Switching Amino-terminal Cytoplasmic Domains of α(1,2)Fucosyltransferase and α(1,3)Galactosyltransferase Alters the Expression of H Substance and Galα(1,3)Gal*

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When α(1,2)fucosyltransferase cDNA is expressed in cells that normally express large amounts of the terminal carbohydrate Galα(1,3)Gal, and therefore the α(1,3)galactosyltransferase (GT), the Galα(1,3)Gal almost disappears, indicating that the presence of the α(1,2)fucosyltransferase (HT) gene/enzyme alters the synthesis of Galα(1,3)Gal. A possible mechanism to account for these findings is enzyme location within the Golgi apparatus. We examined the effect of Golgi localization by exchanging the cytoplasmic tails of HT and GT; if Golgi targeting signals are contained within the cytoplasmic tail sequences of these enzymes then a “tail switch” would permit GT first access to the substrate and thereby reverse the observed dominance of HT. Two chimeric glycosyltransferase proteins were constructed and compared with the normal glycosyltransferases after transfection into COS cells. The chimeric enzymes showed Km values and cell surface carbohydrate expression comparable with normal glycosyltransferases. Coexpression of the two chimeric glycosyltransferases resulted in cell surface expression of Galα(1,3)Gal, and virtually no HT product was expressed. Thus the cytoplasmic tail of HT determines the temporal order of action, and therefore dominance, of these two enzymes.

Glycosylation is a complex form of posttranslational modification of proteins where glycosyltransferases, located in the endoplasmic reticulum and Golgi apparatus, catalyze the sequential transfer of monosaccharides from nucleotide sugars to saccharide acceptors resulting in mature oligosaccharides (1). Thus the large number of both N- and O-linked carbohydrate acceptors resulting in mature oligosaccharides can have different glycosylation patterns generated by the sequential and coordinated action of more than 100 different glycosyltransferases (2). In this process, the product of one transferase serves as the substrate for the next transferase (3), with the differences in the glycosylation pattern being due to variation in the topology and function and the level of expression of the glycosyltransferase within the Golgi complex (3).

Evidence to date based on immunoelectron microscopy and cell fractionation studies suggests that the glycosyltransferases are sequentially distributed within the Golgi compartments in approximately the observed order of glycosylation (4). A number of studies using hybrid molecules have examined mechanisms for the sorting and retention of glycosyltransferases within the Golgi and have identified the transmembrane domains of α(2,6)sialyltransferase, β(1,4)galactosyltransferase, and N-acetylgalcosaminyltransferase I as playing a central role in their Golgi localization (5); however, cytoplasmic tail and stem region sequences in α(2,6)sialyltransferase and N-acetylgalcosaminyltransferase I also contain important structural elements within their cytoplasmic domains that augment the efficiency of Golgi localization of these proteins (5). Thus the sequences that localize glycosyltransferases within different Golgi compartments are poorly defined and cannot be predicted from protein sequences. We have recently reported that simultaneous expression of α(1,2)fucosyltransferase (HT) and α(1,3)galactosyltransferase (GT), both of which can use N-acetyllactosamine to synthesize fucosylated N-acetyllactosamine (H substance) or Galα(1,3)Gal, respectively, does not lead to equal synthesis of each product but rather HT “dominates” over GT, i.e. the activity of HT is given preference over that of GT so that the expression of Galα(1,3)Gal is almost entirely suppressed in the presence of H substance (6). We now address the mechanism of exclusion of Galα(1,3)Gal synthesis by the presence of HT and demonstrate that amino acid sequences in the cytoplasmic domains of these two glycosyltransferases play a central role in the temporal order of action of these enzymes.

EXPERIMENTAL PROCEDURES

Plasmids—The plasmids used were prepared using standard techniques (7); GT encodes the cDNA for the porcine α(1,3)galactosyltransferase (8), and HT encodes the cDNA for the α(1,2)fucosyltransferase (9). Chimeric glycosyltransferase cDNAs were generated by polymerase chain reaction as follows. An 1105-base pair product ht-GT was generated using the primer corresponding to the 5'-end of ht-GT (5'-GCGGATCCATGAATGTCAAAGGAAGACTTGCTTG-3') coding for nucleotides 1–24 of HT (9) followed immediately by nucleotides 68–89 of GT (8) and containing a BamHI site (underlined) and a primer corresponding to the 3'-end of ht-GT (5'-GCGGCCTGGCCTTCCTGC-3') containing complementarity to nucleotides 1102–1127 of GT with an XhoI site downstream of the translational stop site (underlined); an 1105-base pair product gt-HT was generated using primers corresponding to the 5'-end of gt-HT (5'-GCGGATCCATGAATGTCAAAGGAAGACTTGCTTG-3') coding for nucleotides 49–67 of GT followed immediately by nucleotides 86–89 of GT (8) and containing a BamHI site (underlined) and a primer corresponding to the 3'-end of gt-HT (5'-GCGGCCTGGCCTTCCTGC-3') containing complementarity to nucleotides 1075–1099 of HT with an XhoI site downstream of the translational stop site (underlined). Polymerase chain reaction products were restricted with BamHI-XhoI, gel-purified, ligated into a BamHI-XhoI-digested pCDNA1 expression vector

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1 The abbreviations used are: HT, α(1,2)fucosyltransferase; FITC, fluorescein isothiocyanate; GT, α(1,3)galactosyltransferase; H substance, α(1,2)fucosylactosamine.
(Invitrogen) and resulted in two plasmids, pht-GT (encoding the chimeric glycosyltransferase ht-GT) and pgt-HT (encoding the chimeric glycosyltransferase gt-HT), that were characterized by restriction mapping, Southern blotting, and DNA sequencing.

Transfection and Serology—COS cells were maintained in Dulbecco's modified Eagle's Medium (Trace Biosciences Pty. Ltd., Castle Hill, NSW, Australia) and were transfected (1–10 μg of DNA/5 × 10^5 cells) using DEAE-dextran (10). 48 h later, cells were examined for cell surface expression of H substance or Galα(1,3)Gal using FITC-conjugated lectins, IB4 lectin isolated from *Griffonia simplicifolia* (Sigma) detects Galα(1,3)Gal (11) and UEAI lectin isolated from *Ulex europaeus* (Sigma) detects H substance (12). Fluorescence was detected by microscopy.

RNA Analyses—Cytoplasmic RNA was prepared from transfected COS cells using RNAzol (Biotecx Laboratories, Houston, TX). Total RNA was electrophoresed in a 1% agarose gel containing formaldehyde, and the gel was blotted onto a nylon membrane and probed with random primed GT or HT cDNA.

Glycosyltransferase Assays—Forty-eight hours after transfection, cells were washed twice with phosphate-buffered saline and lysed in 1% Triton X-100, 100 mM cacodylate, pH 6.5, 25 mM MnCl₂, at 4°C for 30 min; lysates were centrifuged; and the supernatant was collected and stored at −70°C. Protein concentration was determined by the Bradford method using bovine serum albumin as standard (13). Assays for HT activity (14) and GT assays (15) were performed as described previously (6). All assays were performed in duplicate, and additional reactions were performed in the absence of added acceptor molecules to allow for the calculation of specific incorporation of radioactivity.

**RESULTS**

Expression of Chimeric α(1,3)Galactosyltransferase and α(1,2)Fucosyltransferase cDNAs—We had previously shown that when cDNAs encoding GT and HT were transfected separately, they could both function efficiently, leading to expression of the appropriate carbohydrates, Galα(1,3)Gal for GT and H substance for HT (6). However, when the cDNAs for GT and HT were transfected together, the HT appeared to “dominate” over the GT in that H substance expression was normal, but Galα(1,3)Gal was almost entirely absent. We excluded trivial reasons for this effect and considered that the localization of the enzymes within the Golgi could account for the findings. Thus, if the HT Golgi localization signal placed the enzyme in an earlier temporal compartment of the Golgi than GT, it would have “first use” of the N-acetyllactosamine substrate and, therefore, appear to dominate over GT. To determine if this was the case, two chimeric glycosyltransferases were constructed using polymerase chain reaction wherein the cytoplasmic tails of GT and HT were switched, with the presumption that the Golgi localization signals may be located in any domain of the glycosyltransferase, and the amino terminus of the enzyme was a logical starting point for these studies. The two chimeras constructed are shown in Fig. 1: ht-GT, which consisted of the NH₂-terminal cytoplasmic tail of HT attached to the transmembrane, stem, and catalytic domains of GT; and gt-HT, which consisted of the NH₂-terminal cytoplasmic tail of HT attached to the transmembrane, stem, and catalytic domains of HT. The chimeric cDNAs were subcloned into the eukaryotic expression vector and used in transfection experiments.

The chimeric cDNAs encoding ht-GT and gt-HT were initially evaluated for their ability to induce glycosyltransferase expression in COS cells, as measured by the surface expression of the appropriate sugar, using lectins. Forty-eight hours after transfection, COS cells were tested by immunofluorescence for surface expression of H物质 or Galα(1,3)Gal using FITC-conjugated lectins, IB4 lectin isolated from *Griffonia simplicifolia* (Sigma) detects Galα(1,3)Gal (11) and UEAI lectin isolated from *Ulex europaeus* (Sigma) detects H substance (12). Fluorescence was detected by microscopy.

**FIG. 1. Schematic diagram of normal and chimeric glycosyltransferases.** The diagram shows normal glycosyltransferases porcine α(1,3)galactosyltransferase (GT) and human α(1,2)fucosyltransferase (HT) and chimeric transferases ht-GT, in which the cytoplasmic domain of GT has been completely replaced by the cytoplasmic domain of HT, and gt-HT, in which the cytoplasmic domain of HT has been entirely replaced by the cytoplasmic domain of GT. The protein domains depicted are cytoplasmic domain (CYTO), transmembrane domain (TM), stem region (STEM), and catalytic domain (CATALYTIC). The numbers refer to the amino acid sequence of the corresponding normal transferase.

**FIG. 2. Cell surface staining of COS cells transfected with normal and chimeric transferases.** Cells were transfected with normal GT or HT or with chimeric transferases gt-HT or ht-GT, and 48 h later, they were stained with FITC-labeled lectin IB4 or UEAI. Positive-staining cells were visualized and counted by fluorescence microscopy. Results are from at least three replicates, and values are the means ± S.E.
their expression of Gal\((1,3)\)Gal or H substance (Fig. 2). The staining with IB4 (lectin specific for Gal\((1,3)\)Gal) in cells expressing the chimera ht-GT (30% of cells stained positive) was indistinguishable from that of the normal GT staining (30%) (Fig. 2). Similarly, the intense cell surface fluorescence seen with UEAI staining (the lectin specific for H substance) in cells expressing gt-HT (50%) was comparable with that seen in cells expressing wild-type HT (50%) (Fig. 2). Furthermore, similar levels of mRNA expression of the glycosyltransferases GT and HT and chimeric glycosyltransferases ht-GT and gt-HT were seen in Northern blots of total RNA isolated from transfected cells (Fig. 3). Thus, both chimeric glycosyltransferases are efficiently expressed in COS cells and are functional. Indeed, there was no detectable difference between the chimeric and normal glycosyltransferases.

**Glycosyltransferase Activity in Cells Transfected with Chimeric cDNAs Encoding ht-GT and gt-HT**—To determine whether switching the cytoplasmic tails of GT and HT altered the kinetics of enzyme function, we compared the enzymatic activity of the chimeric glycosyltransferases with those of the normal enzymes in COS cells after transfection of the relevant cDNAs. By making extracts from transfected COS cells and performing GT or HT enzyme assays, we found that N-acetyllactosamine was galactosylated by both GT and the chimeric enzyme ht-GT (Fig. 4A) over a 1–5 mM range of substrate concentrations. Lineweaver-Burk plots showed that both GT and ht-GT have a similar apparent Michealis-Menten constant of \(K_m\) of 2.6 mM for N-acetyllactosamine (Fig. 4B). Furthermore, HT and the chimeric enzyme gt-HT were both able to fucosylate phenyl-\(\beta\)-D-galactoside over a range of concentrations (7.5–25 mM) (Fig. 4C) with a similar \(K_m\) of 2.3 mM (Fig. 4D), in agreement with the reported \(K_m\) of 2.4 mM for HT (9).

**Fig. 3. RNA analysis of transfected COS cells.** Northern blots were performed on total RNA prepared from COS cells transfected: Mock, mock-transfected; GT, transfected with normal GT cDNA; ht-GT, transfected with chimeric transferase ht-GT cDNA; ht-GT + gt-HT, co-transfected with both chimeric transferases ht-GT and gt-HT cDNAs; gt-HT, transfected with chimeric transferase gt-HT cDNA; HT, transfected with normal HT cDNA; and GT + HT, co-transfected with both normal transferases GT and HT cDNAs. Blots were probed with a cDNA encoding GT (top panel), HT (middle panel) or \(\gamma\)-actin (bottom panel).

**Fig. 4. Enzyme kinetics of normal and chimeric glycosyltransferases.** Lineweaver-Burk plots for \((1,3)\)galactosyltransferase and \((1,2)\)fucosyltransferase to determine the apparent \(K_m\) values for N-acetyllactosamine. Experiments were performed in triplicate, and plots shown are of mean values of enzyme activity of wild-type transferases, GT and HT, and chimeric proteins ht-GT and gt-HT in transfected COS cell extracts using phenyl-\(\beta\)-D-galactoside and N-acetyllactosamine as acceptor substrates.
Therefore, the chimeric glycosyltransferases ht-GT and gt-HT are able to utilize N-acetyllactosamine (ht-GT) and phenyl-β-D-galactoside (gt-HT) in the same way as the normal glycosyltransferases. Thus switching the cytoplasmic domains of GT and HT does not alter the function of these glycosyltransferases, and if indeed the cytoplasmic tail is the site of the Golgi localization signal, then both enzymes function as well with the GT signal as with the HT signal.

Switching Cytoplasmic Domains of GT and HT Results in a Reversal of the “Dominance” of the Glycosyltransferases—The cDNAs encoding the chimeric transferases or normal transferases were simultaneously co-transfected into COS cells and, after 48 h, were stained with either IB4 or UEAI lectin to detect Galα(1,3)Gal and H substance, respectively, on the cell surface (Fig. 5). COS cells co-transfected with cDNAs for ht-GT + gt-HT (Fig. 5C) showed 30% cells staining positive with IB4 (Fig. 2) but negligible staining on cells co-transfected with cDNAs for GT + HT (3%) (Fig. 5A). Furthermore, staining for H substance on the surface of ht-GT + gt-HT co-transfectants gave very few cells staining positive (5%) (Fig. 5D) compared with the staining seen in cells co-transfected with cDNAs for the normal transferases GT + HT (50%) (Fig. 5B), i.e., the expression of Galα(1,3)Gal now dominates over that of H. Clearly, switching the cytoplasmic tails of GT and HT led to a complete reversal in the glycosylation pattern seen with the normal transferases, i.e., the cytoplasmic tail sequences dictate the pattern of carbohydrate expression observed.

That exchanging the cytoplasmic tails of GT and HT reverses the dominance of the carbohydrate epitopes points to the glycosyltransferases being relocalized within the Golgi. To address this question, experiments were performed with cDNAs encoding glycosyltransferases with the same cytoplasmic tail; COS cells transfected with cDNAs encoding HT + gt-HT stained strongly with both UEAI (50%) and IB4 (30%) (Fig. 2 and Fig. 5, E and F), the difference in staining reflecting differences in transfection efficiency of the the cDNAs. Similarly, cells transfected with cDNAs encoding GT + ht-GT also stained positive with UEAI (50%) and IB4 (30%) (Fig. 2 and Fig. 5, G and H). Thus, glycosyltransferases with the same cytoplasmic tail lead to equal cell surface expression of the carbohydrate epitopes with no “dominance” of one glycosyltransferase over the other observed, and presumably, the glycosyltransferases localized at the same site appear to compete equally for the substrate.

**DISCUSSION**

Previously we have shown in vitro, using transfected COS and pig kidney cells, and in vivo, using HT transgenic mice, that in cells expressing both HT and GT, the HT takes precedence over GT, favoring the expression of H substance rather than Galα(1,3)Gal (6). We now examine possible mechanisms to explain the exclusion of Galα(1,3)Gal synthesis by HT. Our strategy was based on the observations that HT is located in the medial Golgi (16) and that HT is located in the trans-Golgi (17). Thus, as proteins pass through the Golgi network, they are likely to encounter HT before GT. Golgi localization signals are likely to be in the cytoplasmic tail, transmembrane, or stem regions of the glycosyltransferases (5), and our strategy was, therefore, to begin at the amino-terminal domain of the enzymes, switch their cytoplasmic tails, and determine whether expression of the products on the cell surface was altered.

The chimeric enzymes gt-HT and ht-GT proved to be a very useful way of assessing the role of the cytoplasmic sequences in Golgi localization because of their inherent similarity to the normal GT and HT enzymes with respect to their levels of transcription and transferase activity. In COS cells, the levels of transcription of the cDNAs of chimeric and normal glycosyltransferases were essentially the same (Fig. 3), and the immunofluorescence pattern of COS cells expressing the chimeric glycosyltransferases ht-GT and gt-HT showed the typical staining pattern of the cell surface Galα(1,3)Gal and H substance, respectively (Fig. 2), the pattern being indistinguishable from that of COS cells expressing normal GT and HT. Our studies showed that the $K_m$ of ht-GT for N-acetyllactosamine was identical to the $K_m$ of GT for this substrate, and similarly, the $K_m$ of gt-HT and HT for phenyl-β-D-galactoside was approximately the same (Fig. 4). These findings indicate that the chimeric enzymes are functioning in a cytoplasmic tail-independent manner such that the catalytic domains are functional and are in agreement with those of Henion et al. (18) who showed that an NH$_2$-terminal truncated marmoset GT (this included truncation of the cytoplasmic and transmembrane domains) maintained catalytic activity and confirmed that GT activity is independent of the cytoplasmic domain sequence.

If the Golgi localization signal for GT and HT is contained entirely within the cytoplasmic domains of the enzymes, then switching the cytoplasmic tails between the two transferases should allow a reversal of the order of glycosylation. Co-transfection of COS cells with cDNAs encoding the chimeric glycosyltransferases ht-GT and gt-HT caused a reversal of staining observed with the wild-type glycosyltransferases (Fig. 5), demonstrating that the order of glycosylation has been altered by exchanging the cytoplasmic tails. Furthermore, co-transfection with cDNA encoding glycosyltransferases with the same cyto-
plasmic tails (i.e. HT + ht-GT and GT + gt-HT) gave rise to equal expression of both Galα(1,3)Gal and H substance (Fig. 5). The results imply that the cytoplasmic tails of GT and HT are sufficient for the localization and retention of these two enzymes within the Golgi.

To date, only twenty or so of at least one hundred predicted glycosyltransferases have been cloned, and few of these have been studied with respect to their Golgi localization and retention signals (19). Studies using the elongation transferase N-acetylgalactosaminyltransferase I (20–22) and the terminal sialyltransferase α(2,6)siaryltransferase (23–25) and β(1,4)galactosyltransferase (26–28) point to residues contained within the cytoplasmic tail, transmembrane, and flanking stem regions as being critical for Golgi localization and retention. There are several examples of localization signals existing within cytoplasmic tail domains of proteins including the KDEL and KKXX motifs in proteins resident within the endoplasmic reticulum (29, 30), the latter motif also having been identified in the cis-Golgi resident protein ERGIC-53 (31) and a dileucine-containing peptide motif in mannose-6-phosphate receptors, which directs the receptor from the trans-Golgi network to endosomes (32). These motifs are not present within the cytoplasmic tail sequences of HT or GT or in any other reported glycosyltransferase. To date a localization signal in Golgi resident glycosyltransferases has not been identified, and while there is consensus that transmembrane domains are important in Golgi localization, it is apparent that this domain is not essential in localization of all glycosyltransferases as shown by the study of Munro (33) where replacement of the transmembrane domain of α(2,6)sialyltransferase in a hybrid protein with a polyleucine tract resulted in normal Golgi retention. Dahdal and Colley (34) also showed that sequences in the transmembrane domain were not essential to Golgi retention. Our study concentrated on the role of the cytoplasmic domain sequences of GT and HT in the hierarchy of the action of glycosyltransferases that are simultaneously expressed within the same cell. While Golgi localization has not been formally proved, the data presented herein clearly shows that the cytoplasmic domains of GT and HT play a central role in the temporal order of action of these enzymes. Generation of specific antibody reagents to GT and HT will in the future allow electron microscopic localization of both the normal and chimeric glycosyltransferases to the Golgi.

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