Altered Golgi Localization of Core 2 
\(\beta\)-1,6-N-Acetylglucosaminyltransferase Leads to Decreased Synthesis of Branched O-Glycans*

(Received for publication, May 5, 1997, and in revised form, June 30, 1997)

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Mucin type O-glycans with core 2 branches are distinct from nonbranched O-glycans, and the amount of core 2 branched O-glycans changes dramatically during T cell differentiation. This oligosaccharide is synthesized only when core 2 \(\beta\)-1,6-N-acetylglucosaminyltransferase (C2GnT) is present, and the expression of this glycosyltransferase is highly regulated. To understand how O-glycan synthesis is regulated by the orderly appearance of glycosyltransferases that form core 2 branched O-glycans, the subcellular localization of C2GnT was determined by using antibodies generated that are specific to C2GnT. The studies using confocal light microscopy demonstrated that C2GnT was localized mainly in cis to medial-cisternae of the Golgi. We then converted C2GnT to a trans-Golgi enzyme by replacing its Golgi retention signal with that of \(\alpha\)-2,6-sialyltransferase, which resides in trans-Golgi. Chinese hamster ovary cells expressing wild type C2GnT and the chimeric C2GnT were then subjected to oligosaccharide analysis. The results obtained clearly indicate that the conversion of C2GnT into a trans-Golgi enzyme resulted in a substantial decrease of core 2 branched oligosaccharides.

These results, taken together, strongly suggest that the predominance of core 2 branched oligosaccharides in those cells expressing C2GnT is due to the fact that C2GnT is located earlier in the Golgi than \(\alpha\)-2,3-sialyltransferase that competes with C2GnT for the common acceptor that was formed by another glycosyltransferase. The conversion of C2GnT to a trans-Golgi enzyme renders the chimeric C2GnT much less efficient in synthesizing core 2 branched oligosaccharides, indicating the critical role of orderly subcellular localization of glycosyltransferases.

Leukosialin (CD43) is a major sialoglycoprotein present in leukocytes and heavily glycosylated by mucin-type O-glycans (1–5). This glycoprotein of human origin contains approximately 80 O-linked oligosaccharides in its extracellular domain consisting of 234 amino acids (1, 6). These O-linked oligosaccharides are highly sialylated and have been shown to exhibit an anti-adhesive property (7). It has been also shown that the structure of oligosaccharides attached to leukosialin changes significantly during development of T cells. While resting human T lymphocytes express tetrasaccharides, NeuNacα2→3Galβ1→3(NeuNacα2→6)GalNAc, activated T lymphocytes almost exclusively express branched hexasaccharides, NeuNacα2→3Galβ1→3(NeuNacα2→3Galβ1→4GlcNAc β1→6)GalNAc (8). Moreover, such change is associated with T cell development in thymus; while immature thymocytes in cortical thymus express the hexasaccharides, relatively mature medullary thymocytes express the tetrasaccharides (9).

The conversion of O-glycan biosynthesis is due to the turning on or off of core 2 \(\beta\)-1,6-N-acetylglucosaminyltransferase (C2GnT). 1 It has been demonstrated that activated T lymphocytes express a substantial amount of C2GnT activity, while resting T lymphocytes express negligible C2GnT activity (8). By in situ hybridization of the transcript, it has been shown that immature cortical thymocytes express a substantial amount of C2GnT mRNA, while it was not detected in medullary thymocytes (9). The conversion of O-glycan structures during thymocyte development may be critical for the apoptotic process in thymus, since such a process is modulated by the presence of O-glycans on thymocytes (10).

Expression of the branched hexasaccharide in peripheral blood T lymphocytes has been also observed in patients with immunodeficient syndromes such as Wiskott-Aldrich syndrome (11, 12). It has also been shown that AIDS patients express substantially increased amounts of the hexasaccharide or its monosialylated forms (13, 14). AIDS patients produce antibodies against leukosialin expressing those oligosaccharides, and such antibodies are implicated in causing T lymphocyte depletion, which may be a cause of pathological conditions in these diseases (15). These combined results indicate that it is critical to understand how core 2 branchings are synthesized.

The biosynthesis of oligosaccharides is also controlled by specific localization of glycosyltransferases that add a specific monosaccharide in each reaction (16). If a glycosyltransferase is misplaced, sequential reactions would not take place, since a given glycosyltransferase adds a monosaccharide to a particular acceptor that was formed by another glycosyltransferase that resides in an earlier compartment(s). Although subcellular localization of glycosyltransferases that form N-glycans is relatively well studied (17), very little is known about subcellular localization of glycosyltransferases that form O-glycans.
distribution of glycosyltransferases that form O-glycans (see Ref. 18).

In the present study, we have first determined that C2GnT is localized in cis to medial-Golgi using antibodies specific for C2GnT. We then converted C2GnT into a trans-Golgi enzyme by replacing its domain responsible for Golgi retention with that of Galβ1-4GcNAc α-2,6-sialyltransferase, ST6Gal I (19). Such altered localization of C2GnT was found to result in altered synthesis of oligosaccharides, demonstrating the importance of the orderly presence of glycosyltransferases.

**EXPERIMENTAL PROCEDURES**

**Construction of pGEK-C2GnT**—To prepare antibodies specific to C2GnT, a cDNA encoding the catalytic domain of C2GnT was amplified by PCR using C2GnT cDNA (20) as a template and fused with GST protein. The 5′-primer for PCR is 5′-aatccggatcatCATCATCATCATCATCCgggTCTTCTTTATCATC (BamHI site and 6′-his linker are singly and doubly underlined, respectively, while the italic type corresponds to nucleotides 101–104 of C2GnT). The 3′-primer is 5′-aatccggatctcgggTCAGTGTTTTAATGT-3′ (the last 15 nucleotides correspond to residue 425 to the stop codon). PCR was carried out as described (21), and the amplified DNA was digested with BamHI and EcoRI and cloned into the same sites of pGEX-XG expression vector (Pharmacia). The resultant cDNA encodes a fusion protein composed of GST and a thrombin cleavage site, six histidines, and the catalytic domain (residues 101–428) of C2GnT. Escherichia coli HB101 was transformed with this plasmid vector, and a GST fusion protein was produced after isopropyl-1-thio-β-D-galactopyranoside induction.

**Purification of C2GnT Protein**—HB101 cells were recovered by centrifugation and frozen at −80 °C. After thawing on ice, the pellet was digested with 5 mg/ml lysozyme in 25 mM Tris-HCl, pH 8.0, containing 10 mM EDTA and 1% Triton X-100 (buffer A). After the addition of DNase I (Amersham Corp.), the sample was then sonicated and centrifuged. The resulting pellet was resuspended in 25 mM Tris-HCl, pH 8.0, containing 10 mM EDTA and 1.5% N-lauroylsarcosine (Sigma) (buffer B). The suspended residue was then centrifuged, and the sarcosyl extract was obtained as described (22). Glutathione-Sepharose beads were equilibrated with buffer B and added to the sarcosyl extract. The suspension was mixed gently at 4 °C for 90 min using a rotary mixer and then briefly centrifuged to recover the beads. After washing the beads with buffer C (50 mM Tris-HCl, pH 8.0, containing 150 mM NaCl and 2.5 mM CaCl2), the beads were suspended in 2 ml of buffer C comprising 20 units of thrombin and mixed overnight at room temperature.

Thrombin-released material was recovered in the supernatant after centrifugation of the above mixture. The proteins that remained on beads were then released by SDS-polyacrylamide gel electrophoresis sample buffer, which contained no reducing reagent, and the C2GnT protein fragment was recovered in this extraction and separated from other proteins by SDS-polyacrylamide gel electrophoresis. The purified protein sample, extracted from polyacrylamide gels by electrophoresis, was immunized in rabbits. The antiserum was applied to a protein A-Sepharose column, bound antibodies were eluted with glycine-HCl, pH 2.5, and the eluent was immediately neutralized by the addition of 1.0 M Tris-HCl buffer, pH 8.0. The partially purified antibodies were further applied to a column of Sepharose 4B conjugated to E. coli proteins, and the unbound fraction was used as a purified antibody sample.

**Construction of Vectors Expressing a Chimeric Protein Consisting of C2GnT and ST6Gal I**—A cDNA encoding ST6Gal I was cloned by PCR using a human HL-60 cDNA library (20) as the template. The 5′-primer for this PCR corresponds to nucleotides 15 to 15 with respect to the translation initiation site (23) plus SWaI site. The 3′-primer is 5′-aaccccgctagTCAGTGTTTTAATGT-3′. The SWaI site is underlined, while the italic type corresponds to nucleotides 1201–1221 (nucleotides 1216–1218 encode the stop codon). The PCR product was digested with SWaI and XhoI and cloned into the same sites in the pMSG vector (Pharmacia). A cDNA encoding the cytoplasmic, transmembrane, and stem regions of ST6Gal I was amplified by PCR using the above plasmid vector as a template. The 5′-primer, DS23, corresponds to nucleotides 9 to 11 in relation to the translation initiation site of ST6Gal I, with the BamHI site at the 5′-end. The 3′-primer sequence was 5′-ATCACAATAGGCGCTGGTCAGCTTCT 3′. The last 12 nucleotides of this primer (shown by italics) correspond in antisense to residues 53–56 of C2GnT, and the last 16 nucleotides correspond in antisense to nucleotides 195–210 of ST6Gal I (nucleotides 196–210 encode codons 66–70). This PCR product encodes the first 70 amino acid residues of ST6Gal I plus 4 amino acids in the stem region of C2GnT.

A cDNA encoding the catalytic domain of C2GnT was amplified by PCR. The 5′-primer sequence was 5′-ATGCAACCCAGCCGACTGATGTATAATTGTT-3′. In this sequence, the first 12 nucleotides encode residues 67–70 of ST6Gal I, and the following 20 nucleotides encode residues 53–58 plus the portion of residue 59 of C2GnT. The 3′-primer, DS26, encodes the stop codon plus the following fifteen 3′-untranslated nucleotides of C2GnT sequence with the addition of the xhoI site.

The PCR products of the C2GnT catalytic domain and ST6Gal I sequence were ligated in overlapping at sequence corresponding to Ser-Thr-Gln-Asp-Pro-Ser-Ser-Aasp, which is Ser-Thr-Gln-Asp-Pro-Ser-Ser-Aasp from C2GnT. To make a chimera of the NH2-terminal region of ST6Gal I and the catalytic domain of C2GnT, PCR was carried out using DS23 and DS26 (shown above) as primers and a mixture of the above two PCR products as templates (21). After amplification under the same conditions as described, the PCR product was digested with BamHI and XhoI and then ligated into the same sites of pcDNAI, yielding pcDNAI-ST6Gal I/C2GnT.

**Establishment of CHO Cells Stably Expressing C2GnT and C2GnT Chimeric Protein**—CHO DG44 cells were transfected with pZIPneo-lease alone, with pZIPneo-lease and pcDNAI-C2GnT, or with pZIPneo-lease and pcDNAI-ST6Gal I/C2GnT using LipofectAMINE and were sub- nsequently selected for 148 resistance. Clonal cell lines expressing a substantial amount of either leukosialin (CHO-leu) or both leukosialin and core 2 branched oligosaccharides (CHO-leu-C2GnT, CHO-leu-ST6Gal I/C2GnT) were selected as described (24).

**Double Immunofluorescent Staining and Confoocal Microscopy**—CHO-leu-C2GnT and CHO-leu-ST6Gal I/C2GnT cells were grown on coverslips and fixed in 4% paraformaldehyde in PBS and immersed in 0.05% saponin, 0.1% bovine serum albumin solution in PBS for 10 min at room temperature. They were then incubated with rabbit anti-C2GnT antibodies followed by rhodamine-conjugated goat anti-rabbit IgG as described previously (25). After washing with PBS containing 0.1% bovine serum albumin, they were sequentially washed with PBS containing 1% normal goat serum, 10 μg/ml unconjugated secondary antibody (goat anti-rabbit IgG), and then 100 μg of unconjugated protein A/ml of PBS for 10 min each. The cells were then incubated with rabbit anti-α-mannosidase II antibodies (26) followed by fluorescein isothiocyanate-conjugated goat F(ab′)2 fragment of IgG that is specific to the Fc portion of rabbit IgG (Axell). After washing with PBS containing 0.1% bovine serum albumin followed by PBS, the samples were visualized with a Zeiss Axioplan microscope (25) or Zeiss CSM410 confocal scanning microscope (27) as described. To detect C2GnT and β-1,4-galactosyltransferase in the same sample, pcDNAI-GalT (25) was transiently transfected in the above CHO cells. Simple double immunofluorescent staining was then carried out as described (25), since a mouse monoclonal antibody specific to human β-1,4-galactosyltransferase (29) was available. Controls were performed by omitting the primary antibodies.

**Mouse monoclonal antibodies specific to mouse (and hamster) α-mannosidase II (26) were kindly provided by Dr. Kelley Moremen (University of Georgia). Mouse monoclonal antibody specific to human β-1,4-galactosyltransferase (29) was kindly provided by Dr. Hisashi Narimatsu (Soka University).**

**Indirect Immunoperoxidase Staining for Electron Microscopy**—Staining of specimens was performed as described previously (30, 31). Briefly, frozen sections of human kidney specimens, CHO-leu-C2GnT and CHO-leu-ST6Gal I/C2GnT cells, were fixed with paraformaldehyde-llysine-periodate, and incubation with primary antibody (rabbit anti-human C2GnT IgG, absorbed against E. coli proteins) was performed overnight at 4 °C, followed by three washes in PBS containing 1% egg albumin and 0.075% saponin and incubation with secondary antibodies (goat horseradish peroxidase-conjugated anti-rabbit IgG, Amersham) for 1 h at room temperature. After three washes, bound antibodies were visualized with 0.05% diaminobenzidine (Sigma) and 3% H2O2 in 20 mM Tris-HCl, pH 7.4. After fixation in 2.5% glutaraldehyde in 20 mM phosphate buffer, pH 7.4, and embedding in epon, ultra thin sections were cut and examined in a Jeol 1200 microscope. Controls were performed by either omitting the primary antibody or by replacing it with rabbit preimmune serum.

**Analysis of O-Glycans from CHO-leu, CHO-leu-C2GnT, and CHO-leu-ST6Gal I/C2GnT**—C2GnT was assayed by using the acceptor Galβ1-3GalNAcα3Galβ1-3Galβ1-4GlcNAc (Toronto Chemicals) as described (20).

The CHO cells (~1 × 107 cells) were metabolically labeled with 1Hglucosamine (10 μCi/ml), and the cell residues were subjected to Pronase digestion as described (24). The glycopeptides obtained were...
Core 2 Branching Enzyme with Altered Golgi Localization

RESULTS

Preparation of Antibodies Specific to C2GnT—To determine the subcellular distribution of C2GnT, it was essential to produce antibodies specific to C2GnT. First, the catalytic domain of C2GnT was fused with GST protein and expressed in E. coli. The produced protein was then immunized in rabbits. After two additional boost immunizations, the titer of the antibodies was increased enough to detect C2GnT in CHO cells expressing C2GnT (Fig. 1A). The same antibodies also reacted with COS-1 cells, which transfected with C2GnT cDNA (Fig. 2B) but not with untransfected COS-1 cells (data not shown). Moreover, the antibodies did not react with CHO cells expressing I-branching β-1,6-N-acetylglucosaminyltransferase, which shares homology with C2GnT (33) (Fig. 1C). To confirm that the antibodies reacted with C2GnT, Western blot analysis was performed on the protein products used for immunization. Fig. 2A shows that the antibodies reacted with a fusion protein of ~68 kDa before thrombin digestion (lanes 1 and 2) and reacted with ~36-kDa protein after the digestion (lanes 3 and 4). The results are consistent with the calculated molecular mass for the GST-C2GnT fusion protein (~68 kDa) and C2GnT catalytic domain (36 kDa).

Our preliminary studies on rat tissues showed that kidney had the highest activity of C2GnT. Western blot analysis of human kidney membrane proteins demonstrated that a ~57-kDa protein strongly reacted with the antibodies, while the control experiment gave negative results (Fig. 2B, lanes 5 and 6). Finally, immunoprecipitation of [35S]methionine-labeled CHO cells stably expressing C2GnT produced a specific band at ~60 kDa, which was absent in wild type CHO cells (Fig. 2C, lanes 7 and 8). These results combined clearly indicate that the antibodies generated are specific to C2GnT.

Localization of C2GnT in Golgi Complex—To determine the subcellular distribution of C2GnT, CHO cells were transfected

![Image](image.png)
with pcDNAI-C2GnT and pZipNeo-leu, and those cells stably expressing C2GnT and leukosialin (CHO-leu-C2GnT) were established (24). As shown previously, C2GnT is absent in CHO cells (24, 34); thus, only introduced C2GnT can be detected in CHO cells. When C2GnT is localized differently by the replacement of the Golgi retention signal, such change should be clearly detected. Leukosialin was co-transfected, since those cells expressing core 2 oligosaccharides on leukosialin can be detected by T305 antibody (24).

CHO-leu-C2GnT cells (clone 1) were stained by rabbit antibodies specific to C2GnT followed by rhodamine-conjugated goat anti-rabbit IgG. After chasing the remaining antibodies by protein A, as detailed under “Experimental Procedures,” the same specimens were incubated with rabbit antibodies specific to α-ManII, a glycosidase normally found in cis to medial-Golgi (26), followed by fluorescein isothiocyanate-conjugated goat anti-rabbit IgG. Preliminary experiments showed that the antibodies raised against mouse α-ManII cross-reacted with CHO α-ManII. As shown in Fig. 3 (top left), the majority of C2GnT and α-ManII are overlapping in their distributions, showing strong yellow staining.

In the second set of experiments, pcDNAI-human GalT cDNA (28) was transiently introduced into CHO-leu-C2GnT cells, and the expressed GalT, trans-Golgi enzyme, was similarly visualized by immunofluorescent staining. Since a mouse monoclonal antibody specific to human β-1,4-galactosyltransferase was available (29), the transfected cells were stained with rabbit anti-C2GnT antibodies and rhodamine-conjugated goat anti-rabbit IgG, followed by mouse monoclonal anti-GalT antibodies and goat FITC-conjugated anti-mouse IgG.

The results, as shown in Fig. 3 (bottom left) demonstrated that there was almost no overlap in the distribution of C2GnT and GalT. These results, combined, established that C2GnT is present in the cis to medial-Golgi. The results also demonstrated that two-step immunostaining was specific, since staining for only C2GnT or α-ManII can be seen in certain cells (Fig. 3). These results were confirmed by immunoelectron microscopy using specimens of human kidney and stably transfected CHO cells (data not shown).

Replacement of the Transmembrane Domain of C2GnT with That of ST6Gal I—The next question we asked was whether or not we could shift the Golgi localization of C2GnT by replacing the Golgi retention signal in C2GnT with that of a glycosyltransferase present in the trans-Golgi. For this, we utilized the sequence of ST6Gal I, which was shown to be in the trans-Golgi (35). We thus replaced the transmembrane portion and its flanking sequence of C2GnT with the corresponding sequence of ST6Gal I, resulting in ST6Gal I/C2GnT, as schematically shown in Fig. 4.

After transfecting CHO cells with ST6Gal I/C2GnT cDNA, different clones expressing various activities of C2GnT were chosen. We reasoned that overexpression of a chimeric protein may obscure the change in the subcellular localization brought about by replacing the Golgi retention signal. Fig. 5A shows that CHO cells containing the chimeric protein expressed amounts of C2GnT comparable with those expressed by CHO-leu-C2GnT cells as established previously (see clone M.B., es-
established in Ref. 24, versus clones 2, 6, and 8 of CHO-leu-ST6Gal I/C2GnT).

It was possible, however, that CHO cells expressing the chimeric protein expressed a substantially higher amount of C2GnT that had a lower specific activity. To exclude that possibility, the cell lysates derived from CHO-leu-C2GnT and CHO-leu-ST6Gal I/C2GnT were subjected to immunoprecipitation, and the residual C2GnT activity that remained in the supernatant was measured. As shown in Fig. 5B, the enzymatic activity in CHO cells expressing the wild type or chimeric C2GnT was precipitated in an almost identical manner, indicating that no detectable difference in the specific activity was present in these different CHO cells.

The expression of the chimeric protein was then examined in CHO-leu-ST6Gal I/C2GnT by immunofluorescent staining. As seen in Fig. 3 (top right), the chimeric protein did not overlap with α-Man II. In contrast, the distribution of the chimeric protein and β-galactosyltransferase overlapped appreciably, as shown in Fig. 3 (bottom right). These results indicate that the replacement of the domain responsible for Golgi retention allowed the shift in Golgi localization of C2GnT to the trans-side.

Although we attempted to localize C2GnT and the chimeric protein in CHO cells by immunoelectron microscopy, only C2GnT could be strongly detected in single Golgi cisternae, whereas for the chimeric protein a diffuse, weak signal was obtained (data not shown). Similarly, ST6Gal I introduced into CHO cells was not detected by immunoelectron microscopy in the previous studies, although its product was detected by a lectin, Sambucus nigra agglutinin (36). These results were obtained most likely due to the insufficient amounts of the expressed glycosyltransferases.

Effect of Altered Golgi Localization on Oligosaccharide Synthesis—We then tested if the altered localization of C2GnT affected the biosynthesis of O-glycans, resulting in altered O-glycan products. Mucin-type O-glycans produced from wild-type CHO cells were eluted in two peaks (a and b) after Bio-Gel P-4 gel filtration (Fig. 6A). After desialylation, peak a produced almost exclusively Galβ1→3GalNAcOH (peak 3), and Galβ1→3Galβ1→4GlcNAcβ1→6GalNAcOH (peak 2) was barely produced (Fig. 6B). Moreover, peak b produced only Galβ1→3GalNAcOH (Fig. 6C). The structures of these oligosaccharides were confirmed by exoglycosidase digestion followed by gel filtration as done previously (24). The analysis of peak a, which was derived from CHO-C2GnT cells (clone 8 in Fig. 5), exclusively produced Galβ1→3Galβ1→4GlcNAcβ1→6GalNAcOH, while peak b produced Galβ1→3GalNAcOH as well (Fig. 6D and F) (Table I).

These results are consistent with the previous findings that wild-type CHO cells almost exclusively synthesize disialo or monosialo derivatives of Galβ1→3GalNAc (Fig. 7, left). A large proportion of O-glycans were shifted to those containing core 2 branchings in CHO-C2GnT cells (clone 8 in Fig. 5, right). In contrast, CHO cells expressing the chimeric C2GnT expressed only a small amount of core 2 branched oligosaccharides (see peak 2 in Fig. 6H, which was derived from peak a, Fig. 6G). The majority of the oligosaccharides was either disialo (peak a in Fig. 6G) or monosialo (peak 6 in Fig. 6G) derivative of Galβ1→3GalNAcOH (peak 3 in Fig. 6, H and I) (Table I).

These results clearly indicate that the shifting of C2GnT from the cis-Golgi to the trans-Golgi converted the oligosaccharide biosynthesis as if the oligosaccharides were synthesized in the cells expressing a minimum amount of C2GnT.

DISCUSSION

In the present study, we have prepared polyclonal antibodies specific to C2GnT and studied the subcellular localization of C2GnT and its altered form by using the obtained antibodies.
The antibodies reacted with GST-C2GnT fusion protein produced in E. coli and reacted with a 57-kDa protein in kidney cells and CHO-C2GnT cells (Fig. 2). This molecular mass is consistent with the calculated molecular mass (49,790 kDa), assuming that three N-glycan sites are utilized. The antibodies also stained the Golgi complex in CHO cells where C2GnT was expressed, but not with those expressing IGnT, demonstrating that the prepared antibodies are specific to C2GnT (Fig. 1).

By using the specific antibodies prepared, the subcellular localization of C2GnT was examined by light microscopy and immunoelectron microscopy. Both methods revealed that C2GnT was present in a relatively confined area, which appears to be cis to medial-cisternae of the Golgi complex (Fig. 3).

Among glycosyltransferases involved in O-glycan synthesis, this is only the second enzyme of which subcellular localization was determined. Previously, α-N-acetylgalactosaminytransferase, which adds N-acetylgalactosamine to a polypeptide precursor, was shown to be localized in cis-Golgi (18). The localization of C2GnT in the cis to medial-Golgi is consistent with the previous findings on the biosynthesis of core 2 branched oligosaccharides. It has been shown that T cell activation is associated with the shifting of O-glycans from sialylated Galβ1→3GalNAc to sialylated Galβ1→3Galβ1→4GlcNAcβ1→6GalNAcOH, and Galβ1→3GalNAcOH, respectively. Fraction 20 in D corresponds to the position of Fraction 40 in F.

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charide unless two enzymes differ substantially in the affinity to the acceptor.

In the present study, we then tested the possibility of altering the localization in the Golgi complex by altering the amino acid sequence responsible for the Golgi retention. It has been demonstrated in several laboratories that the transmembrane and its flanking sequences are critical as Golgi retention signals (28, 37–41). We thus replaced those sequences in C2GnT with ST6Gal I, since ST6Gal I was previously shown to be localized in the trans-Golgi (35). Examination of the chimeric protein, ST6Gal I/C2GnT, by confocal microscopy demonstrated that the chimeric protein resided in a relatively broad range of the Golgi complex, and its highest concentration could be observed in trans-Golgi (Fig. 3).

These results indicate that the transmembrane domain and its flanking sequence actually dictate the localization within the Golgi complex. Slightly broader distribution of the chimeric protein indicates, however, that other parts of the molecule such as a catalytic domain might also contribute to a proper localization within the Golgi complex. This may be related to the recent report that the kin recognition between medial-Golgi enzymes is dependent also on the catalytic domains (42).

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**TABLE I**

| Oligosaccharides                                      | CHO    | CHO-C2GnT | CHO-chimera |
|-------------------------------------------------------|--------|-----------|-------------|
| NeuNAc2→3Galβ1→3GalNAc (peak a)                      | 85.4^b| 33.0      | 76.2        |
| NeuNAc2→3Galβ1→3GalNAc (peak b)                      | 11.6  | 0.2       | 4.1         |
| NeuNAc2→3Galβ1→4GlcNAcβ1                              | 1.8   | 5.6       | 1.0         |
| NeuNAc2→3Galβ1→3GalNAc (peak b)                      |        |           |             |
| NeuNAc2→3Galβ1→3GalNAc (peak a)                      | 1.2   | 61.2      | 18.7        |

^a These oligosaccharides were derived from peak a or b in Fig. 6, A, D, and G, and their sources are indicated in parentheses.

^b The ratio of the oligosaccharides were determined after exoglycosidase treatment to convert all of these oligosaccharides to Galβ1→3GalNAcOH.

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**FIG. 7.** Proposed scheme for the subcellular sites of various steps in O-glycan biosynthesis. Resting T lymphocytes synthesize the tetrasaccharide (bottom left), while activated T lymphocytes synthesize the hexasaccharide due to the presence of core 2 β-1,6-N-acetylgalactosaminyltransferase (boxed) (8). α-2,6-Sialyltransferase is also boxed. The subcellular distribution of α-N-acetylgalactosaminyltransferase (18) and core 2 β-1,6-N-acetylgalactosaminyltransferases (the present study) has been established. The intra-Golgi compartmentation of the other glycosyltransferases needs to be established. It appears that chimeric ST6Gal I/C2GnT protein resides mostly in the trans-Golgi, similar to ST6Gal I.
chimeric protein consists of two segments derived from two glycosyltransferases that reside in either the cis- or the trans-Golgi. In the chimeric protein molecules, the combined signal derived from two enzymes is probably less optimal than that derived from two enzymes residing in the same location within the Golgi complex.

The structural analysis of O-glycans synthesized in CHO cells indicates that O-glycans containing α-2,6-linked sialic acid also contain α-2,3-sialic acid, and no structures such as NeuNacα2→6GalNAc and NeuNacα2→3Galβ1→3GalNAc have been reported (24, 34). These results strongly suggest that α-2,6-sialyltransferase in CHO cells requires NeuNacα2→3Galβ1→3GalNAc as an acceptor (43, 44), suggesting that α-2,6-sialyltransferase probably resides in the trans-cisternae of the Golgi complex. Consistent with the altered localization of the chimeric protein in the Golgi, CHO cells expressing the chimeric protein produced much less core 2 branched oligosaccharide than CHO cells expressing wild-type C2GnT (Table I). Before alteration, C2GnT was present in the earlier Golgi compartments than α-2,3-sialyltransferase, which forms the acceptor for α-2,6-sialyltransferase. This α-2,6-sialyltransferase competes for the same acceptor, C-6 of GalNAc (Fig. 7). After the alteration of the Golgi retention signal of C2GnT, the alteration of the Golgi retention signal of C2GnT, the core 2 branching enzyme with altered Golgi localization

Acknowledgments—We thank Drs. Michiko Fukuda and Edgar Ong (The Burnham Institute) for useful discussions and critical reading of the manuscript, Drs. Kelley Moremen (University of Georgia, Athens, GA) and Hisashi Narimatsu (Soka University, Tokyo) for the gifts of antibodies, and Susan Greaney for organizing the manuscript.

REFERENCES

1. Fukuda, M. (1991) Glycobiology 1, 347–356
2. Andersson, L. C., and Gahmberg, C. D. (1978) Blood 52, 57–67
3. Brown, W. R., Barclay, A. N., Sunderland, C. A., and Williams, A. F. (1981) Nature 290, 456–460
4. Carlsson, S. R., and Fukuda, M. (1986) J. Biol. Chem. 261, 12779–12786
5. Remold-O’Donnell, E., Kenney, D. M., Parkman, R., Cairns, L., Sauve, B., and Rosen, F. S. (1984) J. Exp. Med. 159, 1705–1723
6. Remold-O’Donnell, E., and Rosen, F. S. (1990) Immunodefic. Rev. 2, 151–174
7. Ardman, S., Sikorski, M. A., and Staunton, D. E. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 5001–5005
8. Pillar, F., Pillar, V., Fox, R. L., and Fukuda, M. (1988) J Biol. Chem. 263, 15116–15119
9. Baum, L. G., Pang, M., Perillo, N. L., Wu, T., Delegeane, A., Uittenhogaart, C. H., Fukuda, M., and Seilhamer, J. J. (1995) J Exp. Med. 181, 877–887
10. Perillo, N. L., Pace, K. E., Seilhamer, J. J., and Baum, L. G. (1995) Nature 378, 736–739
11. Pillar, F., De Dietz, F., Weinberg, K., Parkman, R., and Fukuda, M. (1991) J. Exp. Med. 173, 1501–1510
12. Higgins, E. A., Siminovich, K. A., Zhanq, D., Brockhausen, I., and Dennis, J. W. (1991) J. Biol. Chem. 266, 6290–6290
13. Saito, O., Pillar, F., Fox, R. L., and Fukuda, M. (1991) Blood 79, 1499–1499
14. Lefevre, J.-C., Giordanengo, V., Limouze, M., Doglio, A., Cucchiari, M., Monpoux, F., Mariani, R., and Peyron, J.-F. (1994) J. Exp. Med. 180, 1609–1617
15. Ardman, S., Sikorski, M. A., Settles, M., and Staunton, D. E. (1990) J. Exp. Med. 172, 1151–1158
16. Badler, J. (1984) in Biology of Carbohydrates (Ginzburg, V., and Robbins, P. W., eds) pp. 199–228, John Wiley & Sons, Inc., New York
17. Roth, J. (1987) Biochim. Biophys. Acta 906, 405–436
18. Roth, J., Wang, Y., Eckhardt, A. E., and Hill, R. L. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 8935–8939
19. Weinstein, J., Lee, E. U., McEntee, K., Lai, P.-I., and Paulson, J. C. (1987) J Biol. Chem. 262, 17735–17743
20. Bierhuizen, M. F., and Fukuda, M. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 9264–9267
21. Ogata, S., and Fukuda, M. (1994) J Biol. Chem. 269, 5210–5217
22. Frankel, S., Sohn, R., and Leinwand, L. (1991) Proc. Natl. Acad. Sci. U. S. A. 88, 1192–1196
23. Wang, X., Vertino, A., Eddy, R. L., Byers, M. G., Jani-Sait, S. N., Shows, T. B., and Lau, J. T. Y. (1993) J Biol. Chem. 268, 4355–4361
24. Bierhuizen, M. F., Maemura, K., and Fukuda, M. (1994) J. Biol. Chem. 269, 4473–4479
25. Williams, M. A., and Fukuda, M. (1990) J Cell Biol. 111, 955–966
26. Moremen, K. W., and Robbins, P. W. (1991) J. Cell Biol. 115, 1521–1534
27. Fukuda, M. N., Sato, T., Nakayama, J., Klier, G., Mikami, M., Aoki, D., and Nozawa, S. (1995) Genes & Dev. 9, 1199–1210
28. Aoki, D., Lee, N., Yamaguchi, N., Dubois, C., and Fukuda, M. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 4319–4323
29. Uemura, M., Sakaguchi, T., Uejima, T., Nozawa, S., and Narimatsu, H. (1992) Cancer Res. 52, 6153–6157
30. Kerjaschki, D., Schulze, M., Binder, S., Kain, R., Ojha, P. P., Susani, M., Horvat, R., Baker, P. J., and Cousser, W. G. (1989) J Immunol. 143, 546–552
31. Kain, R., Matsui, K., Exner, M., Binder, S., Schaffner, G., Sommer, E. M., and Kerjaschki, D. (1995) J Exp. Med. 181, 585–597
32. Pillar, V., Pillar, F., and Fukuda, M. (1990) J. Biol. Chem. 265, 9264–9271
33. Bierhuizen, M. F., Mattei, M. G., and Fukuda, M. (1993) Genes & Dev. 7, 468–478
34. Sasaki, H., Bothner, B., Dell, A., and Fukuda, M. (1987) J Biol. Chem. 262, 12095–12076
35. Roth, J., Taatsjes, D. J., Luocoq, J. M., Weinstein, J., and Paulson, J. C. (1985) Cell 43, 297–295
36. Lee, U. E., Roth, J., and Paulson, J. C. (1989) J Biol. Chem. 264, 13848–13855
37. Munro, S. (1991) EMBO J. 10, 3577–3588
38. Nilsson, T., Luocoq, J. M., Mackay, D., and Warren, G. (1991) EMBO J. 10, 3567–3575
39. Colley, K. J., Lee, E. U., and Paulson, J. C. (1992) J Biol. Chem. 267, 7784–7783
40. Burke, J., Pettitt, J. M., Schachter, H., Sarkar, M., and Gleeson, P. A. (1992) J Biol. Chem. 267, 24433–24440
41. Wang, S. H., Lin, S. H., and Hong, W. (1992) J Cell Biol. 117, 245–258
42. Nilsson, T., Hoe, M. H., Slusarewicz, P., Rabouille, C., Watson, R., Hunte, F., Watzele, G., Berger, E. G., and Warren, G. (1994) EMBO J 13, 562–574
43. Sjoberg, E. R., Kitagawa, H., Gushka, J., van Halbeek, H., and Paulson, J. C. (1996) J Biol. Chem. 271, 7450–7459
44. Tsuji, S. (1996) J. Biochem. (Tokyo) 120, 1–13
45. Hakomori, S. (1985) Cancer Res. 45, 2405–2414
46. Fukuda, M. (1996) Cancer Res. 56, 2237–2244