-associated soluble protein (Kleene *et al.*, 1994). The fermentation technology of *S. cerevisiae* enabled large-scale production of heterologous glycosyltransferase in bioreactors larger than 100 liters (Borsig *et al.*, 1995; Herrmann *et al.*, 1995b).

G. Herrmann and his colleagues realized that in addition to the cell-associated soluble β1,4-GalT the cell walls of intact yeast cells contained 31.5 mU/(g wet cells) β1,4-GalT-activity. For comparison, after disintegration of the cells, soluble β1,4-GalT-activity in yeast cell lysates was 68.1 mU/(g wet cells) (Herrmann *et al.*, 1995a). They used intact yeast cells in the synthesis of N-acetyllactosamine from GlcNAc and UDP-Gal to avoid cell lysis and centrifugation. The whole yeast cells could be lyophilized and reused with no detectable loss of β1,4-GalT-activity and without the need to purify the recombinant glycosyltransferases. Separation of the product from the transferase was done simply by pelleting the cells (Herrmann *et al.*, 1995a).

Among human fucosyltransferases, α1,3-fucosyltransferase III and α1,3-fucosyltransferase VI have been expressed in yeast. Abe *et al.* (2004) fused the ectodomain of human α1,3-fucosyltransferase VI to full-length Hsp150 (also called Pir2p) and detected FucTVI activity in the *S. cerevisiae* cell wall. The ectodomain of FucTVI has been expressed in *P. pastoris* with the aid of the *S. cerevisiae*-derived MFα signal peptide, and one liter of shake flask culture produced 3 U of the enzyme in the medium (Malissard *et al.*, 2000). The ectodomain of FucTIII fused to the prepro-fragment of MFα was expressed in *P. pastoris.* The activity was initially in the cell wall (Lubineau *et al.*, 1998), but after 7 days of fed-batch fermentation, the majority of the enzymatic activity was in the culture medium, and 11.3 units of the enzyme could be harvested from one liter (Gallet *et al.*, 1998).

In conclusion, rat α2,3-sialyltransferase and human α1,3-fucosyltransferase VII were expressed in the yeasts *S. cerevisiae* and *P. pastoris*. Their ectodomains were fused to the Hsp150Δ and MFα carriers. Both carriers effectively promoted the proper folding of the catalytic ectodomains in *S.*

cerevisiae and *P. pastoris*. The fusion proteins were secreted but remained bound to the yeast cell wall, which enabled the use of intact yeast cells as a source of enzymatic activity. Yeasts expressing the two enzymes either in separate strains or in a single strain functioned in tandem, and the intact yeast cells synthesized sLex from lacNAc, CMP-NeuNAc, and GDP-Fuc. The recombinant proteins in *S.cerevisiae* were expressed under the control of the *HSP150* promoter, the strength of which was comparable with that of the *GAL1* promoter.

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