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Genetic engineering of recombinant glycoproteins and the glycosylation pathway in mammalian host cells

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The analysis of many natural glycoproteins and their recombinant counterparts from mammalian hosts has revealed that the basic oligosaccharide structures and the site occupancy of glycosylated polypeptides are primarily dictated by the protein conformation.

The equipment of many frequently used host cells (e.g. BHK-21 and CHO-cells) with glycosyltransferases, nucleotide-sugar synthases and transporters appears to be sufficient to guarantee complex-type glycosylation of recombinant proteins with a high degree of terminal α2-3 sialylation even under high expression conditions. Some human tissue-specific terminal carbohydrate motifs are not synthesized by these cells since they lack the proper sugar-transferring enzymes (e.g. α1-3/4 fucosyltransferases, α2-6 sialyltransferases). Glycosylation engineering of these hosts by stable transfection with genes encoding terminal human glycosyltransferases allows to obtain products with tailored (human tissue-specific) glycosylation in high yields.

Using site-directed mutagenesis, unglycosylated polypeptides can be successfully converted in N- and/or O-glycoproteins by transferring glycosylation domains (consisting of 7-17 amino acids) from donor glycoproteins to different loop regions of acceptor proteins.

The genetic engineering of glycoproteins and of host cell lines are considered to provide a versatile tool to obtain therapeutic glyco-products with novel/improved in-vivo properties, e.g. by introduction of specific tissue-targeting signals by a rational design of terminal glycosylation motifs.

Keywords: **glycosylation engineering, human fucosyltransferases, human sialyltransferases, N- and O-glycosylation, host cell specificity, recombinant glycoprotein expression**

Introduction

The importance of the posttranslational modification of polypeptides with *N*- or *O*-linked oligosaccharides is well documented by their implication in numerous biological phenomena [1]. Consequently, it has already been recognized in the early eighties [2] that only mammalian host cells meet the criteria for an appropriate biotechnological development of recombinant glycotherapeutics to be used in humans. This has led to the attractive new research area of the biotechnology of mammalian cells as factories for medicinal glycoproteins.

Protein-linked oligosaccharides control the intracellular and tissue targeting of polypeptides, their half-life *in vivo* and their dynamic interaction with other proteins inside the cells or in body fluids. Carbohydrate structures of glycoproteins are typically polypeptide-specific and it has been shown that each individual glycosylation site of a glycoprotein may contain its own characteristic pattern of oligosaccharide chains [3]. Apart from the 3D-domain structure that governs its decoration with glycans, also the tissue or cell type that synthesizes a glycoprotein plays an important role in the phenomenon of microheterogeneity of protein glycans. This is a result of the regulated expression of a characteristic set of glycosidase and terminal glycosyltransferase genes which is different in the various cells/tissues of an organism [4] and may also vary with the physiological conditions of an organism or the differentiation state of cells. For example, human transferrin secreted from liver cells into the blood stream contains oligosaccharides usually found on serum glycoproteins, mostly afuco diantennary oligosaccharides with terminal α2,6-linked NeuAc, whereas the same protein isolated from human cerebrospinal fluid carries asialo and asialo-agalacto diantennary forms of proximally fucosy-

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lated chains with bisecting GlcNAc typical for glycoproteins synthesized in brain tissues [5–7] due to the different expression levels of the pertinent terminal glycosyltransferases.

Concepts for the biotechnological production of recombinant glycoprotein therapeutics or recombinant retrovirus vectors and *ex vivo* expansion of human primary cells for medicinal treatment must take into consideration different interactions of differently glycosylated cell/virus surface glycoconjugates or soluble glycotherapeutics with cellular receptors and subsequent altered modulation of intracellular signalling cascades. While during the past 12 years much work has been published on the structural characterization of recombinant glycoproteins expressed from various mammalian and nonmammalian expression systems, a great deal of efforts is presently going into attempts to improve recombinant host cell lines, and here especially mammalian cells, for the manufacturing of glycoprotein pharmaceuticals and retrovirus vectors with novel *in vivo* properties. It seems promising to explore the advantages of new generations of products with improved *in vivo* stability and carbohydrate-based tissue-targetable addressing signals. For this, the host cell lines must be improved by genetic engineering with newly introduced glycosyltransferases. The transferases must be stably directed into the proper subcellular compartment for their efficient function in the glycosylation pathway of the host. Here we are reporting on some of these aspects of the work from our laboratory at the German Center for Biotechnology (GBF) during the past 10 years.

I. Recombinant expression of human therapeutic glycoproteins

Mammalian host cells

Recombinant mammalian host cells cultured in large bioreactor systems are currently used to generate human glycoprotein pharmaceuticals which can be obtained from natural sources in only minute quantities. Amongst the first recombinant pharmaceutical glycoproteins produced from mammalian host cells were the secretory polypeptides interleukin-2 (IL-2), interferon-β (IFN-β and interferon-γ (IFN-γ) [8–10]. Over the past 12 years the literature reporting on the glycosylation analysis of recombinant glycoproteins from different hosts has accumulated tremendously. In most of these studies Chinese hamster ovary (CHO) and baby hamster kidney cells (BHK-21) have been used as expression systems and most of our present knowledge about the culture conditions that can affect the fine structural characteristics of recombinant glycoproteins produced in large scale processes has been obtained from studies with these two hosts cells [11–14]. In our hands, a constitutively secreted glycoprotein expressed at a level of 0.1 μg/ml from BHK-21 or CHO cells has the same carbohydrate structure as has the protein expressed at a 200-fold higher level, and as a general rule, it appears that the glycosylation machinery of the host cell itself is not a bottleneck for an efficient posttranslational modification of a polypeptide with carbohydrates. However, problems might eventually be encountered when a recombinantly expressed protein has an abnormal half-life in different cellular subcompartments of the host. It should be noted that in rare cases the selection procedures used for the isolation of transfected high expression cell clones may lead to the detection of a variant cell clone with aberrant glycosylation capacity, as has been recognized in our laboratory with a BHK-21 cell line that showed a complete loss of carrying out complex-type glycosylation of a recombinantly expressed protein [15].

As has become clear from the work of others and our own investigations, CHO and BHK-21 cells show basically the same characteristics for the glycosylation of recombinant *N*- or *O*-glycoproteins. The structural features detected in recombinant glycoproteins expressed from murine and hamster cell lines are summarized in Table 1. In principle, the antennarity and the LacNAc content of *N*-linked oligosaccharides of a given recombinant glycoprotein expressed in CHO, BHK-21 or the murine cell lines will be the same and this is also true for characteristics of

Table 1. Structural features of N-linked oligosaccharides from recombinant glycoproteins expressed in mammalian host cells. Data are based on structural analysis of the recombinant human glycoproteins IFN-β, Epo, AT III, IL-6, tissue-plasminogen activator and β-TP as well as recombinant humanized antibodies, soluble receptor proteins and *N*-glycosylation mutants of human IL-2.

	host cell line			
carbohydrate structure	*CHO*	*BHK-21*	*C127*	*Ltk⁻*
proximal fucose	+	+	+	+
Fuc(α1-2)Gal-R*	+	+	?	?
α2,6-NeuAc	−	−	+	+
α2,3-NeuAc	+	+	+	+
NeuAc(α2-8)NeuAcα2-3-R	+	+	−	+
NeuGly*	+	+/−	+	+
tri/tetra-antennarity	+	+	+	+
Gal(β1-4)GlcNAc repeats	+	+	+	+
Gal(β1-3)GlcNAc-R	−	+	−	−
sulfated glycans	+	+	+	+
Gal(α1-3)Gal	+	−	+	+
branched repeats	?	−	+	−
mannose 6-phosphate*	+	+	?	?
bisecting GlcNAc	−	−	+	+
GalNAc(β1-4)GlcNAc	−	+**	−	−

*detectable only in trace amounts
**detected in large amounts in the BHK-21A variant cell line [16,17]

the oligosaccharide pattern at individual glycosylation sites. However, in view of the pronounced higher microheterogeneity of terminal carbohydrate motifs in recombinant products obtained from the murine host cells (α2,3- vs. α2,6-NeuAc, NeuGly, Gal(α1→3)Gal, Gal(β1→3)GlcNAc-R, sulfated structures and branched repeats in L*tk*$^-$ and C127 cells), the two hamster cell lines seem to provide a more favourable expression host cell system when low glycoform heterogeneity is required.

It should be emphasized that in most publications on carbohydrate structures of recombinant glycoproteins the work has been performed with purified glycoprotein preparations destined for pharmaceutical use. Consequently, these preparations represent a subfraction of the total product secreted by the host cell and they are enriched in glycoforms which are believed to be most effective for *in vivo* application in humans. One such example is recombinant human erythropoietin (EPO) from BHK-21 or CHO cells where only a highly sialylated subfraction (based on the isoelectric focussing pattern) of the total recombinant glycoprotein hormone that is secreted by the producer cells is manufactured for medical treatment. This subfraction represents only about 20–25% of the total EPO secreted from the host cell lines. Therefore, for a complete description of the glycosylation characteristics of any host cell, it is indispensible to purify the product quantitatively, *e.g.,* by immunoaffinity chromatography using polypeptide-specific antibodies that guarantee >90% final yield.

Significant advances in the sensitivity of carbohydrate structural analysis has been achieved during the past three years. Especially in mass spectrometry (on-line ESI-MS, nanospray tandem mass spectrometry (ESI-MS/MS) and improved MALDI/TOF techniques), very sensitive instrumentation for glycosylation analysis has been made available to a broader group of research units, and thus has led to a broader use of complementary tools by academic researchers and in industrial laboratories. This is of outstanding importance in the area of glycobiology and glycotechnology where the combined methods of molecular biology, protein biochemistry, cell biology and analytical know-how are required to understand in detail the basic mechanisms and the role of modification of proteins and lipids with carbohydrate in both, health and different states of disease.

Production of secretory glycoproteins in insect cells using the recombinant baculovirus expression system

Some 10 years ago it has been proposed to use insect cells infected with recombinant baculoviruses for production of large amounts of recombinant glycoproteins. However, it has become clear that the insect expression system has its limitations for the production of mammalian-type modified glycotherapeutics. We found that secretory glycoproteins that contain complex-type *N*-glycans when expressed in mammalian host cell lines are modified only with the short oligomannosidic Man(α1→6)Man(β1→4)GlcNAc(β1→4)[Fuc(α1→6)]GlcNAc and Man(α→3)[Man(α→6)]Man(β1→4) GlcNAc(β1→4)[Fuc(α1→6)]GlcNAc *N*-glycans when expressed from Sf21 or Sf9 (*Spodoptera frugiperda*) cells [18]. *N*-glycan structures most similar to those synthesized in Sf9 or Sf21 cells were also detected in the products secreted from SPC-Bm36 (*Bombyx mor*i) cells (see Table 2). SPC-Bm36 cells produce *N*-glycosylated proteins with higher amounts of dimannosyl- over trimannosyl-oligosaccharides and only 60% α1,6-fucosylation of the proximal GlcNAc. A model glycoprotein with a potential *O*-glycosylation motif [18] expressed from SPC-Bm36 cells was found to be unglycosylated, modified with GalNAc or with Gal(β1→3)GalNAc in a ratio of 1:3:5 that is different in the same protein when synthesized from Sf21 cells, where a ratio of 1:4:4 was detected [18]. Therefore, it appears that SPC-Bm36 cells generally underglycosylate *N*- and *O*-glycoproteins.

N-glycan structures of glycoproteins expressed from BTI-Tn-5B1-4, "High Five" (*Trichoplusia ni*) cells are essentially the same as those from Sf21 cells and are present in a similar ratio. However, a considerable proportion of the oligosaccharides was found to be *difucosylated,* containing an additional fucose in α1,3-linkage to the proximal GlcNAc. The enzymatic activity involved in the biosynthesis of this structural motif has also been described for a *Mamestra brassica* cell line, IZD Mb0503 [19]. Surprisingly, the analysis of a *N*-glycosylated protein variant expressed in BTI-EaA (*Estigmene acrea*) cells revealed the presence of fucosylated trimannosyl-oligosaccharides containing 1,2, or small amounts of even 3 terminal GlcNAc-residues as detected by methylation analysis and ESI-MS/MS of the pertinent tryptic glycopeptide [20]. However, no indication for galactosylated oligosaccharides was detected by the complementary analytical techniques applied in our laboratory. Such complex-type *N*-glycan structures were not detected on recombinant glycoproteins expressed in any other baculovirus-infected insect cell line shown in Table 2. These data clearly confirm our previous results [18] and those of others [21,22] that insect cells lines are incapable of synthesizing sialylated lactosamine complex-type *N*-glycans or sialylated core 1 *O*-glycans and therefore are not suitable for the production of recombinant pharmaceutical glycoproteins for clinical use.

An further disadvantage of the insect cell expression system is that recombinant baculovirus vector-driven high expression of proteins is run as a batch-culture process and cells die after infection within the productive phase. Only about 20% of the total recombinant protein synthesized by the host cells is secreted into the supernatant. The remainder is found denatured as inclusion body-like aggregates

Table 2. Glycosylation characteristics of recombinant glycoproteins secreted from 6 different lepidopteran cell lines. Data were obtained by detailed carbohydrate analysis of human β-TP, IFN-β and different human IL-2 *N*-glycosylation variants secreted from baculovirus-infected cells. Cell cultures were run in tissue culture flasks and in bioreactors, and the products were analysed from harvests after 2 days, 4 days, and 7 days post infection. M = mannose, Gn = *N*-acetylglucosamine, F = fucose

<table>
<tr><th></th><th colspan="10">N-glycan structures of secreted recombinant glycoproteins</th></tr>
<tr><th>Insect cell line</th><th>M
\
M—Gn—Gn</th><th>M
\
M—Gn—Gn
/
M</th><th>M F
\ |6
M—Gn—Gn</th><th>M F
\ |6
M—Gn—Gn
/
M</th><th>M
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M F
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M M—Gn—Gn
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M</th><th>M F
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M—Gn—Gn
|3
F</th><th>M F
\ |6
M—Gn—Gn
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M F</th><th>M F
\ |6
M—Gn—Gn
/
Gn—M</th><th>Gn—M F
\ |6
M—Gn—Gn
/
Gn—M</th><th>Gn
\
Gn—M F
\ |6
M—Gn—Gn
/
Gn—M</th></tr>
<tr><td>Spodoptera frugiperda (Sf21)</td><td>+</td><td>+</td><td>+++</td><td>+++</td><td>+</td><td>−</td><td>−</td><td>−</td><td>−</td><td>−</td></tr>
<tr><td>Spodoptera) frugiperda (Sf9</td><td>+</td><td>+</td><td>+++</td><td>+++</td><td>+</td><td>−</td><td>−</td><td>−</td><td>−</td><td>−</td></tr>
<tr><td>Trichoplusia ni (BTI Tn5B1-4, “High-Five”)</td><td>+</td><td>+</td><td>++</td><td>++</td><td>−</td><td>++</td><td>++</td><td>−</td><td>−</td><td>−</td></tr>
<tr><td>Bombyx mori (SPC-Bm36)</td><td>+++</td><td>+</td><td>+++</td><td>+</td><td>−</td><td>−</td><td>−</td><td>−</td><td>−</td><td>−</td></tr>
<tr><td>Mamestra brassicae (IZD Mb0503)</td><td>−</td><td>−</td><td>++</td><td>++</td><td>−</td><td>+++</td><td>++</td><td>−</td><td>−</td><td>−</td></tr>
<tr><td>Estigmene acrea (BTI-EaA)</td><td>−</td><td>−</td><td>++</td><td>++</td><td>−</td><td>−</td><td>−</td><td>++</td><td>++</td><td>+</td></tr>
</table>

inside the cell and is partially released into the medium at later states of infection from dying cells [23]. Nevertheless, the baculovirus expression system is the preferred system when the production of several 100 mg of a protein is required for research purposes within a short time and a mammalian/human-type of glycosylation is not of primary importance.

II. Genetic engineering of new glycoproteins and their recombinant expression in animal cells

Engineering of proteins with new glycosylation properties

The successful modification of a polypeptide with newly introduced *N*- or *O*-glycosylation properties may increase its solubility or influence its *in vivo* biological properties (e.g., activity, antigenicity, rate of clearance). In other cases, the simple deletion of a glycosylation site with pronounced carbohydrate microheterogeneity might also be of advantage for a final clinical application of the product. It is now known from the 3D-structures of many glycoproteins that *N*- or *O*-glycosylation motifs are mostly found in loop-regions of polypeptides. According to our experience, a single amino acid exchange creating a new potential consensus tripeptide Asn-Xxx-Ser/Thr is often not sufficient for *N*-glycosylation to occur, even when present in loop regions. The concept of polypeptide-specific and glycosylation site-specific modification of proteins with carbohydrates has led us to investigate by using site-directed mutagenesis the introduction of individual *glycosylation domains* from donor glycoproteins with known glycosylation characteristics into suitable locations of model acceptor proteins (IL-2, IFN-β. This approach aimed at the definition of short peptide domains that should result in predictable oligosaccharide structures when the constructs are expressed from a given host cell. The insertion of short (8–15 residues) peptide sequences containing an Asn-Xxx-Thr/Ser sequence has been proved to be successful, and, most importantly, was found not to severely affect the overall 3D-structure as shown for the biological activity of the resulting human IL-2 and IFN-β variants [23,24]. We have analyzed the carbohydrate structure of the different resulting chimers after expression from BHK-21 cells as shown in Figure 1. The single *N*-glycosylation site of human IFN-β contains preponderantly diantennary complex-type oligosaccharide chains when expressed from CHO or BHK-21 cells [9,25], as is the case for all four *N*-glycosylation sites of human antithrombin III (AT III) [26]. The three *N*-glycosylation sites of human EPO from the same host cells contain preponderantly tetraantennary chains with 1-3 *N*-acetyllactosamine repeats [27–30], with Asn_{86} (site III) bearing the most homogenous oligosaccharide population. As mentioned above, the introduction of a new *N-glycosylation site* into the IL-2 polypeptide by substitution of $Thr_3 \rightarrow Asn$ (APNSSSTKKT$_{10}$..) does not result in any modification with *N*-glycans when the construct is expressed from BHK-21 or L*tk*$^-$ cells [24]. However, the transfer of the human IFN-β *N-glycosylation domain* SSSTGWNETIV(GG) to the N- or C-terminus of IL-2 yielded proper *N*-glycosylation with diantennary complex-type chains [31,32], as is the case for wild-type IFN-β from these hosts [9,25].

Similarly, the peptide comprising the *N*-glycosylation domain III of human AT III was found to be modified with diantennary *N*-glycans when inserted at position 80 within the loop region between helices B′ and C of human IL-2 as is depicted in Figure 1. However, the introduction of *N*-glycosylation domain III of human EPO at the same location resulted in oligosaccharides with significantly higher antennarity. These investigations indicate that *N*-glycosylation domains can successfully be transferred from one protein to a loop region or the N- or C-terminus of another protein. Our data allow for the conclusion that in several cases the characteristic antennarity of the donor *N*-glycosylation domain is preserved when inserted into the newly constructed mutant glycoprotein [11,23,24,31,32].

Engineering of O-glycosylated proteins

All mammalian cell lines frequently used for recombinant protein expression (*e.g.* the cell lines in Table 1) modify *O*-glycosylation sites with preponderantly core 1 *O*-glycans containing one or two NeuAc [27,29,33]. In secretory glycoproteins like human IL-2 or EPO, which are *O*-glycosylated at a single hydroxyamino acid, all recombinant host cells recognize specifically the same Ser or Thr that is modified in the natural protein even when it is part of a hydroxyamino acid cluster [11,24], as is the case for human IL-2 where specifically Thr_3 within the N-terminal sequence H_2N-APT$_3$SSSTKKT$_{10}$ . . . is modified by NeuAc(α2→3)Gal(β1→3)[NeuAc(α2→6)]$_{0-1}$GalNAc chains [33]. As is summarized in Table 3, mutant IL-2 proteins with substitution of $Thr_3 \rightarrow Ser$ or containing Thr at position 5 are not *O*-glycosylated [24]. The deletion of Thr_3 or its substitution with Ala or Ser abolishes *O*-glycosylation completely, whereas the exchange of Ser_4 or Ser_6 to Thr results in detectable *O*-glycosylation. The peptide sequences APTPP, APTAPPT (present in human plasminogen) or the artificial sequence APTPPP can be used to introduce novel *O*-glycosylation sites into human IL-2 or human IFN-β at different positions of the polypeptide chain [11,18,24,32], and the resulting proteins are efficiently *O*-glycosylated in BHK-21 cells and L*tk*$^-$ cells, as is indicated also in Figure 1. Thus, these sequence motifs can be considered to constitute general transferable *O*-glycosylation recognition domains when introduced into loop regions of polypeptides although no consensus sequence for the attachment of *O*-glycans to polypeptides has yet been identified.

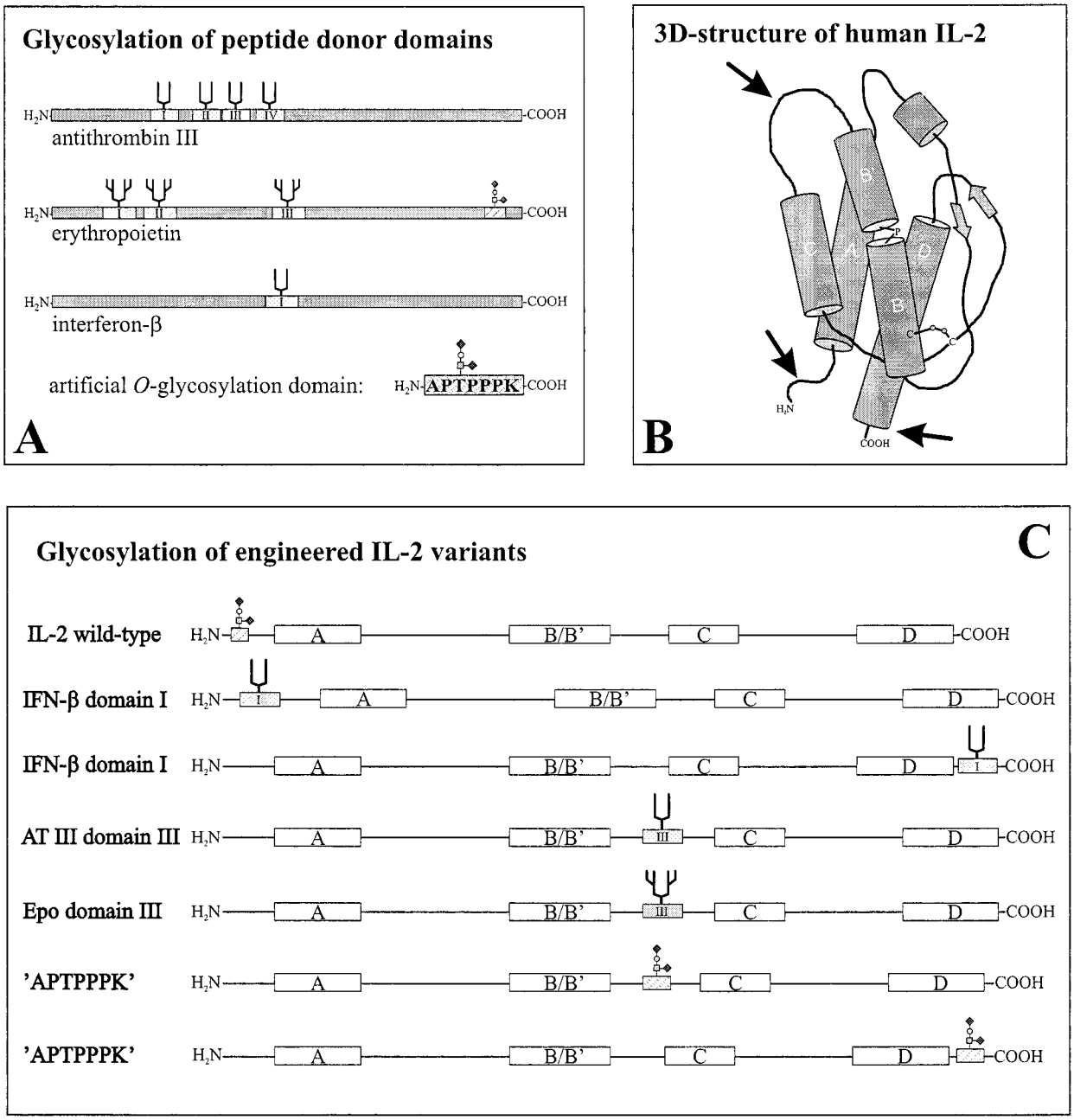

Figure 1. Genetic engineering of glycoproteins with defined glycosylation characteristics: Insertion of *N*- and *O*-glycosylation domains into human IL-2. *panel A,* glycosylation domain donor glycoproteins with known glycosylation characteristic; *panel B,* 3D-structural model of human IL-2 based on NMR data; arrows indicate acceptor sites used for insertion of glycosylation domains; *panel C,* glycosylation acceptor characteristics of newly introduced domains as identified by analysis of the resulting IL-2 variants. Bars A, B/B', C and D indicate helical domains of human IL-2 as shown in Figure 1*B*

Table 3. Mutation analysis of the *O*-glycosylation acceptor properties of the N-terminus of human IL-2. Data were corroborated by immunoprecipitation of stably (BHK-21) as well as transiently (L*tk*⁻) transfected mammalian host cells and N-terminal sequencing of the purified proteins.

N-terminal sequence	*O-glycan attached at position*
APTSSSTKKT . . .	3
APASSSTKKT . . .	no
APSSSSTKKT . . .	no
APSSSTKKT . . .	no
APTTSSTKKT . . .	3↑
APTSTSTKKT . . .	3↓
APTSSTTKKT . . .	3↑
APNSSSTKKT . . .	no
APSTSSTKKT . . .	no
APSSTSTKKT . . .	no
APSSSTTKKT . . .	no
APTAPPTKKT . . .	3↑, 7
APTPPSTKKT . . .	3↑
APTPPPTKKT . . .	3↑

III. Construction of host cell lines with novel glycosylation characteristics

In vivo specificity of glycosyltransferases

In vitro assays of glycosyltransferases with small acceptor substrates may yield some preliminary information about acceptor substrates properties recognized by the enzymes and are indispensible for the evaluation of glycosyltransferase levels in cells/tissues and the control of enzyme purification. A final description and comparison of the *in vivo* specificity of the individual glycosyltransferases, however, can only be achieved by structural analysis of the cellular product(s). As shown in Figure 2, we suggest the recombinant expression of the full length form of human glycosyltransferases along with a suitable reporter glycoprotein (here human β-TP) at a constant expression level in a heterologous mammalian host cell line that is devoid of the pertinent enzyme activity. This is considered to represent a valuable model and should enable the comparison of the *in vivo* specificities of different members of a glycosyltransferase family [16,17,34,35] and allow the selection of the optimal enzyme suitable for the glycosylation engineering of host cell lines for the production of a new generation of glycotherapeutics with defined altered glycosylation characteristics. Basic information can be expected from such

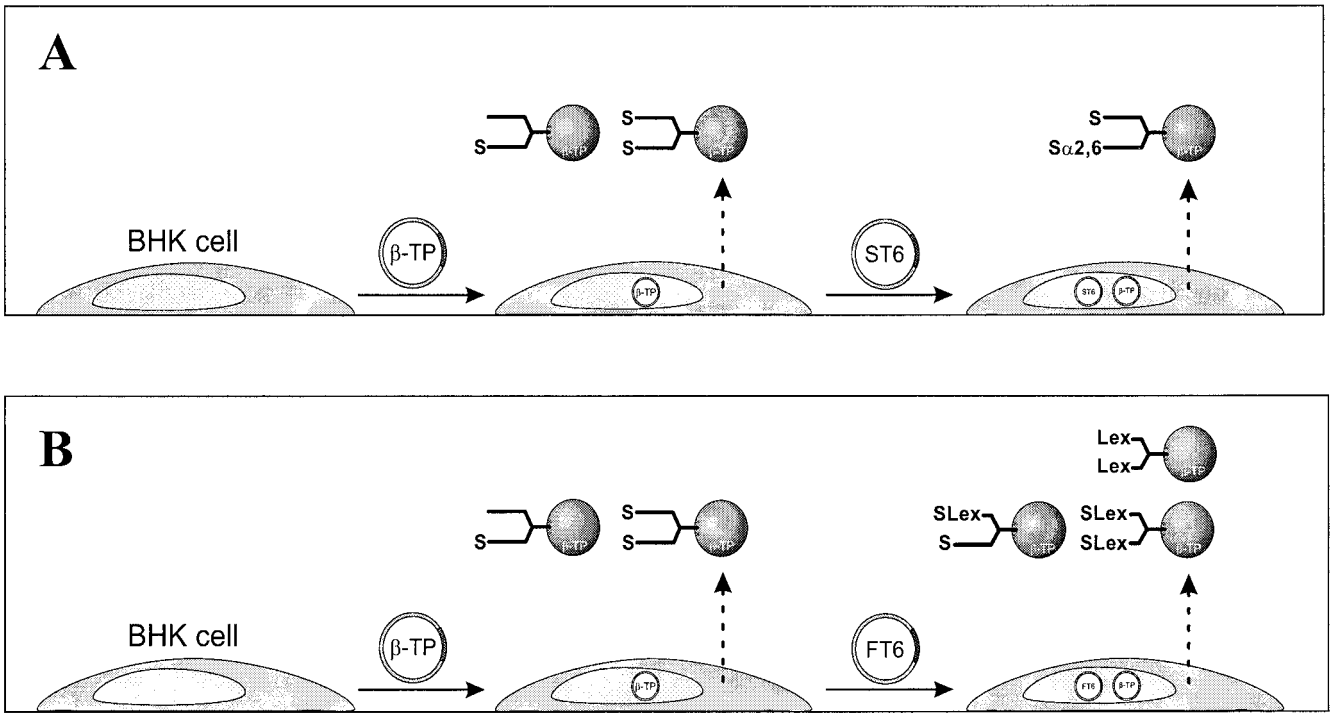

Figure 2. Genetic engineering of new BHK host cells by transfection with human glycosyltransferase genes. In order to generate expression of a reporter glycoprotein, BHK-21 cells were transfected with a plasmid encoding human β-TP. The recombinant β-TP secreted from such cell lines is glycosylated host cell-type-specifically with α2,3-di- or monosialo diantennary complex-type *N*-glycans (with proximal fucose) as indicated by "S" attached to the structural symbols. *panel A,* cotransfection with the human ST6Gal I gene leads to secretion of β-TP containing α2,6-linked NeuAc; *panel B,* cotransfection with human α1,3-fucosyltransferase VI (FT6) results in β-TP modified with sLex- or Lex-containing oligosaccharides

studies concerning the intracellular organization of the protein glycosylation machinery and the temporal and spatial distribution of the transferases in the *in vivo* biosynthetic compartments. Prerequisites for such an *in vivo* assay system are:

i. Reproducible transfection procedures using high expression vectors and rapid selection/isolation of stably transfected cells
ii A constant level of acceptor substrate expression (reporter glycoconjugate) by the host cells
iii. A defined expression level of the recombinant enzymes (20- to 50-fold higher expression levels of the wild-type forms are achieved in transfected cells when compared to the levels in primary cells/tissues)
iv. A simple, fast and quantitative purification procedure for the product (preferably secreted into the medium)
v. Application of fast and sensitive carbohydrate structural analytical micromethods (MS and MS/MS-techniques, HPAE-PAD)

It should be emphasized that the 3D-structure of the glycoprotein substrate and thus the accessibility of its oligosaccharide moieties under the intracellular environmental conditions is also of importance. In addition, it is conceivable that cell surface membrane glycoproteins might be recognized differently than are obligate secretory glycoproteins. However, for a given model glycoprotein with defined structural characteristics, the above approach by analysis of a reporter glycoconjugate from stably transfected cells should yield precise information on the *in vivo* substrate specificity of the individual members of a family of enzymes acting on the same precursor substrate. Transient expression experiments are of limited value, since cell damage and cell leakage resulting from the transfection procedures is considered to lead to artefacts.

Engineering of cells by stable transfection with human α1,3/4-fucosyltransferases genes

Analysis of the in vivo acceptor substrate specificity of fucosyltransferases by glycosylation analysis of coexpressed recombinant human β-trace protein.

The α1,3/4-fucosyltransferases III-VII [36–41] add fucose to the GlcNAc residue in sialylated or unsialylated Gal(β1→3)GlcNAc-R or Gal(β1→4)GlcNAc-R type structures of glycoconjugates. They are thus involved in the regulation of the synthesis of the Lewis X (Lex) and sialyl Lewis X (sLex) type ligands that are involved in inflammation-induced adhesion of neutrophils, monocytes, T cells and platelets to selectins [42–45]. Fucosylated glycoconjugates play also a central role in other important biological phenomena like differentiation and tumorigenesis, and elevated levels of peripherally fucosylated serum glycoproteins have been detected in humans associated with inflammatory processes [46,47].

Many natural human tissues/cells express more than one fucosyltransferase at the same time and therefore it is difficult to obtain homogenous enzyme preparations from natural tissues or body fluids for the unequivocal assessment of the specificity of the individual enzymes. The cloning of the α1,3/4-fucosyltransferases III-VII (FT3-FT7) and their expression in recombinant form has provided a tool to isolate pure enzyme preparations for studying their substrate specificity *in vitro.* However, several questions concerning the implication of each of the individual fucosyltransferases in the generation of selectin ligands are still not resolved.

Mammalian glycosyltransferases are Golgi-resident type II transmembrane proteins, and according to current opinion, their transmembrane region is responsible for the retention of the enzymes in the proper Golgi compartment. Many transferases contain *N*-glycosylation sites in their stem region and/or their catalytic domain; however, no information is available if, or to what extent, *N*-glycosylation is involved in the *in vivo* activity or specificity of glycosyltransferases. A number of publications have appeared that describe the recombinant expression of human fucosyltransferases [48–52] mostly as soluble forms lacking the cytoplasmic, the transmembrane and some part of the stem region. In several cases, recombinant chimers containing N-terminally fused polypeptide fragments (*e.g.,* of protein A) have been constructed to facilitate recombinant enzyme purification. According to the data published so far, FT7 has been reported to fucosylate exclusively α2,3-sialylated *N*-acetyllactosamine-type structures *in vitro* and is inactive with neutral acceptors [41,51,52]. FT4 acts almost exclusively on unsialylated Gal(β1→4)GlcNAc-R (type **II**) structures [48,53], whereas *in vitro,* FT5 and FT6 have been reported to act on both, α2,3-sialylated as well as unsialylated type **II** acceptors [48,49,54]. FT3 has been reported to mainly transfer Fuc in α1,4-linkage onto GlcNAc in type **I** chains [34,48,54]. Activity with type **I** acceptors has also been found for human FT5 [48], while FT4, FT6 and FT7 are not active with Gal(β1→3)GlcNAc-R substrates [41,48,54].

Human β-TP is a 168 amino acid protein which contains two *N*-glycosylation sites that are occupied with almost exclusively diantennary complex-type chains [5,16,35,55]. Similar to human transferrin described above, β-TP isolated from human cerebrospinal fluid exhibits "brain-type" glycosylation characteristics, *i.e.,* mainly truncated asialo chains, bisecting GlcNAc, complete proximal and some peripheral fucosylation besides small amounts of α2,3/6-sialylated *N*-glycans [5,7]. Recombinant human β-TP expressed from wild-type BHK-21B cells is also modified with almost exclusively diantennary oligosaccharides at each of its two *N*-glycosylation sites, however, as shown in Figure 3, the oligosaccharide pattern here is very homogenous, the structures contain either two or one α2,3-linked NeuAc and only small amounts of asialo chains are present [16,35].

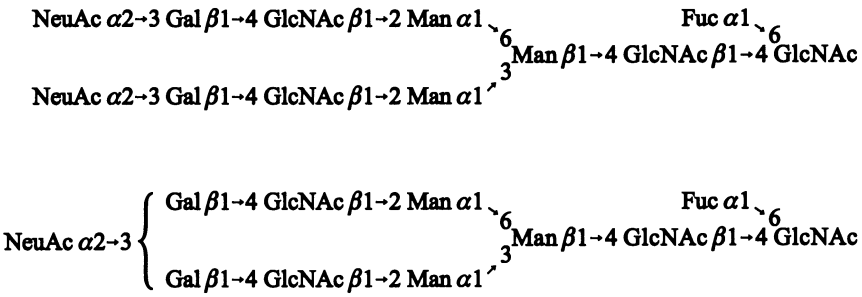

Figure 3. *N*-glycan structures of recombinant human β-TP secreted from *wild-type BHK-21* cells contain exclusively α2,3-linked NeuAc.

Coexpression of β-TP as a reporter glycoprotein from cells transfected with a human α1,3/4-fucosyltransferase therefore should yield oligosaccharides with Lewis X or sialyl Lewis X motifs or mixtures of the two motifs depending on the *in vivo* specificity of the transfected fucosyltransferase gene. In total 12 different diantennary *N*-linked oligosaccharides can be expected in β-TP from BHK-21 cells expressing human FT6 (four each of asialo, mono- and disialo chains with no, one or two peripheral fucose residues, respectively).

Human FT6 synthesizes preponderantly α1,3-difucosylated structures on diantennary chains *in vivo*

When incubated *in vitro* with soluble FT6 in the presence of GDP-Fuc, we found efficient fucosylation of β-TP, with a roughly 50% modification of the α2,3-monosialylated oligosaccharide with one α1,3-linked Fuc, and with 33% and 6% modification of the α2,3-disialylated oligosaccharides with one or two α1,3-linked Fuc residues, respectively [35]. This result obtained for the complex type *N*-glycans is in agreement with published reports for the *in vitro* specificity of recombinant human FT6, which indicate that the enzyme can form Lex as well as sLex motifs with small type **II** oligosaccharides [49,54]. When β-TP is coexpressed from BHK-21 cells together with human FT6 (see Table 4), about 50% of all *N*-glycans contain α1,3-linked Fuc (*cf.* Fig. 4*A*). However, the sialylation degree of the *N*-glycans is significantly lower when compared to *N*-glycans of β-TP from wild-type BHK cells (Fig. 4*B*), and also in contrast to the *in vitro* modified β-TP, most of the oligosaccharides are found to be modified *in vivo* with *two* peripheral Fuc (see

Table 4. Fucosyltransferase activities of stably transfected BHK-21 cell lines. Measurements were performed using the substrate GDP-[^{14}C]Fuc and Gal(β1→4)GlcNAc—O—$(CH_2)_8$—$COOCH_3$ as an acceptor. A dash indicates incorporation of radioactivity at background levels. *FT3 activity was detected by using the type **I** Gal(β1→3)GlcNAc-O-$(CH_2)_8$-$COOCH_3$ acceptor; **FT7 was determined with native bovine fetuin. *In vitro* activity values for FT5 and FT7 are very low; however, from standard transfection procedures used, and in view of the *in vivo* fucosylation efficiency (see Fig. 5), an expression value similar to those detected for the other fucosyltransferases is assumed.

Cell line	*Total activity (cells + culture medium) $\mu U \times 10^{-6}$ cells $\times$ 48 h^{-1}*	*% of total activity accumulated in the culture medium after 48 hours*
BHK-21B (wild-type)	—	—
FT3	—	—
FT3*	100	78%
FT4	75	27%
FT5	1	0%
FT6 (BHK21-B)	170	74%
FT6 (BHK-21A)	210	88%
FT6 (CHO DHFR-)	370	81%
s-FT6(I)	175	92%
s-FT6(II)	2970	91%
BT-FT6	9400	96%
FT7	—	—
FT7**	3	0%

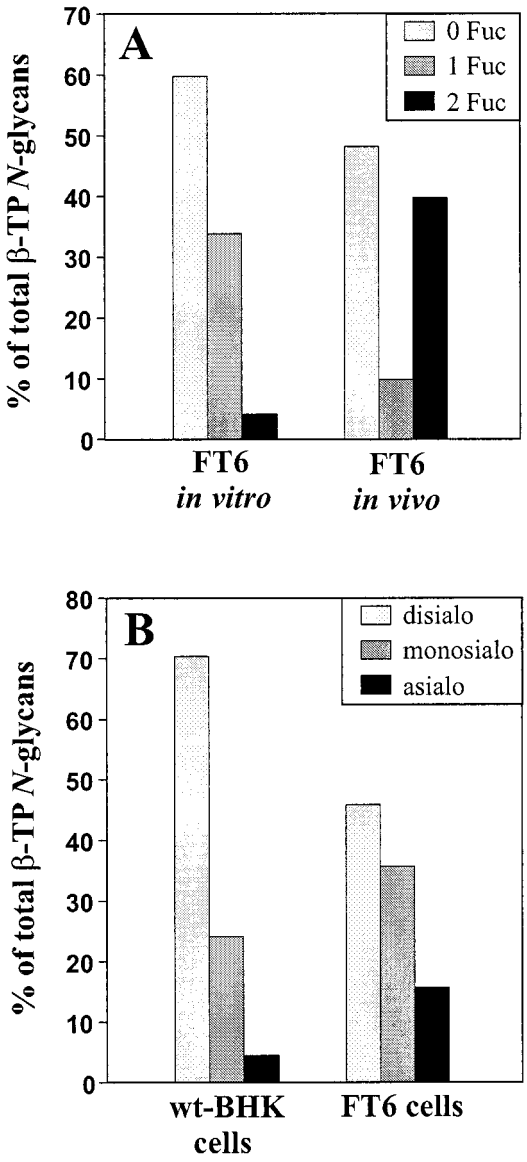

Figure 4. Comparison of β-TP oligosaccharides after *in vitro* or *in vivo* modification with human FT6. For *in vitro* fucosylation, purified β-TP expressed from BHK-21 cells was incubated with recombinant soluble human FT6 (s-FT6) in the presence of GDP-Fuc. *In vivo* data were obtained following coexpression of β-TP and full-length FT6 genes in BHK-21 cells (*cf.,* Fig. 2). *panel A,* percentage of nonfucosylated, α1,3-mono- and α1,3-difucosylated *N*-glycans; *panel B,* percentage of disialo, monosialo and asialo diantennary chains in β-TP *N*-glycans

Table 5. In vivo fucosylation characteristics of human α1,3/4-fucosyltransferases. Values represent the percentage of all diannten-nary oligosaccharide forms comprising >90% of total N-glycans isolated from β-TP expressed in each individual FT-transfected BHK-21 cell line. Structural analysis was performed by HPAE-PAD mapping, MALDI/TOF-MS, ESI-MS/MS and methylation analysis. The two isomeric monosialo/monofucosylated structures from FT3, FT5 and FT6 cells were not resolved.

	β-TP N-glycan structures (%)										
BHK cell line	S / S	S		SLex / SLex	SLex / S	SLex / Lex	SLex		Lex / S	Lex / Lex	Lex
wild-type	75	20	5	—	—	—	—		—	—	—
FT3	59	20	2	3	13	—	—	3	—	—	—
FT4	40	9	—	—	5	4	—		21	17	3
FT5	46	18	2	8	12	5	—	7	—	2	—
FT6	29	16	3	13	5	15	—	5	—	13	—
FT7	43	22	4	11	11	—	9		—	—	—
s-FT6(I)	59	35	6	—	—	—	—		—	—	—
BT-FT6	55	17	2	5	7	6	—	3	—	4	1

also Table 5). The ratio of sLex:Lex antennae in the total *N*-glycan mixture is 1.1:1. The lower sialylation degree of β-TP from FT6 cells compared to wild-type BHK-21 cells can be explained by an *in vivo* competition of the recombinantly expressed FT6 with the endogenous α2,3-ST(s), ST3Gal III and/or ST3Gal IV, for the common asialo oligosaccharide substrate. This phenomenon has been observed previously for products from recombinant host cell lines transfected with α1,3-galactosyltransferase [56] or FT4 [57]. The α2,3-STs have been reported to be unable to sialylate Gal(β1→4)[Fuc(α1→3)]GlcNAc-R motifs [41,50,57]. Obviously, as is the case for *in vitro* incubation conditions, FT6 can act also *in vivo* on both, α2,3-sialylated as well as unsialylated *N*-linked oligosaccharides. To our knowledge, this is the first publication describing quantitatively and in detail the *in vivo* substrate specificity of a recombinant fucosyltransferase expressed in a stably transfected heterologous host cell line [35].

All human α1,3/4-fucosyltransferases synthesize sLex structures *in vivo*

The differences of the *in vitro* and *in vivo* results obtained for FT6 led us to compare the *in vivo* properties of all human α1,3/4-fucosyltransferases. For this, we have also constructed stable BHK-21 cell lines (see Table 4) expressing human FT3, FT4, FT5 or FT7 together with human β-TP as a reporter glycoprotein, and for each individual cell line, β-TP was purified from the culture supernatant and subjected to oligosacharide structural analysis using MALDI/TOF-MS, ESI-MS/MS and HPAE-PAD mapping. About 30–50% of the *N*-linked oligosaccharides of β-TP secreted from the new cell lines were α1,3-fucosylated except for the FT3 cell line in which case only 19% of the structures were fucosylated. A comparison of all β-TP *N*-glycan structures formed by the α1,3/4-FT-transfected cells is presented in Table 5 and gives an overview of the *in vivo* substrate specificity of the five known human α1,3/4-FTs with protein-bound complex-type *N*-linked oligosaccharides [35].

FT7 cells (*i.e.,* BHK-21 cells coexpressing FT7 and β-TP) exclusively synthesize sLex structures. We have confirmed this also for the monosialylated oligosaccharide fraction that did not contain any α1,3-difucosylated structure. The single Fuc was exclusively present as the sLex and *not* as the Lex motif (determined by ESI-MS/MS, see. ref. [35]), and the small amount of asialo oligosaccharides did not contain any Lex epitopes. This *in vivo* specificity with complex-type *N*-glycans is in agreement with recent work published on the *in vitro* activity of the enzyme with small oligosaccharide substrates [41,50,51].

The vast majority of the peripherally fucosylated product in β-TP oligosaccharides from **FT4 cells** were found to contain the Lex motif which result is compatible with published data on the *in vitro* activity of FT4 with low molecular weight compounds. From the fragmentation pattern of reduced and permethylated chains using ESI-MS/MS, we could show that the monosialylated α1,3-monofucosylated *N*-glycan contains preponderantly the Lex motif [35]. However, a significant amount (11%) of mono-sLex was also observed in the disialo oligosaccharide fraction which contradicts published data on the *in vivo* specificity of the enzyme as measured by E-selectin binding studies [41,53,58,59]. However, no α1,3-difucosylated disialo structure was observed, supporting the view of the preferential action of FT4 on nonsialylated Ga1(β1→4)GlcNAc-R structures.

FT5 cells secrete β-TP with oligosaccharides modified preponderantly with the sLex motif, but also Lex-containing structures are formed. They were detected as α1,3-difu-

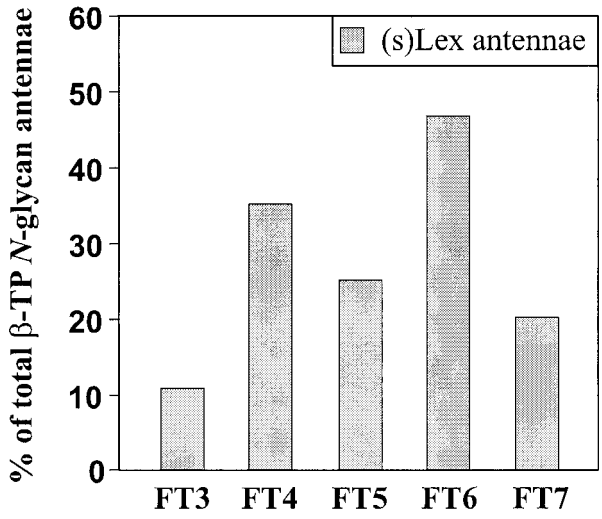

Figure 5. Comparison of fucosylated oligosaccharide antennae of β-TP secreted from cell lines cotransfected with human fucosyltransferases III–VII (compare also Table 4)

cosylated asialo and α1,3-difucosylated monosialo oligosaccharides. In contrast to the situation found for the monosialo-monofuco oligosaccharides from FT7 and FT4 cells, the α1,3-monofucosylated monosialo oligosaccharides from FT5 cells were found to consist of a mixture of Lex and sLex containing structures.

In a previous study [34], we have reported that human **FT3** from BHK-21 cells does not fucosylate type **II** *N*-acetyllactosamine structures in several glycoproteins when incubated *in vitro*. Furthermore, we could clearly demonstrate that, with bovine fetuin as a substrate, only the triantennary oligosaccharide containing one type **I** branch (Gal(β1→3)GlcNAc) is modified with α1,4-linked Fuc *in vitro,* although an 8-fold higher type **II** acceptor concentration (Gal(β1→4)GlcNAc-R branches) was present during the experiment [34]. Similarly, we could show that no sLex or Lex *in vitro* activity was present in extracts of FT3 cells when using low molecular weight type **II** oligosaccharide acceptors (see also Table 4). These findings have been further confirmed in a recent publication from our group [54] describing the failure to *in vitro* fucosylate diantennary type **II** oligosaccharides with large amounts of a purified recombinant FT3 preparation. For the *in vivo* activity of the Golgi form of the enzyme, almost no fucosylation of asialo branches was observed in β-TP oligosaccharides from FT3-transfected cells. Using ESI-MS/MS and methylation analysis, we confirmed that only the sLex and no sLea structure was present in the oligosaccharides of coexpressed β-TP. Since only 11% of the total oligosaccharide antennae of β-TP from FT3 cells were modified with peripheral fucose compared to up to 50% of the total antennae in β-TP from cells transfected with FT4-FT7 (*cf.* Fig. 5), we conclude that FT3 preferentially acts *in vivo* as a type **I** chain-specific transferase which is in agreement with the *in vitro* data published previously [34,54].

The results of our *in vivo* specificity studies of recombinant human α1,3/4-FTs indicate that each of the enzymes exhibits a specific fucosylation characteristics with type **II** complex *N*-glycan chains on coexpressed human β-TP as is exemplified by the different sLex/Lex ratios: FT7 (only sLex) > FT3 (14:1) > FT5 (3:1) > FT6 (1.1:1) > FT4 (1:7) [35]. Furthermore, from the results obtained, recombinant human FT6 turns out to have a high *in vivo* preference to form α1,3-difucosylated structures with all three, asialo, mono- and disalo diantennary acceptor oligosaccharides. A similar high preference for the synthesis of α1,3-difucosylated diantennary glycans is only detected for FT4 with the asialo structures (compare Table 5). Apart from its strict specificity towards α2,3-sialylated antennae, FT7 appears to have very similar preference for both, α1,3-mono- and α1,3-di-Fuc-transfer onto *N*-linked oligosaccharides, whereas FT3 and FT5 predominantly attach a single peripheral Fuc residue to diantennary *N*-glycans.

Human FT6 requires Golgi membrane localization for its *in vivo* activity

In a previous publication we have reported that the wild-type Golgi form of human FT3 is intracellularly cleaved in stably transfected BHK-21 cells and the catalytically active fragment can be detected by *in vitro* assays and Western blotting in the cell supernatant [34]. Similar observations have been published for α2,6-sialyltransferase [60], β1,4-galactosyltransferase [61], α1,3-galactosyltransferase [62], polypeptide α1→O GalNAc-transferase [63], G_M2 synthase (β1,4-GalNAc-transferase) [64] and FT6 [65]. The enzymes responsible for this proteolytical cleavage have been proposed to be cathepsin-like proteases or serine proteases, respectively. We have found that recombinant human FT6 is secreted by two different BHK-21 cell lines and from CHO DHFR cells [35], as shown in Table 4. While FT7 and FT5 were found to be resistant to proteolysis in transfected BHK-21 cells, we have also observed secreted forms of FT4 in supernatants of cells transfected with wild-type human FT4. It is important to consider the proteolytically cleaved enzyme forms when describing the *in vivo* specificity of glycosyltransferases, since it is known that also from natural cells/tissues considerable amounts of soluble forms have been detected in the medium of cells or in body fluids in certain diseases [66,67].

We have addressed the question of a possible contribution to the *in vivo* activity of soluble forms by engineering of cells that express variants of human FT6 lacking the cytoplasmic and transmembrane domain and part of the stem region (s-FT6), as depicted in Figure 6. In addition, we have constructed a chimeric secretable protein (BT-FT6) by fu-

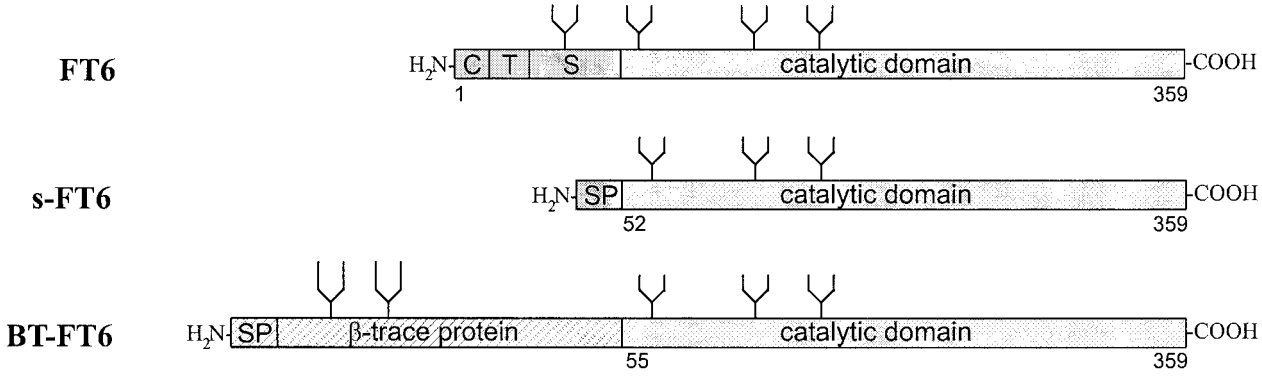

Figure 6. Schematic representation of the protein domain structures of wild-type human FT6 and soluble variants. C, T, S denote the *c*ytoplasmic, *t*ransmembrane and *s*tem regions; SP = signal peptide; the number of potential *N*-glycosylation sites are indicated by symbols

sion of the full-length human β-TP sequence to the N-terminus of the catalytic domain of human FT6 which resulted in a 70-fold overexpression of catalytic activity. Our results obtained with the coexpression of s-FT6 together with β-TP indicate that enzymes secreted along the secretory pathway can be considered to not contribute to the *in vivo* functional activity of the enzyme, since only after about 20-fold overexpression of s-FT6 (cell line s-FT6(II), compare Table 4), we were able to detect very small amounts of fucosylation of β-TP, and even 70-fold overexpressed BT-FT6 results in a low α1,3-fucosylation of β-TP, as shown in Table 5. Cho and Cummings [68] found by lectin binding that a recombinant, soluble α1,3-galactosyltransferase (lacking the transmembrane and cytoplasmic domain) is functionally active *in vivo* when expressed at slightly higher levels than the full-length form of the enzyme. The reason for this discrepancy is unknown; however, that truncated forms of glycosyltransferases in general do not contribute significantly to the *in vivo* specificity of the enzyme towards secreted glycoproteins is supported by our finding that a recombinant soluble form of human ST6Gal I does not modify co-secreted β-TP in BHK-21 cells. This finding is confirmed by a recent publication [69] where the authors describe that soluble forms of recombinant β1,4-GalNAc-transferase or ST6Gal I were not at all or significantly less efficient *in vivo* than their membrane-bound counterparts. It should be emphasized that in natural tissues or cells transferase expression levels are much lower than those that can be achieved by transfection of cells with corresponding plasmids when the gene is under the control of a strong promoter.

Interestingly, in addition to the very low fucosylation efficiency of the high enzyme activity expressing s-FT6(II) cell line in our studies, also the fucosylation pattern of β-TP oligosaccharides was different with a higher proportion of α1,3-monofucosylated structures observed over the α1,3-difucosylated oligosaccharides which are the major *N*-glycans synthesized by cells transfected with the full-length form of FT6 [35]. This then supports the view of the importance of the *c*ytoplasmic, *t*ransmembrane and *s*tem region (*CTS*-region) not only for the *in vivo* functional activity of glycosyltransferases, but also for their *in vivo* substrate specificities. In this context, it seems attractive to speculate that the CTS-region is also involved in the addressing of glycosyltransferases into different subcompartments of the biosynthetic glycosylation pathway of cells. The CTS polypeptide domains would be responsible for the targeting of the recombinantly expressed FT6 and FT4 to subcompartments where they can compete with the BHK cell endogenous ST3Gal III/IV for the same acceptor substrate since the sialylation state is lower for the *N*-glycans of β-TP secreted from the transfected cells (and as mentioned above, this indicates that ST3Gal III and ST3Gal IV do not recognize Lex motifs *in vivo*). The targeting properties of the FT6 CTS-region should result in an intracellular broader distribution of this enzyme functional activity and its overlapping with ST3Gal III/IV. FT4 should be targeted into an earlier Golgi subcompartment before the α2,3-STs modify the acceptor Gal(β1→4)GlcNAc-R substrate. Likewise, the CTS-region should direct FT7 and FT5 into a later functional compartment than the BHK cell endogenous

α2,3-STs which must provide the properly sialylated oligosaccharide precursor substrates.

Engineering of cells by stable transfection with human Gal(β1→4)GlcNAc-R α2,6- sialyltransferase

BHK-21 cells and CHO cells do not express *N*-glycan-specific α2,6-sialyltransferases (see Table 1). Therefore, glycoproteins with this typical human serum-type sialylation characteristic are not synthesized by these host cell lines. The stable transfection of cells with plasmids encoding human ST6Gal I [70] seems to be an attractive way to manipulate host cell lines for the production of this human serum-type carbohydrate structural motif. This experimental approach is justified by considering data published for the *in vitro* specificity of ST6Gal I as well as structural studies on natural glycoproteins. However, it has to be considered that the newly introduced enzyme competes with the host cell endogenous α2,3-STs (ST3Gal III/IV) for the same precursor substrate Gal(β1→4)GlcNAc-R as has been discussed above for human FT6 and FT4. In order to evaluate such a competition, we have stably transfected BHK-21B cells with a plasmid encoding the Golgi-resident form of human ST6Gal I and have coexpressed human β-TP as a secretory model glycoprotein.

As depicted in Figure 3, human β-TP from wild-type BHK cells contains simple diantennary chains which are α2,3-disialylated, monosialylated, or asialo in a ratio of 70:25:5 [16,35]. BHK-21B cells were stably transfected with plasmids encoding human β-TP and human ST6Gal I (*cf.* Fig. 2) and were used for the subsequent production of β-TP. Isolation of the secreted β-TP from cell supernatants was performed by a single step using immunoaffinity chromatography with a polypeptide-specific monoclonal antibody raised against β-TP [55]. Careful structural analysis of the *N*-linked oligosaccharides of the recombinant β-TP by complementary techniques (MALDI/TOF-MS, HPAE-PAD and NMR methods [16]) was performed and we found that monosialylated and disialylated glycans were present in a ratio of 1:5. As shown in Figure 7, 60% of the disialylated oligosaccharides contained both, α2,3- and α2,6-linked NeuAc. The α2,6-linked NeuAc was preferentially attached to the Gal(β1→4)GlcNAc(β1→2)Man (α1→3) branch. This indicates that the newly introduced ST6Gal I competes with the endogenous ST3Gal III/IV. Since the ST6Gal I from bovine colostrum has been reported to act preferentially on the Man-3 branch of diantennary glycans [71], we could confirm the specificity of the human enzyme by our *in vivo* experiments. Similarly, we showed successful α2,6-sialylation of di- and triantennary oligosaccharides of recombinant human AT III secreted by BHK-21B cells coexpressing ST6Gal I (a ratio of α2,3- to α2,6-linked NeuAc of 1:2 was determined here by integration of the NMR signals of the axial and equatorial H-3 protons of NeuAc in the total oligosaccharide mixture [16]).

Human ST6Gal I tranfers NeuAc in α2,6-linkage to GalNAc(β1→4)GlcNAc-R motifs *in vivo*

We have previously identified a BHK-21A cell clone which synthesizes large amounts of GalNAc(β1→4)GlcNAc-R motifs in addition to the common type **II** Gal(β1→4) GlcNAc-R structures on secretory glycoproteins [16,17]. Recombinant glycoproteins secreted from this host cell line are found to be undersialylated because the endogenous ST3Gal III/IV do not recognize terminal GalNAc(β1→4) GlcNAc residues as a substrate. This offered the possibility to investigate if the transfection of this cell line with human ST6Gal I would result in a higher degree of sialylation of recombinant EPO, since from *in vitro* experiments it has been postulated that the enzyme also recognizes LacdiNAc

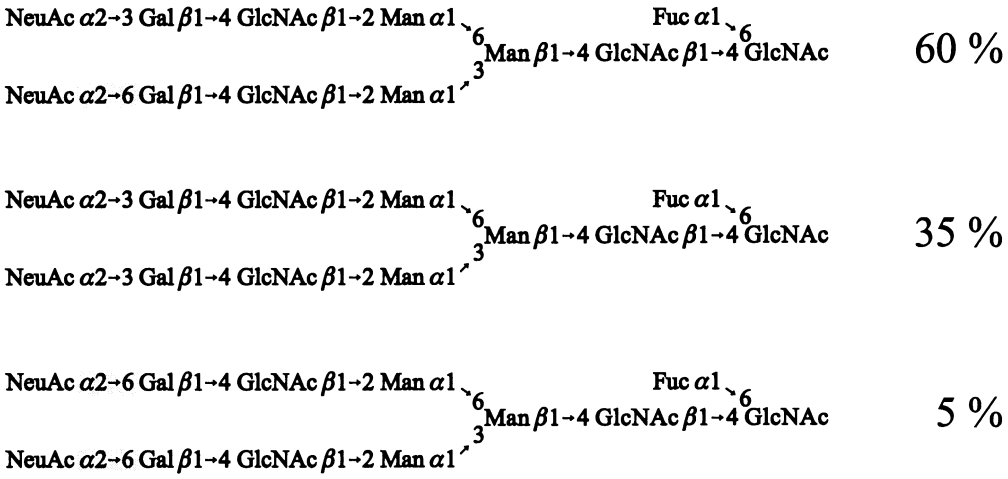

Figure 7. Disialylated *N*-linked oligosaccharide chains of recombinant β-TP expressed from ST6Gal I-transfected BHK-21B cells contain large amounts of α2,6-linked NeuAc.

motifs [72,73]. Human EPO expressed from wild-type BHK-21A cells contains large amounts of the unsialylated diantennary oligosaccharides with two terminal GalNAc(β1→4)GlcNAc motifs as well as tri- and tetrantennary chains with 1,2 or 3 GalNAc substitutions [17]. When cells are cotransfected with ST6Gal I (15-fold higher expression based on mRNA level and 5-fold higher enzyme activity with Gal(β1→4)GlcNAc-O-$(CH_2)_8COOCH_3$ as an acceptor over the endogenous α2,3-STs), we found asialo, α2,6-mono and α2,6-disialylated derivatives of the biantennary di-GalNAc oligosaccharides in a ratio of 0.1:1:0.2 based on the MALDI/TOF signals obtained from the reduced and permethylated native glycan pool of the purified EPO product (signals for $[M + K^+]$ at m/z 2360, 2721 and 3083, respectively) and methylation data [17]. This indicates that the GalNAc(β1→4)GlcNAc-R branches are efficiently recognized as substrates for ST6Gal I *in vivo*. The cell line therefore allows for the production of recombinant glycoproteins with a *human-type* NeuAc(α2→6)GalNAc(β1→4)GlcNAc-R structural motif (see Fig. 8) that is frequently found in glycoproteins secreted from human kidney tissue [74–76].

Taken together, the expression of recombinant human α2,6-sialyltransferase increases the sialylation state of glycoproteins secreted from BHK-21A and BHK-21B cells as we have shown for human β-TP, AT III and EPO [16,17]. However, the human ST6Gal I has its limitations since the enzyme has a high preference for the Man-3 branch of oligosaccharides and, due to the competition with the endogenous α2,3-STs, the final products contain mixtures of α2,3/6-sialylated oligosaccharides. Also many natural human glycoproteins have both NeuAc linkages, and at present it is unknown if therapeutic glycoproteins that are exclusively modified with α2,6-linked NeuAc would be advantageous over those with a mixture of α2,3/6-linked NeuAc.

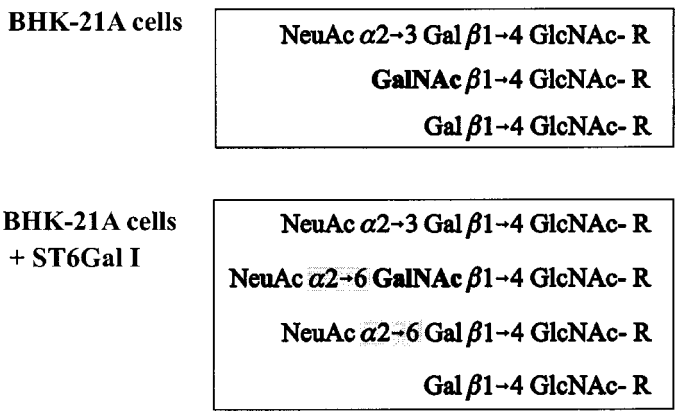

Figure 8. Terminal structural motifs in oligosaccharides of recombinant EPO from wild-type BHK-21A cells and human ST6Gal I-transfected BHK-21A cells

Future perspectives

By using recombinant DNA technology, we are now able to efficiently manipulate the glycosylation capacity of cells to be used as new stable cell factories for biotechnological processes. However, we need to understand in much more detail basic regulatory phenomena underlying the complex interaction of the intracellular enzyme machinery that is involved in the biosynthesis of glycoconjugates. All theories and current models of compartmentalization of the cellular glycosylation pathways rely on experimental data obtained by immuno-localization of the enzymes [77–79]. In our opinion, more detailed knowledge about the *in vivo* functional localization of the glycosylation machinery of cells is required and this has to be approached experimentally.

The importance of the CTS-region of glycosyltransferases not only for their *in vivo* function, but also for their *in vivo* specificity has been addressed for the human α1,3/4-fucosyltransferases in this review. In this context, it is noteworthy that polysialyltransferase (ST8Sia IV) from humans or CHO cells possesses a very short transmembrane domain of only 13 amino acid residues [80,81] which apparently does not fit to the lipid bilayer thickness model proposed for the intracellular targeting of transmembrane proteins [78,82]. Nevertheless, according to the concept of a sequential action of glycosyltransferases, ST8Sia IV should be localized within a late Golgi compartment where it can get access to its α2,3-sialylated precursor substrate, and therefore, the CTS-region of this transferase attached to the catalytic domain of, *e.g.,* FT6 should direct this enzyme into a location where it should synthesize higher amounts of sLex with coexpressed β-TP. Our results, however, obtained for this ST8Sia IV-FT6 fusion protein coexpressed together with human β-TP in BHK-21 cells, showed an oligosaccharide pattern for the reporter glycoprotein that was almost indistinguishable from that obtained from cells cotransfected with wild-type FT6 [83].

Other important considerations include:

- identification of *in vivo* functional cellular subcompartments at the molecular level of transferases and nucleotide sugar transporters
- intracellular turnover and the posttranslational modification of the enzymes
- dynamics of acceptor substrate and transferase transport within Golgi subcompartments
- role of splicing variants of glycosyltransferase/glycosidase genes

This is of general significance in all cases where recombinant glycobiology is used not only for the construction of "improved" cell factories for the production of more efficient and safer glycotherapeutics or recombinant retroviral vectors for gene therapy with oligosaccharide-based addressing signals, but also when it is intended to modify by

genetic/metabolic engineering the surface of *ex vivo* propagated human primary cells destined for *in vivo* therapy in clinics.

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References

1 Varki A (1993) *Glycobiology* **3:** 97–130.
2 Berman PW, Lasky LA (1985) *Trends Biochem Tech* **3:** 51–3.
3 Kobata A (1992) *Eur J Biochem* **209:** 483–501.
4 Schachter H (1994) In *Molecular Glycobiology* (Fukuda M, Hindsgaul O, eds.), pp 88–162. Oxford, UK: Oxford University Press.
5 Hoffmann A, Nimtz M, Wurster U, Conradt HS (1994) *J Neurochem* **63:** 2185–196.
6 Hoffmann A, Nimtz M, Getzlaff R, Conradt HS (1995) *FEBS Lett* **359:** 164–68.
7 Pohl S, Hoffmann A, Rüdiger A, Nimtz M, Jaeken J, Conradt HS (1997) *Glycobiology* **7:** 1077–84.
8 Conradt HS, Lindenmaier W, Ausmeier M, Hauser H, Dittmar KEJ (1986) *Carbohydr Res* **49:** 443–50.
9 Conradt HS, Egge H, Peter-Katalinic J, Reiser W, Siklosi T, Schaper K (1987) *J Biol Chem* **262:** 14600–605.
10 Mutsaers JHGM, Kamerling JP, Devos R, Guisez Y, Fiers W, Vliegenthart JFG (1986) *Eur J Biochem* **156:** 651–54.
11 Conradt HS, Hofer B, Hauser H (1990) *Trends Glycosci Glycotechnol* **2:** 168–81.
12 Goochee CF, Monica T (1990) *Bio/Technology* **8:** 421–27.
13 Goochee CF, Gramer MJ, Andersen DC, Bahr JB, Rasmussen JR (1991) *Bio/Technology* **9:** 1347–55.
14 Jenkins N, Parekh RB, James DC (1996) *Nature Biotechnol* **14:** 975–81.
15 Grabenhorst E, Nimtz M, Conradt HS, unpublished results.
16 Grabenhorst E, Hoffmann A, Nimtz M, Zettlmeiμ G, Conradt HS (1995) *Eur J Biochem* **232:** 718–25.
17 Schlenke P, Grabenhorst E, Nimtz M, Conradt HS (1998) *Cytotechnology,* in press.
18 Grabenhorst E, Hofer B, Nimtz M, Jäger V, Conradt HS (1993) *Eur J Biochem* **215:** 189–97.
19 Ackermann M (1995) Doctoral thesis, Technical University of Braunschweig, Germany.
20 Ackermann M, Nimtz M, Grabenhorst E, Conradt HS, Jäger V (1995) In *The Baculovirus and Insect Cell Gene Expression Conference,* Pinehurst, NC, USA (abstract).
21 Knepper TP, Arbogast B, Schreurs J, Deinzer ML (1992) *Biochemistry* **31:** 11651–59.
22 James DC, Freedman RB, Hoare M, Ogonah OW, Rooney BC, Larionov OA, Dobrovolsky VN, Lagutin OV, Jenkins N (1995) *Bio/Technology* **13:** 592–96.
23 Grabenhorst E (1994) Doctoral thesis, Technical University of Braunschweig, Germany.
24 Dittmar KEJ, Conradt HS, Hauser H, Hofer B, Lindenmaier W (1989) In *Advances in Protein Design* (Blöcker H, Collins J, Schmidt RD, Schomburg D, eds.), GBF-Monographs vol. 12, pp 145–56. Weinheim, New York: VCH Publishers.
25 Kagawa Y, Takasaki S, Utsumi J, Hosoi K, Shimizu H, Kochibe N, Kobata A (1988) *J Biol Chem* **263:** 17508–15.
26 Zettlmeissl G, Conradt HS, Nimtz M, Karges P (1989) *J Biol Chem* **264:** 21153–59.
27 Sasaki H, Bothner B, Dell A, Fukuda M (1987) *J Biol Chem* **262:** 12059–76.
28 Takeuchi M, Takasaki S, Miyazaka H, Kato T, Hoshi S, Kochibe N, Kobata A (1988) *J Biol Chem* **263:** 3657–63.
29 Nimtz M, Wray V, Augustin M, Klöppel K-D, Conradt HS (1993) *Eur J Biochem* **213:** 39–56.
30 Hokke CH, Bergwerff AA, van Dedem GWK, Kamerling JP, Vliegenthart JFG (1995) *Eur J Biochem* **228:** 981–1008.
31 Grabenhorst E (1991) Diploma thesis, Technical University of Braunschweig, Germany.
32 Conradt HS, Gawlitzek M, Grabenhorst E, Hoffmann A, Nimtz M, Oltmanns-Bleck F, Pohl S (1997) In *Medicinal Chemistry: Today and Tomorrow* (Yamazaki M, ed) pp 205–12. Blackwell Science.
33 Conradt HS, Nimtz M, Dittmar KEJ, Lindenmaier W, Hoppe J, Hauser H (1989) *J Biol Chem* **264:** 17368–73.
34 Costa J, Grabenhorst E, Nimtz M, Conradt HS (1997) *J Biol Chem* **272:** 11613–21.
35 Grabenhorst E, Nimtz M, Costa J, Conradt HS (1998) *J Biol Chem,* in press.
36 Kukowska-Latallo JF, Larsen RD, Nair RP, Lowe JB (1990) *Genes Dev* **4:** 1288–1303.
37 Goelz S, Hession C, Goff D, Griffiths B, Tizard R, Newman B, Chi-Rosso G, Lobb R (1990) *Cell* **63:** 1349–56.
38 Weston BW, Nair RP, Larsen RD, Lowe JB (1992) *J Biol Chem* **267:** 4152–60.
39 Koszdin KL, Bowen BR (1992) *Biochem Biophys Res Commun* **187:** 152–57.
40 Weston BW, Smith PL, Kelly RJ, Lowe JB (1992) *J Biol Chem* **267:** 24575–84.
41 Sasaki K, Kurata K, Funayama K, Nagata M, Watanabe E, Ohta S, Hanai N, Nishi T (1994) *J Biol Chem* **269:** 14730–37.
42 Varki A (1994) *Proc Natl Acad Sci USA* **91:** 7390–97.
43 Lasky LA (1995) *Ann Rev Biochem* **64:** 113–39.
44 McEver RP, Moore KL, Cummings RD (1995) *J Biol Chem* **270:** 11025–28.
45 Kansas GS (1996) *Blood* **88:** 3259–87.
46 de Graaf TW, van der Stelt ME, Anbergen MG, van Dijk W (1993) *J Exp Med* **177:** 657–66.
47 Brinkman-van der Linden EC, de Haan PF, Havenaar EC, van Dijk W (1998) *Glycoconj J* **15:** 177–82.
48 de Vries T, Srnka CA, Palcic MM, Swiedler SJ, van den Eijnden DH, Macher BA (1995) *J Biol Chem* **270:** 8712–22.
49 de Vries T, Palcic MP, Schoenmakers PS, van den Eijnden DH, Joziasse DH (1997) *Glycobiology* **7:** 921–27.
50 Shinoda K, Morishita Y, Sasaki K, Matsuda Y, Takahashi I, Nishi T (1997) *J Biol Chem* **272:** 31992–97.
51 Britten CJ, van den Eijnden DH, McDowell W, Kelly VA, Witham SJ, Edbrooke MR, Bird MI, de Vries T, Smithers N (1998) *Glycobiology* **8:** 321–27.
52 Natsuka S, Gersten KM, Zenita K, Kannagi R, Lowe JB (1994) *J Biol Chem* **269:** 16789–94.
53 Kumar R, Potvin B, Muller WA, Stanley P (1991) *J Biol Chem* **266:** 21777–83.

54 Nimtz M, Grabenhorst E, Gambert U, Costa J, Wray V, Morr M, Thiem J, Conradt HS (1998) *Glycoconj J* **15:** 873–83.
55 Hoffmann A, Nimtz M, Conradt HS (1997) *Glycobiology* **7:** 499–506.
56 Smith DF, Larsen RD, Mattox S, Lowe JB, Cummings RD (1990) *J Biol Chem* **265:** 6225–34.
57 Sueyoshi S, Tsuboi S, Sawada-Hirai R, Dang UN, Lowe JB, Fukuda M (1994) *J Biol Chem* **269:** 32342–50.
58 Lowe JB, Kukowska-Latallo JF, Nair RP, Larsen RD, Marks RM, Macher BA, Kelly RJ, Ernst LK (1991) *J Biol Chem* **266:** 17467–77.
59 Gersten K, Natsuka S, Trinchera M, Petryniak B, Kelly RJ, Hiraiwa N, Jenkins NA, Gilbert DJ, Copeland NG, Lowe JB (1995) *J Biol Chem* **270:** 25047–56.
60 Weinstein J, Lee EU, McEntee K, Lai P-H, Paulson JC (1987) *J Biol Chem* **262:** 17735–43.
61 Masri KA, Appert HE, Fukuda MN (1988) *Biochem Biophys Res Commun* **157:** 657–63.
62 Larsen RD, Rajan VP, Ruff MM, Kukowska-Latallo J, Cummings RD, Lowe JB (1989) *Proc Natl Acad Sci USA* **86:** 8227–31.
63 Homa FL, Hollander T, Lehman DJ, Thomsen DR, Elhammer AP (1993) *J Biol Chem* **268:** 12609–16.
64 Jaskiewicz E, Guofen Zhu, Bassi R, Darling DS, Young WW Jr (1996) *J Biol Chem* **271:** 26395–403.
65 Borsig L, Katopodis AG, Bowen BR, Berger EG (1998) *Glycobiology* **8:** 259–68.
66 Kaplan HA, Woloski BMRNJ, Hellman M, Jamieson JC (1983) *J Biol Chem* **258:** 11505–9.
67 Uejima T, Uemura M, Nozawa S, Narimatsu H (1992) *Cancer Res* **52:** 6158–63.
68 Cho SK, Cummings RD (1997) *J Biol Chem* **272:** 13622–28.
69 Zhu G, Allende ML, Jaskiewicz E, Qian R, Darling DS, Worth CA, Colley KJ, Young WW Jr (1998) *Glycobiology* **8:** 831–40.
70 Grundmann U, Nerlich C, Rein T, Zettlmeissl G (1990) *Nucl Acids Res* **18:** 667.
71 Joziasse DH, Schiphorst WECM, van den Eijnden DH, Van Kuik JA, Van Halbeek H, Vliegenthart JFG (1987) *J Biol Chem* **262:** 2025–33.
72 Nemansky M, van den Eijnden DH (1992) *Biochem J* **287:** 311–16.
73 Hokke CH, van der Ven JGM, Kamerling JP, Vliegenthart JFG (1993) *Glycoconj J* **10:** 82–90.
74 Weisshaar G, Hiyama J, Renwick AGC, Nimtz M (1991) *Eur J Biochem* **195:** 257–68.
75 Yan SB, Chao YB, van Halbeek H (1993) *Glycobiology* **3:** 597–608.
76 Bergwerff AA, Van Oostrum J, Kamerling JP, Vliegenthart JFG (1995) *Eur J Biochem* **228:** 113–26.
77 Colley KJ (1997) *Glycobiology* **7:** 1–13.
78 Munro S (1998) *Trends Cell Biol* **8:** 11–15.
79 Lowe M, Nakamura N, Warren G (1998) *Trends Cell Biol.* **8:** 40–44.
80 Nakayama J, Fukuda MN, Fredette B, Ranscht B, Fukuda M (1995) *Proc Natl Acad Sci USA* **92:** 7031–35.
81 Eckhardt M, Mühlenhoff M, Bethe A, Koopman J, Frosch M, Gerardy-Schahn R (1995) *Nature* **373:** 715–18.
82 Bretscher MS, Munro S (1993) *Science* **261:** 1280–81.
83 Grabenhorst E, Nimtz M, Conradt HS, manuscript in preparation.

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