Exploring the Acceptor Substrate Recognition of the Human β-Galactoside α2,6-Sialyltransferase*

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Human β1,4-galactoside α2,6-sialyltransferase I (ST6GalI) recognition of glycoprotein acceptors has been investigated using various soluble forms of the enzyme deleted to a variable extent in the N-terminal half of the polypeptide. Full-length and truncated forms of the enzyme have been investigated with respect to their specificity for a variety of desialylated glycoproteins of known complex glycans as well as related proteins with different carbohydrate chains. Differences in transfer efficiency have been observed between membrane and soluble enzymatic forms, indicating that deletion of the transmembrane fragment induces loss of acceptor preference. No difference in substrate recognition could be observed when soluble enzymes of similar peptide sequence were produced in yeast or mammalian cells, confirming that removal of the membrane anchor and heterologous expression do not alter enzyme folding and activity. When tested on free oligosaccharides, soluble ST6GalI displayed full ability to sialylate free N-glycans as well as various N-acetyllactosaminyl substrates. Progressive truncation of the N terminus demonstrated that the catalytic domain can proceed with sialic acid transfer with increased efficiency until 80 amino acids are deleted. Fusion of the ST6GalI catalytic domain to the N-terminal half of an unrelated transferase (core β1,3-N-acetyllactosaminyltransferase) further showed that a chimeric form of broad acceptor specificity and high activity could also be engineered in vivo. These findings therefore delineate a peptide region of 50 amino acids within the ST6GalI stem region that governs both the preference for glycoprotein acceptors and catalytic activity, thereby suggesting that it may exert a steric control on the catalytic domain.

Glycosyltransferases belong to a family of hundreds of enzymes that catalyze the transfer of a sugar from a sugar nucleotide donor to glycoproteins and glycolipids in eukaryotic cells and to polysaccharides in bacteria. Many of these enzymes are in charge of assembling cell-surface glycoconjugates, for which the extent of sialylation has been increasingly recognized as the molecular determinant of a wide array of biological recognition processes as different as viral and bacterial adhesion, immune response, and neuronal outgrowth (1, 2). These events equally involve all types of glycoconjugates and different glycosidic linkages, but the key specificity of such complex molecular recognition is still unclear to us. It thus appears of primary importance to elucidate how sialic expression at the cell surface is controlled by glycosyltransferases. Especially within a transferase family sharing similar catalytic properties, the determinants governing specificity for their substrates and their underlying respective acceptor(s) need to be identified to understand the structural information that may be encoded by these enzymes through such a remarkable diversity.

Sialyltransferases constitute a family of at least 15 distinct glycosyltransferases that catalyze the transfer of sialic acid from CMP-NeuAc to glycoproteins or glycolipids during biosynthesis in animals and man (3, 4). A number of them have been cloned across the animal kingdom, including bacteria, suggesting a broad function throughout evolution (1). In eukaryotes, they share with the other glycosyltransferases a typical type II architecture with a short cytoplasmic N-terminal tail, a transmembrane fragment followed by a stem of variable length, and a C-terminal catalytic domain facing the lumen of the Golgi apparatus (5). Unlike other glycosyltransferase families, they display significant sequence homology in their catalytic domain, viz. three conserved peptide sequences designated as sialyl motifs L and S (6) and VS (7). Mutagenesis of the L motif, consisting of ~50 amino acids present in the middle of the catalytic domain, showed that it is largely involved in donor recognition (8), whereas the S motif, located closer to the C terminus, contributes to the binding of both donor and acceptor substrates (9). Sialyltransferases are generally named and classified according to the nature of the monosaccharide substrate and the type of linkage formed (10). Within a subfamily catalyzing the same glycosidic bond, sialyltransferase members may exhibit very low amino acid sequence identity as well as major differences in acceptor substrate recognition, i.e. in their requirement for simple or more complex acceptor structures (11). A typical example demonstrating such keen specificity in substrate recognition resides in the ST6GalNAc1 group.

The abbreviations used are: ST6GalNAc, β1,3N-acetyllactosaminyl α2,6-sialyltransferase; ST6Gal, β1,4-galactoside α2,6-sialyltransferase; LacNac, N-acetyllactosamine; CHO, Chinese hamster ovary; SNA, S. nigra agglutinin; PBS, phosphate-buffered saline; FITC, 2,7-dimethoxy-3-(4′-imidazolyl)acridine; BAPTA, 1,2-bis(o-aminophenoxy)ethane-N,N′,N′′,N′′-tetraacetic acid; pHStat, titration apparatus with pH meter equipped with astatimetric electrode; ST6GalNAcI, recombinant ST6GalNAc.; BSA, bovine serum albumin; age, age of the culture; ml, milliliter; μm, micrometer; pm, picometer; nM, nanomolar; μM, micromolar; nM, nanomolar; μM, micromolar.

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ST6GalNAcI, likely to be involved in the synthesis of the sialosyl-Tn antigen, exhibits the broadest specificity on (NeuAco2,3)o-1(3Galβ1,3)o-1GalNAc-O-Ser/Thr, whereas ST6GalNAcIII was shown to be specific for NeuAco2,3Galβ1,3GalNAcα/β-R without discriminating between α- and β-linked GalNAc (11, 12). As a result, this enzyme can synthesize the GD1a antigen (12). ST6GalNAcII presents an intermediate specificity and is active on (NeuAca2,3)o-1Galβ1,3GalNAcO-Ser/Thr (13), and isoforms I and II are considered to be responsible for mucin synthesis. The recently cloned ST6GalNAcIV displays a similar substrate specificity to that of ST6GalNAcIII, but prefers O-glycans to glycolipids (14). Another candidate for GD1a synthezae activity has been identified in mouse and is referred to as ST6GalNAcV (15). Comparison of peptide sequences reveals interesting features since ST6GalNAcIII appears to be virtually devoid of a stem region, whereas ST6GalNAcI exhibits the longest variable region. It thus appears that in this group, the broader the acceptor specificity, the longer the stem region.

Other examples showing distinct acceptor preferences can be found among sialyltransferase families. Among the six enzymatically distinct human α2,3-sialyltransferases cloned to date, galactoside α2,3 sialyltransferase ST3Gal and ST3GalII display similar catalytic activity for Galβ1,3GalNAc substrates common to O-linked glycans and glycolipids. ST3GalII prefers glycoproteins to glycolipids, whereas ST3GalIII prefers glycolipids (16). In addition, ST3GalIII was found to utilize Galβ1,3GlcNAc more efficiently than Galβ1,4GlcNAc in vitro (17). Other studies also demonstrated that mouse α2,8 sialyltransferase II requires the presence of internal fucose on N-glycans as well as the polypeptide region to sialylate variants of the neural adhesion molecule at special IgG-like domains (18). It is thus believed that these enzymes should display specific structural features to transfer sugars to appropriate acceptors and to exhibit such exquisite specificity.

Very recently, three x-ray crystallographic structures of eukaryotic glycosyltransferases have been reported (19–21) showing common structural features, which are as follows: (i) the occurrence of a structurally very similar catalytic domain consisting of a three-layer αβα-fold and (ii) the presence of a large pocket on one face capable of accommodating both the donor and acceptor substrates, surrounded by conserved motifs identified for a given glycosyltransferase family (22). It appears that the recognition of donor substrate is mediated by residues that are found in the most conserved regions, but very little experimental background has so far delineated those residues that specify acceptor recognition. For several α2,3-fucosyltransferases, a region located in a hypervariable segment at the N-terminal end of the catalytic domain was shown to account for differences in acceptor recognition (23–25). It has also been demonstrated that soluble forms of some transferases are less efficient in vivo than their membrane-bound counterparts in glycosylating endogenous acceptors (26), whereas no difference was observed for other classes of enzyme (27). These findings suggest that acceptor recognition may reside in variable regions, and as a result, we investigated whether or not it could be located within the stem region. We therefore compared the activity of membrane and soluble forms of human ST6GalI (EC 2.4.99.1) for glycoprotein acceptors of known glucosyl structure and show here that deleting the stem region of this transferase results in loss of acceptor preference together with increased transfer efficiency. We thus tentatively conclude that, in vivo, the juxtamembrane region of ST6GalII restricts enzyme specificity and activity.

**EXPERIMENTAL PROCEDURES**

**Materials**—Asialofetuin and human protein acceptors, neuraminidase, CMP-NeuAc, p-nitrophenyl phosphate, and cadicrylic acid were purchased from Sigma. Synthetic acceptor substrate (LacNAc-O-space-biotin) was purchased from Syntone. Biotinylated Sambucus nigra lectin was lectin from EY Laboratories. Streptavidin–alkaline phosphatase conjugates were purchased from Jackson Immunoresearch Laboratories, Inc. Restriction enzymes, purified rut liver α2,6-sialyltransferase, and T4 K polymerase (high fidelity) were from Roche Molecular Biochemicals. FLAG-cytomegalovirus plasmid was from Eastman Kodak Co. pDNA3 was from Invitrogen. Fluorescent-assisted carbohydrate electrophoresis gels were from Bio-Rad. All culture reagents were from Life Technologies, Inc. Oligonucleotide primers were from and plasmid sequencing was carried out by Eurogentec.

**Cloning of Human ST6GalII and Expression of Soluble and Membrane Variants**—The cDNA encoding ST6GalII was isolated from an HL-60 cDNA library and further cloned in the FLAG-cytomegalovirus expression vector containing the preprotrypsinogen signal peptide upstream from the FLAG sequence. Sequencing of the insert showed the same sequence as the one published earlier (28). The full-length sequence was also deleted of the same N-terminal region for the soluble form to be constructed in stably transfected CHO-K1 cells.

Various truncated forms of human ST6GalII lacking the transmembrane fragment were cloned into the pFLAG expression vector. The Δ1–28 form of ST6GalII was stably transfected in CHO-K1 cells for comparison with the membrane ST6GalII form, and a chimeric form was constructed with the N-terminal (amino acids 1–52) membrane portion of the rat core 2 β1,6-GlcNAc-transferase and Δ1–70 of human ST6GalII and also tagged at the N terminus. The six soluble forms of ST6GalII deleted to a variable extent (Δ1–28, Δ1–35, Δ1–48, Δ1–60, Δ1–80, and Δ1–100) were transiently expressed in COS cells as summarized in Scheme 1.

The cell culture media were collected over a 48–72-h period and further concentrated 10-fold by Centriprep centrifugation. Each batch was assayed for activity on asialofetuin to calibrate enzyme production and stored in small aliquots of similar activity at −20 °C. When necessary, the amount of the tagged protein was further estimated by competitive immunossays using anti-FLAG antibodies. The stable cell line was found to secrete ∼10 ng of FLAG-ST6GalII/106 cells/h. The specific activity of this enzymatic form was estimated to be 7.5 unit/mg of protein.

Human soluble ST6GalII was produced in Pichia pastoris after truncation of the N terminus and the transmembrane fragment as described (29). This deletion was therefore very similar to the FLAG-ST6GalII construct. The activity present in the supernatant was 0.8 milliunits/mg of yeast protein.

**Transfection**—Transfection of cells was carried out using FLAG-cytomegalovirus plasmids containing the different ST6GalII constructs and the LipofetAMINE (Life Sciences) procedure as recommended by the manufacturer. Semiconfluent cells were routinely transfected with

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**Scheme 1. Scheme of various human ST6GalII constructs.** The N-terminal peptide portion of human ST6GalII was progressively truncated up to residue 100, which was predicted to correspond to the boundary between the stem and the catalytic domain (indicated by shadow). The nomenclature used indicates the number of amino acids deleted. The chimeric construct C2GnT-ST6GalIIΔ1–70 (where C2GnT is core 2 β1,6 N-acetylgalcosaminyltransferase) was obtained by joining the N-terminal peptide segment of human core 2 β6-N-acetylgalcosaminyltransferase (amino acids 1–52) to the truncated ST6GalII form (Δ1–70). The checked boxes indicate the transmembrane domain (TMD).
~5 μg of plasmid DNA. Stably transfected CHO-K1 cells were selected using Geneticin at a concentration of 500 μg/ml over a time period of at least 4 weeks. Transient expression was routinely performed in COS cells using a lower selection pressure of the antibiotic.

**Solid-phase Assay for ST6GalI Acceptor Specificity**—The preference of ST6GalI for N-glycan acceptors was studied according to a solid-phase procedure described earlier by us (30) using the sialic acid-specific lectin S. nigra (SNA) as reported by Mattos et al. (31). Briefly, coating of microtiter plates was carried out using increasing amounts of protein acceptors in 100 μl of 50 mM PBS (pH 7.5) overnight at 4 °C. After saturating wells with 2% bovine serum albumin and washings, coating supernatants were treated with neuraminidase with 0.5% paraformaldehyde in calcium- and magnesium-containing PBS and incubated with 50 μg/ml lectin for 60 min on ice. For intracellular distribution of FLAG-tagged enzymes, FITC-SNA-stained cells were fixed with 0.5% paraformaldehyde in PBS, permeabilized with 0.5% Triton X-100 in PBS containing 1% fetal calf serum, and incubated with 10 μg/ml anti-FLAG monoclonal antibody M2 for 1 h. They were then rinsed three times and incubated with rhodamine isothiocyanate-conjugated goat anti-mouse IgG antibody for 1 additional h, and staining was visualized by confocal microscopy on a Leica instrument (Uniblitz Sutter Instrument Co.). Confocal images were processed with Metamorph Imaging System Version 3.5. Volumes were originally raytraced as 24-bit TrueColor images and transferred to Adobe Photoshop as 24-bit RGB TIFF files.

**Differential Activity of Full-length and Soluble Forms of ST6GalI**—The specificity of the rat enzyme has been extensively characterized (5–9); and since sequence analysis of rat liver and human ST6GalI showed 88% identity, it is assumed that the specificity of both enzymes is very similar. Of particular interest is the earlier observation by us that recognition of the human enzyme for LacNAc residues includes the branch downstream of the α-linked mannose (35), confirming the earlier observation that, like the rat enzyme, ST6GalI can discriminate glycans on the basis of their branching pattern (36). Preliminary testing showed that the purified liver preparation was more stable than the native form of the human enzyme extracted from transfected cells using a detergent. We therefore used the rat liver transferrase to compare the acceptor preference of the same soluble forms of human ST6GalI expressed in two different systems for various glycoproteins of known glycan structure.

First, we investigated the influence of the branching pattern on enzyme specificity based on the earlier results that fetuin, human transferrin, and human α1-acid glycoprotein are efficient acceptors for the native enzyme (37). These glycoprotein acceptors all contain complex-type N-glycans with terminal LacNAc groups, but differ in branching pattern and sialylated bonds. Human transferrin contains biantennary glycans sialylated in the α2,6-position; fetuin contains 2,2,4-triantennary glycans with both α2,3- and α2,6-linked sialic acids; and α1-acid glycoprotein contains 2,2,4,6-tetraantennary N-glycans with both α2,3- and α2,6-sialic acid. The presence of α2,6-linkages was confirmed by SNA binding to native preparations, and loss of lectin binding confirmed the removal of sialic acid by the neuraminidase treatment (data not shown).

We thus first compared the specificity for these acceptors of native and soluble forms of ST6GalI produced in mammalian cells and yeast. When the desialylated glycoproteins were incubated with recombinant ST6GalI at concentrations of 50 nM, the reaction products were probed by lectin binding (Fig. 1). Efficient transfer was recorded in all cases, with affinity values similar to those reported previously, with the membrane enzyme having a slightly better recognition of asialofetuin compared with other desialylated glycoproteins (Fig. 1A). These results with the rat transferrase are similar to those previously...
reported (37), and this assay has been repeated here for comparative purposes. Interestingly, the soluble forms of similar length produced in CHO cells (Fig. 1B) or yeast (Fig. 1C) equally recognized these acceptors, independently of the expression system. These findings show that the soluble enzymes did not differ in binding asialoglycoprotein acceptors and therefore suggest that differential acceptor recognition by the full-length transferase has been altered by truncation of the membrane anchor.

Transfer Efficiency of Soluble FLAG-ST6GalI for the Glycoprotein Hormone Family—To understand whether or not the enzyme may recognize a determinant present in the polypeptide backbone that could influence branch specificity, we investigated a family of structurally related proteins sharing bi- or triantennary glycans differing in terminal glycosylation. Glycoprotein hormones are heterodimers sharing a common α-subunit and differing in their β-subunit (38, 39). Placental hCG contains biantennary glycans; urinary hCG contains mostly triantennary oligosaccharides with α2,3-sialic acid as terminal sugars; and follicle-stimulating hormone is known to display triantennary glycans terminating in both α2,3- and α2,6-sialic acids. Pituitary hTSH contains biantennary glycans with low α2,3-sialic acid content (38, 40).

When incubated in the presence of soluble FLAG-ST6GalI, both urinary hCG (Fig. 2A) and human pituitary follicle-stimulating hormone (hFSH) (Fig. 2B) behaved as efficient acceptors in their native state, indicating that their glycans display sites available for receiving additional sialic acid. As anticipated, desialylated forms were found to be better acceptors, demonstrating that their branching pattern has no influence in the transfer reaction and that the soluble enzyme does not discriminate LacNAc groups originally sialylated in the α2,3-position. It was concluded that like the plasma proteins tested above, these glycoprotein acceptors do not appear to contain any structural features governing sialic acid transfer in the α2,6-position to specific lactosaminyl groups. These data are in total agreement with previous structural work on hCG remodeling using soluble bovine ST6GalI (41).

Pituitary hTSH was anticipated to be a very poor acceptor because of its high content of sulfated sugar (Fig. 3A). However, when recombinant hTSH was tested as an acceptor, the native preparation could readily be over-sialylated to the same extent as the hormone treated with neuraminidase (Fig. 3B). In this regard, this glycoprotein hormone reacted similarly to asialofetuin and asialo-follicle-stimulating hormone, indicating that the recombinant product could be extensively sialylated in vitro. Similar data were obtained with the ST6GalIΔ1–28 form produced in yeast (data not shown), confirming again that...
heterologous expression per se does not affect the catalytic properties of the transferase. Rather, it is likely that the transferase deleted of its membrane portion is no longer able to recognize features present in the various acceptors necessary to specify sialic acid transfer.

**Sialic Acid Transfer to Free N-Glycans and Derivatized Disaccharides**—To further investigate to what extent soluble forms of ST6GalI are able to sialylate N-linked glycans, we analyzed if these enzymatic forms could achieve the completion of complex oligosaccharides. Glycans released from glycoprotein acceptors by endoglycosidase treatment were incubated with the soluble forms produced in CHO cells or yeast and further derivatized for separation on gels according to their sialic acid content. Fig. 4 presents evidence that the glycans originally present in asialofetuin (lane 2) could be sialylated to a similar extent as the most negatively charged originally present in the fetuin preparation (lane 5). Urinary hCG glycans (lane 4) were similarly completed (lane 3) by the enzyme, further confirming that the protein acceptor is totally dispensable for sialic acid transfer. Whether these glycans were of low sialic acid content. These data thus confirmed that the soluble catalytic domain of ST6GalI is able to recognize a substrate as small as a disaccharide. They are in good agreement with earlier observations on the full-length transferase showing that, in vitro, the 6-OH of galactose and the amide group of the GlcNAc residue are the only groups essential for efficient transfer of sialic acid (42).

**Effect of Deleting the Stem Sequence of ST6GalI on Acceptor Preference**—Various protein sequence analysis methods were used to tentatively determine the boundary between the stem and the catalytic domain to identify the minimal size of the catalytic domain of the transferase. Comparison of known ST6GalI peptide sequences from different species showed the highest similarity in a region starting at about residue 100 down to the C-terminal end. In addition, predictive methods based on hydrophobic cluster analysis indicated that this region is composed of alternating, regular secondary structure elements, whereas the preceding segment (amino acids 50–100) appeared to be less organized and composed mostly of coiled regions. We therefore decided to progressively delete the N terminus up to residue 100 to assess to what extent this segment is involved in enzyme activity and to elucidate whether or not this portion may interact with the catalytic domain and affect acceptor preference. It was expected that selective acceptor recognition would be abolished, whereas cat-

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**2 J. C. Michalski and C. Ronin, unpublished data.**
alytic properties would be maintained. To compare the activity of the truncated ST6GalI variants, the activities of all enzymatic forms were matched using asialofetuin under conditions in which sugar transfer was displayed as suboptimal lectin binding and increased as a function of time. Under these conditions, sialic acid transfer was quantitatively similar for all dose-response curves and independent of enzyme concentration in the medium. Since multiple β-Gal residues are present in different glycan structures for each glycoprotein acceptor, no classical kinetic parameters can be accurately measured in this approach, but only relative activity.

Five additional deletions in the stem regions were carried out and tested for activity; but for clarity, only those starting from amino acids 228 to 260 are shown in Fig. 7. Four variants with deletions over the first 80 amino acids were found to be enzymatically active, whereas truncation of 100 amino acids abolished activity. Of interest, the transfer reaction was found to be increased as the deletion augmented from D1–28 to D1–60 (Fig. 7, A–C). For all three glycoproteins, optimal transfer was reached at lower acceptor concentration for the shortest enzymatic variant. Additional truncation between amino acids 260 and 280 did not further modify this property, but a more extensive deletion of 100 amino acids led to inactivation (data not shown). Half-maximal activity of the five active variants over the three acceptors indicated that transfer efficiency was augmented by a factor of 3–5 when the stem region was shortened (Table I). However, since these acceptors differ in LacNAc content, no conclusion could be drawn as to whether the removal of the N-terminal region affected acceptor recognition and/or the catalytic properties of the transferase.

To obtain a better understanding of the structural basis of increased transfer efficiency, we determined the kinetic parameters of the shortest and longest soluble truncated forms in
liquid-phase assays using a radioactive donor and similar enzyme concentrations. Kinetic analyses were performed using both a synthetic acceptor (Galβ1,4-GlcNAc-O-spacer-biotin) and a glycoprotein acceptor (asialofetuin). As shown in Table II, there appeared to be no difference in affinity between both enzymatic forms for the common donor and the two acceptors. They both exhibited \( K_m \) values of \( \sim 18 \mu M \) for CMP-NeuAc, 1 mM for LacNAc, and 150 mM for asialofetuin, in the same order of magnitude as those reported in the literature for the rat enzyme (42). Thus, removing \( \sim 60 \) amino acids upstream from the catalytic domain did not alter the recognition parameters, but rather modified the velocity of sialic acid transfer. Catalytic efficiency was found to be augmented by a factor of 3 for CMP-NeuAc, by a factor of 36 for LacNAc, and by a factor of 10 for asialofetuin. To ensure that truncation did not modify biosynthesis and secretion of the variants during transfection, Western blot analysis of the intracellular content was carried out using the same amount of total proteins and showed no difference in the various truncated forms of ST6GalI. Similarly, cell supernatants showed no significant variation in the secretory rate of the variants in the medium (data not shown). Gel electrophoresis carried out under mild reducing conditions did not display FLAG-tagged proteins of high molecular weight, ruling out that enzyme activity can be related to association with unknown proteins or enzyme oligomerization. In addition, confocal microscopy using FITC-SNA binding further showed that each transfected cell exhibited a higher content of sialylated glycoconjugates at the cell surface for the Δ1–80 variant compared with the Δ1–28 variant, further indicating that both forms were also differentially active on endogenous acceptors.

Altogether, these data demonstrate that releasing ST6GalI as a soluble enzyme significantly altered the conformation of the transferase to broaden its acceptor specificity and to accelerate the transfer reaction. It is therefore proposed that in the full-length enzyme, the stem region interacts with the catalytic domain to expose a specific recognition site for glycoprotein acceptors. Removing this modulatory region probably released steric constraints and induced a conformational change that opened up the active site and thus facilitated sugar transfer.

To elucidate whether or not membrane anchorage can indeed contribute to such an interaction, a chimeric protein was constructed by fusing the shortest and most active truncated variant to an unrelated N-terminal moiety of another transferase. The catalytic domain of ST6GalI was thus extended with the N-terminal portion of the core 2 (β6-GlcNAc-transferase tagged with the FLAG epitope (Scheme 1), and the chimeric form was stably transfected in CHO cells. The activity was then compared for these full-length membrane enzymes both \( \text{in vivo} \) and \( \text{in vitro} \). Fig. 8 (A and B, panels 2) shows the subcellular immunolocalization of these transferases using anti-FLAG antibodies. Both enzymes were localized to the Golgi apparatus very similarly. The respective activities of the full-length forms were then compared \( \text{in vivo} \) and \( \text{in vitro} \). As shown in Fig. 8 (A and B, panels 1), both ST6GalII and the chimeric transferase proved to be able to sialylate endogenous cell-surface glycoconjugates to a large extent. However, full-length ST6GalII led to SNA binding distributed in the Golgi and periplasmic compartment, whereas the cell surface exhibited spotted labeling (Fig. 8B, panel 1). It was concluded that the chimeric form was less restrictive and more efficient in sialylating cell-surface acceptors than the wild-type enzyme.

To tentatively compare the acceptor recognition of the full-length, chimeric, and secreted forms of ST6GalII, detergent extracts of different CHO clones were assayed in solid-phase assays for their respective activity for asialofetuin (Fig. 9A) or asialotransferrin (Fig. 9B). We observed that human full-length ST6GalII had lower activity for transferrin like the rat enzyme presented in Fig. 1A. The chimeric enzyme in which the N-terminal anchor and the stem region were substituted generated a transfer activity equally efficient for both fetuin and transferrin acceptors. The half-maximal transfer rate was found to be similar to that for the corresponding truncated variant, again reproducing increased efficiency compared with that of the full-length enzyme. These findings further support the conclusion that the membrane anchor does not participate by itself in acceptor specificity, but may rather constrain the stem region through lipid packing and somehow sustain its interaction with the catalytic domain of the transferase.

### Table I

| Glycoprotein acceptor | EC\(_{50}\) |
|-----------------------|-----------|
|                       | µg/ml     |
| **Transferrin**       |           |
| WT ST6GalII           | 2.8       |
| ST6GalIIΔ1–28         | 1.8       |
| ST6GalIIΔ1–35         | 1.6       |
| ST6GalIIΔ1–42         | 1.0       |
| ST6GalIIΔ1–60         | 1.0       |
| C2-ST6GalII           | 0.5       |
| **Fetuin**            |           |
| WT ST6GalII           | 2.2       |
| ST6GalIIΔ1–28         | 1.0       |
| ST6GalIIΔ1–35         | 1.8       |
| ST6GalIIΔ1–42         | 0.5       |
| ST6GalIIΔ1–60         | 0.5       |
| C2-ST6GalII           | 0.4       |
| **Acid glycoprotein** |           |
| WT ST6GalII           | 4.8       |
| ST6GalIIΔ1–28         | 1.0       |
| ST6GalIIΔ1–35         | 1.0       |
| ST6GalIIΔ1–42         | 0.8       |
| ST6GalIIΔ1–60         | 0.8       |
| C2-ST6GalII           | 0.6       |

**WT**, wild-type.

### Table II

| **CMP-NeuAc** | **Galβ1,4GlcNAc-R** | **ASF** |
|---------------|---------------------|---------|
| \( K_m \)     | \( V_m \)           | \( K_m \) | \( V_m \) | \( K_m \) | \( V_m \) | \( K_m \) | \( V_m \) |
| mM            | nmol/min/ml         | mM      | nmol/min/ml | mM      | nmol/min/ml | mM      | nmol/min/ml |
| FLAG-ST6GalIIΔ1–28 | 0.018 | 2.6     | 144 | 1     | 0.5     | 0.5       | 0.15 | 4.4       | 29.3    |
| FLAG-ST6GalIIΔ1–80 | 0.018 | 7.5     | 417 | 1.1   | 20      | 18        | 0.14 | 45        | 321     |

\(^a\) R, O(CH\(_2\)_4-NH-CO(CH\(_2\)_9-NH-biotin; ASF, asialofetuin.

\(^b\) Expressed as picomoles of sialic acid transferred to acceptor at 37 °C/min/ml of concentrated medium from transfected COS cells.

3 J.-C. Guillemot, C. Lamagna, and C. Ronin, manuscript in preparation.

**TABLE I**

Comparative efficiencies of membrane and deleted forms of human ST6GalI for glycoprotein acceptors

Efficiency is the half-maximal rate of transfers of sialic acid to various desialylated glycoproteins under conditions in which all the transferase variants exhibited transfer similar to asialofetuin (see "Experimental Procedures").
DISCUSSION

This study was aimed at exploring the acceptor preference of the human ST6GalI sialyltransferase and infers that recognition of the glycan, followed by sugar transfer, is governed by a short peptide sequence present in the juxtamembrane portion of the protein. Among glycosyltransferases, the a2,6-sialyltransferase has been extensively characterized because this enzyme terminates most of the N-glycans in human serum proteins, thereby controlling both their duration in blood and metabolic clearance (1). Like most of the glycosyltransferases, the rat liver transferase is a type II membrane Golgi protein of a well characterized domain structure. It has been widely assumed that the stem region protrudes into the lumen because proteolytic degradation has been shown very early on to release an active soluble form missing 62 amino acids (36). Within a homologous glycosyltransferase group, the length and primary structure of catalytic domains are relatively well conserved, and variability in the molecular size of these enzymes is often attributable to differences in the length of the stem region. This polypeptide portion tethers the catalytic domain to the membrane anchor and generally displays highly variable amino acid sequence. It is thought to consist of a flexible stretch of little secondary organization. The longest stem reported so far (416 amino acids) was observed in N-acetylgalactosaminyltransferase, a region rich in hydroxyamino acids containing many potential glycosylation sites (43). Conversely, ST6GalNAcIII was found to have a very short stem region, if any (12). Earlier on, it was proposed that the stem region may contribute to Golgi localization and/or enzyme oligomerization (44). It has also been very recently shown that the ST6GalI stem region is directly involved in Golgi retention and contains at least two possible cleavage sites responsible for enzyme secretion (45). However, because this portion has been found to be dispensable for enzyme activity in vitro, it has not been considered to be functionally related to the catalytic properties of the transferase, but rather to facilitate its access to the glycan acceptor by acting as a spacer arm.

Until recently, very little information was available concerning the acceptor preference of glycosyltransferases, but ongoing cloning of many new enzymes and characterization of their catalytic activity unraveled a very complex redundancy. It appears that each class of transferases can be divided in subfamilies displaying several activities capable of constructing the same sugar linkage on distinct glycoconjugates and that exhibit high but not necessarily exclusive preference for an acceptor. To date, a key step is therefore to understand the structural features that allow each glycosyltransferase within a family to discriminate among their glycoprotein or glycolipid acceptors. Consequently, it is also an intriguing question to understand how a cell equipped with a wide array of such enzymes can control the many combinatorial possibilities of glycosylation of its cell-surface glycoconjugates and/or secretory glycoproteins. Since full-length and soluble transferases transfected in mammalian cell lines were found to be differentially active at glycosylating cell proteins (26), it has been suggested that membrane anchorage and intracellular localization are also important for enzyme activity. As an example, transfecting the full-length rat a2,6-sialyltransferase in mammalian cells was shown to result in limited addition of a2,6-linked sialic acid, indicating a weak competition with endogenous enzymes (46). Indeed, the mode of action of glycosyltransferases in vivo is far from being fully identified, but it is widely admitted that the remarkable diversity of the cell glycosylation machinery should contribute to the synthesis of glycan structures with exquisite specificity. This study has deciphered the minimal catalytic domain of ST6GalI and showed that it is active in vivo in both membrane and soluble forms. Preliminary work has also revealed that all truncated variants of ST6GalI (Δ1–28 to Δ1–80) are fully active on cell-surface acceptors in vivo and clustered in the Golgi apparatus together with endogenous sialylated acceptors. These findings further confirm that discrimination of cell-surface glycoconjugates heavily relies on intracellular compartmentalization of the transferase. While this work was in progress, a second Golgi retention signal was reported for ST6GalI (45), which is in total agreement with our observation that, in the soluble enzymes, Golgi localization and activity are still interdependent for the newly synthesized glycoproteins trafficking in the trans-Golgi network to acquire their final glycan structure.

Using site-directed mutagenesis, several laboratories have elucidated the location of substrate-binding sites of sialyl- and fucosyltransferases over the last few years. Structure-function
relationships of the rat liver enzyme ST6GalII have been well advanced, especially the role of the two sialyl motifs in catalysis (6, 8, 9). In this regard, it was shown that LacNAc was less effective than glycoprotein acceptors, partly because enzyme specificity extends over glycan branching (35, 36, 41). Although the S sialyl motif is involved in the recognition of both the donor and the lactosaminyl substrates (9), it is anticipated that regions located elsewhere in the protein should also contribute to the display of glycan specificity through more distal interactions. In this regard, recent work on homologous α3-fucosyltransferases demonstrated that residues important for discriminating related substrates are located within a hyper-variable segment of the catalytic domain close to the stem region (23–25). Using subdomain swapping with human α1,3-fucosyltransferases III and VI, a hyper-variable region of 11 amino acids (positions 103–153) at the N terminus of the catalytic domain has been found to contain structural information for Galβ1,3GlcNAc-R versus Galβ1,4GlcNAc specificity (23). When the stem region was deleted in human fucosyltransferases V (amino acids 76–374) and III (amino acids 62–361), which then exhibited 93% identity, two regions in the N-terminal half of the catalytic domain were also identified to induce an α1,3- to α1,4-switch, representing a difference in 8 and 12 amino acids between the two protein sequences, respectively (24). These studies therefore delineate the existence of discrete subsites within this family of enzymes sharing similar α1,3/4-activity that are responsible for the various aspects of enzyme specificity. Recently, site-directed mutagenesis demonstrated that, in bovine fucosyltransferase β-encoded α1,3-fucotransferase, a single amino acid substitution at position 115 is sufficient to switch the substrate specificity, whereas a mutation at position 116 regulates the transferase efficiency of bovine α1,3-fucosyltransferase and human α1,3/4-fucosyltransferase III (25). This work lends further support to the possibility that, in fucosyltransferases, the stem region may participate in transferase activity by creating a highly specific acceptor site in the spatial vicinity of the catalytic domain. Such a modulatory region allows several transferses involved in Lewis antigen synthesis to discriminate structurally related disaccharide substrates while catalyzing a similar fucose transfer through highly homologous catalytic domains.

Regarding ST6GalII, our data also favor the participation of the stem region in the selective recognition of glycan acceptors. Removal of this region proved to alter interactions with the catalytic domain that are essential for restricting access to acceptor subsite and for accommodating well defined glycans. This in turn affects the catalytic domain probably through further conformational change to readily proceed with sugar transfer, as if the enzyme has been turned on to a more open functional state. Since constructing a chimeric molecule containing the stem and anchor portions of an unrelated transferase (core 2 βGlCNAc-transferase) showed that this latter transmembrane fragment does not reverse this effect, we also concluded that the role of the stem region may be specific for the transferase.

While this work was in progress, the crystal structures of three eukaryotic glycosyltransferases were elucidated (19–21). These proteins were crystallized in the presence of UDP, thereby revealing the portion of the protein interacting with the nucleotide moiety and the involvement of the DXD motif previously identified in many glycosyltransferases (22). Despite the lack of sequence identity, these enzymes adopt a similar topology (α/βα/βα-fold), with the key amino acids involved in UDP binding apparently located at equivalent positions. Since only the catalytic domains of these glycosyltransferases have been crystallized, there is still no indication of a possible role of the stem region in acceptor recognition. In addition, whether or not sialyltransferases can adopt a similar topology is still difficult to assess for at least two reasons: (i) they use a different sugar donor (CMP); and (ii) they do not contain the DXD, motif but instead contain other specifically conserved motifs (6–7, 22). Nevertheless, since acceptor-binding subsites may involve regions upstream of the catalytic domain for at least α3-fucosyltransferase and, as reported here, for ST6GalII, there are definitely structural features to be searched in the variable region that control the transfer reaction. Even though the stem region is predicted to be highly flexible, it often contains cysteine residues as well as several N- and/or O-glycosylation sites, which can contribute to a local conformation stabilized by membrane anchorage. Since truncated variants exhibited unchanged affinity for the donor and acceptor substrates, but displayed increased velocity parameters, it was concluded that they lost peptide elements crucial for the active site that possibly maintain the catalytic domain in a closed state. Alternatively, removal of this modulatory region may induce local changes that facilitate positioning of critical amino acids within the catalytic site to proceed with the transfer reaction. Further work based on crystallization of the full-length transferase in the presence of its acceptor will thus be required to delineate both the location of the acceptor-binding site and the catalytic mechanism. Accordingly, variation in stem length and sequence within a glycosyltransferase subfamily may reflect variation in acceptor preference among transferases sharing similar properties like glycan specificity, recognition of the underlying glycosylation site, and/or the nature of the glycoconjugate acceptor.

Whether or not the stem region plays a physiological role in regulating glycosyltransferase action is still unknown. Since enzyme diversification occurs during evolution (2), it may very well be that modulatory sites could have been added to restrain catalytic properties of transferases to well defined classes of glycoconjugates and to create acceptor preference. Such a fine mechanism may allow site-specific sialylation like that found in the glycoprotein hormone family, for which alternate sulfate signaling and sialylation control hormonal activity and pulsatility (38). Also, the α2,8-sialyltransferase subfamily was found to exhibit exquisite glycosylation very specific for neural cell adhesion molecules during development (47) since polysialylation by α2,8 sialyltransferase II and III demonstrated to require core fucosylation of neural-cell adhesion molecule variants, showing the highest preference of all transferases known to date, i.e. for both the glycan and protein acceptors (18). Furthermore, mice produce four isoforms of α1,3-galactosyltransferase that differ in the length of this region, indicating that polymorphism of a transferase polypeptide may physiologically occur, possibly introducing new regulatory features (48). Such a length polymorphism has also been described to occur in ST3GalIII (49). Moreover, it has been found that two naturally occurring α2,8-sialyltransferases differing by a single amino acid in their catalytic domain can differ in their enzymatic properties (50). If these findings prove to be applicable to all classes of glycosyltransferases, such functional complexity may very well have important implications during biosynthesis of cell-surface glycoconjugates in coordinating the action of multiple enzymes in a tissue-specific manner and in displacing the physiological balance in favor of those enzymes overexpressed under pathological conditions.

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Exploring the Acceptor Substrate Recognition of the Human $\beta$-Galactoside $\alpha$ 2,6-Sialyltransferase

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