Hamster UDP-N-Acetylg glucosamine:Dolichol-P N-Acetylg glucosamine-1-P Transferase Has Multiple Transmembrane Spans and a Critical Cytosolic Loop*

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UDP-GlcNAc:dolichol-P GlcNAc-1-P transferase (GPT) is an endoplasmic reticulum (ER) enzyme responsible for synthesis of GlcNAc-P-P-dolichol, the committed step of dolichol-P-P-oligosaccharide synthesis. The sequence of hamster GPT predicted multiple transmembrane segments (Zhu, X., and Lehrman, M. A. (1990) J. Biol. Chem. 265, 14250–14255). GPT has also been predicted to act on the cytosolic face of the ER membrane, based on topological studies of its substrates and products. In this report we test these predictions by: (i) immunofluorescence microscopy with antibodies specific for native GPT sequences or epitope tags inserted into GPT, after selective permeabilization of the plasma membrane with digitonin; (ii) insertion of Factor Xa cleavage sites; (iii) in vitro translation of GPT; and (iv) site-directed mutagenesis.

The loops between the 1st and 2nd and between the 9th and 10th predicted transmembrane spans of GPT were found to be cytosolic. In contrast, the loop between the 6th and 7th transmembrane spans, as well as the carboxyl terminus, were luminal. Thus, hamster GPT must cross the ER membrane at least three times, consistent with previous computer-assisted predictions. There was no apparent N-glycosylation or signal sequence cleavage detected by in vitro translation. The cytosolic loop between the 9th and 10th transmembrane spans is the largest hydrophilic segment in GPT and, as judged by site-directed mutagenesis, has a number of conserved residues essential for activity. Hence, these results directly support the hypothesis that dolichol-P-P-oligosaccharide assembly is initiated in the cytosol and that a downstream intermediate must translocate to the luminal face of the ER membrane.

Many functions have been attributed to the asparagine-linked class of oligosaccharides on glycoproteins (1), such as directly mediating specific interactions with endoplasmic reticulum (ER) chaperones (2, 3), and there is considerable interest in the mechanisms governing their synthesis. UDP-GlcNAc:

dolichol-P GlcNAc-1-P transferase (GPT) is responsible for the synthesis of GlcNAc-P-P-dolichol, the first intermediate in the synthesis of dolichol-P-P-oligosaccharides, which serve as precursors of asparagine-linked glycans (reviewed in Refs. 4 and 5). GPT is well known as a site of action for tunicamycin (Tn), although at least one other site of action exists (6). GPT is found in all eukaryotes and has been purified from bovine mammary gland (7) and cloned from hamster cells (8, 9), Saccharomyces cerevisiae (the ALG7 protein) (10, 11), Schizosaccharomyces pombe (12), Leishmania (13), and mouse (14). Although computer-assisted analysis of the hamster sequence predicted that GPT might contain as many as 10 transmembrane segments (8), there has been little direct information regarding the topological structure of this critical enzyme.

GPT is of interest because it is one of a small group of glycosyltransferases that diverge from the common type II model typical of glycosyltransferases found in the Golgi apparatus. Type II glycosyltransferases have small cytosolic amino-terminal domains, single transmembrane segments, luminal stem regions, and large carboxyl-terminal luminal domains responsible for catalysis (15). Transferases that now appear to have alternate topological arrangements include the ALG5 protein (glucose-P-dolichol synthase) (16), which may have multiple transmembrane spans, and UDP-GlcNAc:phosphatidyl-inositol α-GlcNAc transferase, which is likely to be composed of at least three genetically defined subunits (17).

Interest in the structure of GPT also stems from current hypotheses regarding the topological orientation of dolichol-P-P-oligosaccharide synthesis. The most widely accepted model for the topology of oligosaccharide assembly dictates: (i) cytosolic assembly of GlcNAc-P-P-dolichol, mannose-P-dolichol, and glucose-P-dolichol; (ii) cytosolic conversion of GlcNAc-P-P-dolichol to Manα,GlcNAc2-P-P-dolichol by nucleotide sugar donors; (iii) flipping of mannose-P-dolichol, glucose-P-dolichol, and Manα,GlcNAc2-P-P-dolichol to Glcα,Manα,GlcNAc2-P-P-dolichol with mannose-P-dolichol and glucose-P-dolichol as donors (reviewed in Refs. 5, 18, and 19).

Evidence in support of cytosolic assembly of GlcNAc-P-P-dolichol includes the observations that GPT in intact microsomes is active toward exogenous dolichol-P carried by liposomes (20), that the product of the subsequent reaction, GlcNAc2-P-P-dolichol, is accessible to a membrane-impermeant galactosyltransferase (21), and that inhibition of an ER UDP-GlcNAc transporter does not inhibit GPT activity in intact microsomes (21). Although these approaches provide valuable topological information, such experiments are subject to the possibility that the enzyme substrates or products under
analysis may have relocated during the experiment. This could be due to the action of unknown flipases or transporters or, in the case of dolichol-P, the ability of the substrate to alter membrane structure (22) to give a misleading result. For example, a careful study involving galactosyltransferase as an impermeant probe with intact microsomes concluded that GlcNAc₂-P-P-dolichol was luminal, not cytosolic (23). In addition, Man₃GlcNAc₂-P-P-dolichol and Man₂GlcNAc₂-P-P-dolichol clearly have access to the luminal space (24). Hence, studies of dolichol-P-P-oligosaccharide topology that focus on enzyme substrates and products may be affected by factors that are difficult to control.

An approach that is not subject to such limitations is to define the topology of the membrane-bound enzymes involved in dolichol-P-P-oligosaccharide synthesis and to identify the topological locations of critical residues. In this regard, GPT has received considerable attention, because it forms the first intermediate. For example, the sensitivity of GPT to intact microsomes from either rat (25) or embryonic chick (20, 26) liver to proteases has been studied. GPT appears to have one or more cytosolic elements that are sensitive to trypsin and Pronase (20, 26), although in some cases these enzymes failed to affect GPT (25, 26), suggesting lot-to-lot variation. These data suggested that the catalytic site of GPT faced the cytosol. However, as noted (25), such studies can be difficult to interpret, because an enzyme with a luminal catalytic site could still exhibit “cytosolic” protease sensitivity if the enzyme has an essential cytosolic domain or cytosolic accessory protein.

The ability to express recombinant GPT (27) raises the possibility of other methods of topological mapping. However, in initial studies we found that many of the conventional methods used to map the topologies of proteins with multiple membrane spans failed with hamster GPT. GPT becomes unstable after truncation (28), precluding reliable analyses of fusions with marker proteins, and yields a complex pattern of products on digestion with trypsin and other common proteases. Currently there are no sensitive immunoprecipitation protocols for GPT, which could be used with sequence-specific antibody antibodies, and insertion of N-glycosylation sequons has yielded negative results.

This report describes the topological mapping of hamster GPT by insertion of diagnostic epitope tags and protease recognition sites and a mutagenic analysis of conserved residues found facing the cytosol.

**EXPERIMENTAL PROCEDURES**

**Materials**—UDP-[³H]GlcNAc (26 Ci/mmol) was from DuPont. Translation-grade [¹⁴C]smethionine (1000 Ci/mmol) and [¹⁴C]methylated proteins were from Amersham Corp. T7 RNA polymerase, ribonuclease inhibitor, trypsin, RNAsin, nuclelease-free rabbit reticulocyte lysate, yeast α-mating factor mRNA (0.1 mg/ml), and canine pancreatic microsomal membranes (2 eq/ml) were from Promega. Protein A-agarose and Mutα-Gene 2 Kits were from Bio-Rad. Endoglycosidase H was from Genentech. Genitc (G418 sulfate) and powdered cell culture media were from Life Technologies, Inc. Serum was from Atlanta Biologicals. Geneticin (G418 sulfate) and powdered cell culture media were from Boehringer, Genzyme. Geneticin (G418 sulfate) and powdered cell culture media were from New England Biolabs, Fisher, or Boehringer–Mannheim. Mouse monoclonal antibodies were from Eastman Chemicals (anti-DKDDDKDD peptide (FLAG) IgG, catalog number 1B13010), StressGen (anti-KSEKDELP peptide (carboxyl terminus of BIP) IgM, catalog number SPA-827), or Sigma (antivimentin IgM, catalog number V-5255, a gift from Dr. George Bloom, University of Texas, Southwesten Medical Center). Fluorescein isothiocyanate-labeled antibodies used for immunofluorescence microscopy were obtained from Sigma (goat anti-mouse IgG, catalog number F-5262, and goat anti-rabbit IgG, catalog number F-8857) or Zymed (goat anti-mouse IgM, a gift of Dr. George Bloom).

**Cell Culture**—CHO-K1 cells were normally maintained in Ham’s F-12 medium buffered at pH 7.2 with 15 mM Na-HEPES with 2% fetal bovine serum and 8% calf serum as described (29). COS-6 cells were cultured in Dulbecco’s modified Eagle’s medium containing 10% fetal calf serum.

**Transient and Stable Transfection**—Stable transfection of normal GPT cDNA and various mutants in plasmid pJB20 (30) was performed by the calcium phosphate procedure as described earlier (27). Stable transfectants were selected with 1 μg/ml Geneticin (for vector) or with Tn (T7765, Sigma) for GPT transfectants (27); primary selections were performed with 1 μg/ml Tn, and individual colonies were screened with higher concentrations to obtain the highest expressers. Transfectants were subcloned by limiting dilution.

Transient transfection of COS-6 cells by a minor modification of the DEAE-dextran method (8) was also performed with cDNA subcloned into pJB20.

Protein concentrations were determined immediately after membrane preparation (29). The membranes were stored at 4 °C and used within 1 h. 

GPT assays were performed with UDP-[³H]GlcNAc as described (29).

**Determination and Interpretation of Cellular Resistance to Tunica-mycin**—As described earlier (29), individual transfectants were plated in media containing various concentrations of Tn, in 2-fold increments, and stained after 7 days of incubation. The highest concentration of Tn that had no visible effects on colony size or number was taken as the Tn resistance of that transfectant. For any given Tn resistance value-transfectant combination, we have found that Tn concentrations that are 2–4-fold higher cause complete inhibition of the growth of that transfectant.

Tn resistance is a linear function of GPT expression (27). In addition, some GPT mutants lack enzymatic activity but are still able to confer cellular resistance to Tn (for example, see Table I and “Discussion”), indicating that such enzymes are properly folded and probably confer resistance to Tn by a buffering effect. The endogenous GPT in CHO transfectants contributes to the total GPT activity and Tn resistance. Thus, for stable transfectants of either normal or mutated GPT, the ratio of GPT enzyme activity/Tn resistance was determined by first subtracting the contribution of the endogenous GPT as determined with vector-transfected cells. This was done as indicated in the descriptions accompanying Fig. 1 and Table I.

**Selective Permeabilization of the Plasma Membrane and Indirect Immunofluorescence Confocal Microscopy**—Selective permeabilization of CHO cells with digitonin was performed in a manner similar to that described earlier (31, 32). Unless specified, all steps were carried out at room temperature with gentle agitation. Cells grown on coverslips at 37 °C for 24–48 h were washed three times with PBS and then fixed in 2% paraformaldehyde in PBS for 30 min. Excess paraformaldehyde was neutralized with 100 mM NH₄Cl in TBS (20 mM Tris-Cl, 150 mM NaCl, pH 7.4) for 10 min and briefly washed three times with PBS. For selective permeabilization of the plasma membrane, the cells were incubated on ice for 12 min with 10 μg/ml of digitonin in 0.5% sucrose, 0.1 M KCl, 2.5 mM MgCl₂, 1 mM Na₂EDTA, and 10 mM Na-PIPES, pH 6.8. For permeabilization of plasma and intracellular membranes, the cells were treated with 0.1% Triton X-100 in PBS at room temperature for 5 min. After permeabilization by either method, cells were washed three times with TBS and incubated for 1 h in blocking buffer (TBS containing 10% goat serum and 1 μg/ml CaCl₂). The solution was then replaced with blocking buffer with primary antibody (anti-BIP, 0.2 μg/ml; anti-FLAG, 5 μg/ml; anti-loop 1-2 (DEAE-IgG fraction; Ref. 27), 15 μg/ml; or antivimentin, 1:200 dilution) and incubated for 4 h. The cells were then washed four times with TBS containing 1 μg/ml CaCl₂ and incubated for 1 h with appropriate fluorescent secondary antibodies (anti-mouse IgM, 1:200 dilution; anti-mouse IgG, 1:300; or anti-rabbit IgG, 1:200 dilution) in 5% bovine serum albumin in TBS containing 1 μg/ml CaCl₂. After six washes in TBS containing 1 μg/ml CaCl₂, the coverslips were rinsed twice in H₂O and mounted on glass slides with Citifluor glycerol. Cells were viewed and photographed with a Bio-Rad MRC 600 laser confocal microscope as described earlier (27).

**Treatment of Microsomes with Factor Xa and Trypsin**—Freshly prepared microsomal membranes (28) were suspended at a concentration of 250 μg/ml membrane protein in 20 μl of a solution of 50 mM Tris-Cl, pH 8.0, 100 mM NaCl, 2 mM CaCl₂, 0 or 0.2% Nonidet P-40, and 0 or 75 ng/ml Factor Xa for 1.5 h at 4 or 23 °C with gentle shaking. Samples were then treated with SDS-PAGE sample buffer as described (27), supplemented with 4 M urea and 10% mercaptoethanol, incubated for 20 min at 23 °C, and subjected to electrophoresis on a 13% SDS-poly-
acrylamide gel. GPT was detected by immunoblotting with an antipeptide (His-tag) antibody directed against residues 42–56 in loop 1/2 and visualized by enhanced chemiluminescence as described (27), except that the primary and secondary antisera were used at dilutions of 1:1,000 and 1:10,000, respectively.

Microsomes were treated with 250 units/ml trypsin (T-8253, Sigma) in place of Factor Xa in a similar manner, except that the trypsin was present for only the final 20 min of incubation, after which 50 μM aprotinin, 300 μM leupeptin, and 940 μg/ml soybean trypsin inhibitor were added. Controls in which inhibitors preceded the trypsin demonstrated their effectiveness (data not shown). Samples were mixed with SDS-PAGE sample buffer supplemented with 10% mercaptoethanol, boiled for 5 min, and subjected to electrophoresis on a 13% SDS-polyacrylamide gel and immunoblotted as described (27). Anti-KSEKDEL IgG was detected with 0.5 μg/ml primary antibody, and a peroxidase-labeled goat anti-mouse IgG (Amersham) was used as second antibody at a dilution of 1:2,000.

Cell-free Transcription—Transcription was carried out essentially as described (33). The 1.6-kilobase EcoRI-PstI fragment of hamster GPT cDNA was subcloned into pBSM13+ or pTZ18U and then linearized with PstI to give a transcription encoding the full-length polypeptide. Transcripts were carried out with T7 RNA polymerase at 37°C.

Cell-free Translation—Translations were performed in a nuclease-treated rabbit reticulocyte system using 17.5 μl of lysate, 0.5 μl of 1 mM amino acids (minus methionine), 15 μCi of [35S]methionine, and 1 μl of mRNA (typically 100 ng) in a final volume of 25 μl. Where indicated the translation reaction was supplemented with 2 μg (1 μl) of canine pancreatic ribonuclease. Unless indicated otherwise, translations were performed for 15 min at 30°C. In some cases microsomal vesicles were isolated posttranslationally by centrifugation at 150,000 × g for 10 min through a 0.5 M sucrose cushion containing 50 mM Na-HEPES, pH 7.5, and 100 mM KCl.

Characterization of Cell-free Translation Products—Immunoprecipitation of translation products was carried out in 0.5 ml of buffer A (50 mM Tris-Cl, pH 7.4, containing 0.15 M NaCl, 2 mM EDTA, and 1% (w/v) SDS, 10% (w/v) glycerol, and 0.002% bromophenol blue). Unless stated otherwise, samples were treated with 50 μl protein A-agarose in place of Factor Xa in a similar manner, except that the trypsin was replaced with 250 units/ml trypsin (T-8253, Sigma) in place of Factor Xa in a similar manner, except that the trypsin was present for only the final 20 min of incubation, after which 50 μM aprotinin, 300 μM leupeptin, and 940 μg/ml soybean trypsin inhibitor were added. Controls in which inhibitors preceded the trypsin demonstrated their effectiveness (data not shown). Samples were mixed with SDS-PAGE sample buffer supplemented with 10% mercaptoethanol, boiled for 5 min, and subjected to electrophoresis on a 13% SDS-polyacrylamide gel and immunoblotted as described (27). Anti-KSEKDEL IgG was detected with 0.5 μg/ml primary antibody, and a peroxidase-labeled goat anti-mouse IgG (Amersham) was used as second antibody at a dilution of 1:2,000.

Factor Xa Sites—The cleavage recognition sequence for Factor Xa (Ile-Arg) was introduced in loops 6/7 and 9/10 by replacing residues Leu315–Glu341–Gly342–Asp343 and Ala357–Glu358–Arg359–Leu360, respectively.

FLAG Epitopes—A FLAG epitope was attached to the carboxyl terminus of GPT in two steps. First, a silent mutation at Asp397 was made by replacing nucleotide 1368 (T) with C, creating a unique AatII site (GAGCTC) just before the stop codon. Next, an oligonucleotide cassette encoding the FLAG epitope followed immediately by a termination codon was made by hybridizing CGACTACAAGGACGACGTAAGTTA and AGCTCAGCTCGTACTGCTTGTACGCACTTTG. This cassette had a 5’-AatII-cohesive end and a 3’-HindIII-cohesive end and was inserted between the AatII site at nucleotides 1386–1371 and a unique HindIII site in the polynucleotide of pBR20 (30). This resulted in a mutant with all 408 residues of GPT followed by the FLAG epitope and a termination codon.

Unique BamHI sites for FLAG epitope insertion were introduced into loops 6/7 and 9/10 by replacement of nucleotides 792–795 (TGAC) with ATTG and 1123–1128 (CTCTCT) with GGATCC, resulting in the amino acid changes Asp376→Ser and Leu385→Gly, respectively. A double stranded synthetic DNA cassette with BamHI ends, encoding the FLAG epitope (Asp-Tyr-Lys-Asp-Asp-Lys) flanked by 5 glycine residues on each side, was cloned in frame into the BamHI site. Insertion mutants were identified by screening for a BpmI site introduced in the cassette by replacing the BamHI-compatible sequence GATCC with GATCT at the upstream BamHI site.

Region 1 (Arg301–His302–Arg303) Mutants of Loop 9/10—The Arg301→Asn double mutant was made by replacement of nucleotides 1048–1050 (CGA) with AAT and nucleotides 1054–1059 (CGATA) with AATTAT.

Region 2 (Leu366–Ile367–Leu368–Leu369–Leu370–Leu371) Mutants of Loop 9/10—The triple mutant Hele363→Ala/Hele364→Ala/Leu366→Ala was created by replacement of the nucleotides 1231–1248 (ACCCCTACATCATCTGTA) with ACGGCCCGTATA, which also introduced silent mutations for Thr362 and Leu366→Asn. The Hele364→Ala double mutant Leu365→Ala/Ile364→Ala/Leu366→Ala was created by replacement of the nucleotides 1231–1239 (ACCCCTACAT) with ACGGCCGCTATA. The single mutant Leu366→Ala was made by changing nucleotides 1054–1056 (COC) to AAG, in conjunction with alterations of residues 1048–1050 (CGA) to AAG to create a unique AvrII site with a silent mutation for Arg303.

Region 2 (Leu366–Ile367–Leu368–Leu369–Leu370–Leu371) Mutants of Loop 9/10—The triple mutant Hele363→Ala/Hele364→Ala/Leu366→Ala was created by replacement of the nucleotides 1231–1248 (ACCCCTACATCATCTGTA) with ACGGCCCGTATA, which also introduced silent mutations for Thr362 and Leu366→Asn. The Hele364→Ala double mutant Leu365→Ala/Ile364→Ala/Leu366→Ala was created by replacement of the nucleotides 1231–1239 (ACCCCTACAT) with ACGGCCGCTATA. The single mutant Leu366→Ala was made by changing the nucleotides 1243–1248 (TTGCTA) to GCGTTA, which also introduced silent mutations for Arg303 and a unique MluI site.

RESULTS

Transmembrane Topology of GPT Assessed by Mapping Natural and Artificial Epitopes in Loops 1/2, 6/7, and 9/10, and at the C Terminus—Hamster GPT resides in the ER membrane and has a predicted molecular weight of 46,112. We combined two microscopic techniques used previously to map the topological orientations of membrane proteins: (i) microscopy of epitope-tagged plasma membrane proteins in intact or fully permeabilized cells, using antibodies specific for the epitope tag (34, 35); and (ii) microscopy of ER membrane proteins, using antibodies against native epitopes, after permeabilization of both the plasma and ER membranes with Triton X-100 or only the plasma membrane with digitonin (31, 32). In the latter strategy cytosolic epitopes are detected in cells permeabilized with either digitonin or Triton X-100, but luminal ER epitopes are detected only with Triton X-100.
analyzed as follows. Terminal FLAG (in microsomal protein/lane. (Fig. 1)

Factor Xa insertion (with pJB20 vector (Fig. 1). Loop 9/10 FLAG proteins had a ratio of enzyme activity (Fig. 1) with tunicamycin (Fig. 1). Loop 9/10 FLAG had relatively low enzyme activity as judged by enzyme assays after treatment with either digitonin or Triton X-100, but proteins reactive with anti-KSEKDEL were detected only with Triton X-100.

COS-6 transfections (data not shown). All of the other stable transfectants described in this study gave results comparable with those presented here.

Representative micrographs of key experiments with GPT are shown in Fig. 3. Both anti-FLAG and anti-loop 1/2 antibodies were used. By the criteria discussed above, loops 1/2 and 9/10 were found to be cytosolic, whereas loop 6/7 and the carbonyl terminus were luminal. Thus, GPT is a polytopic enzyme with a minimum of three transmembrane spans. The orientations of loops 1/2, 6/7, and 9/10 and that of the carbonyl terminus are in full agreement with the computer-assisted predictions reported earlier (8). Clearly, the actual orientations of the other predicted loops remain to be determined (see “Discussion”).

Factor Xa Cleavage Site Insertions—Due to particular interest in loop 9/10 (see below and Table I), stable transfectants were generated with Factor Xa cleavage sites (IEGR) inserted into loop 9/10 and, for comparison, loop 6/7, to independently confirm the results of Fig. 3. By the same criteria described above for the FLAG constructs, the Factor Xa constructs were judged to be folded normally (Fig. 1). Consistent with the results of FLAG insertions, only the loop 9/10 Factor Xa mutant was specifically degraded, yielding a discrete product, when intact microsomes were treated with Factor Xa at 23 °C in the absence of detergent (Fig. 4A, upper panel, lane 6).

Fig. 4B shows that the intact microsomes did not become permeabilized during the 23 °C incubation. In this experiment an antibody directed against the carbonyl terminus of BiP was used that could recognize multiple luminal ER proteins. Since this ratio is independent of expression level for native GPT (27), the loop 9/10 FLAG protein behaved normally. This contrasted with the ratios obtained for inactivating mutations above for the FLAG constructs, the Factor Xa constructs were judged to be folded normally (Fig. 1). Consistent with the results of FLAG insertions, only the loop 9/10 Factor Xa mutant was specifically degraded, yielding a discrete product, when intact microsomes were treated with Factor Xa at 23 °C in the absence of detergent (Fig. 4A, upper panel, lane 6).

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To demonstrate that overexpression of GPT did not cause an ER permeabilization artifact, antibodies to the carbonyl-terminal peptide (KSEKDEL) of BiP, a luminal ER protein, and vimentin, a cytoskeletal protein, were tested with cells expressing a loop 6/7 FLAG mutant (Fig. 2). Vimentin was detected after treatment with either digitonin or Triton X-100, but proteins reactive with anti-KSEKDEL were detected only with Triton X-100.
Topological analysis of GPT. Untransfected CHO-K1 cells (CHO), or CHO-K1 cells stably transfected with normal GPT (Tn-10; Ref. 27) or GPT with a FLAG tag inserted into loops 6/7 (L 6/7 FLAG) or 9/10 (L 9/10 FLAG) or at the carboxyl terminus (C-term FLAG) were permeabilized with digitonin (DIG) or Triton X-100 (TX) and stained with antibodies specific for the FLAG epitope (anti-FLAG) or residues 42–56 in loop 1/2 of GPT (anti-L 1/2; Ref. 27) as described under “Experimental Procedures.” Weak outlines of cells are visible in panels A, upper panel, lanes 5 and 6. Interestingly, a higher molecular mass form of this mutant of approximately 66 kDa, possibly an SDS-resistant oligomer, was efficiently degraded yielding a product of approximately 62 kDa. As evidenced by multiple exposures of this and other experiments (data not shown), the corresponding oligomers of the other GPTs shown in the figure were not degraded. This suggests that the conformation or accessibility of loop 9/10 may differ depending on the oligomeric state of GPT.

Since the loop 6/7 mutant was not cleaved by Factor Xa in the absence of detergent (Fig. 4A, upper panel, lane 4), these results were also consistent with the luminal orientation identified for loop 6/7 (Fig. 3). To demonstrate that loop 6/7 could, in fact, be recognized by Factor Xa, microsomes were permeabilized with detergent and treated with Factor Xa at 4 °C (Fig. 4A, lower panel). Both the loop 6/7 (lane 4) and loop 9/10 (lane 6) mutants yielded discrete fragments. For this experiment it was necessary to incubate at 4 °C, because at 23 °C it was found that all GPTs were completely degraded by a nonspecific activity present in the microsomal preparation. We could not identify a protease inhibitor that prevented this degradation without also inhibiting Factor Xa (data not shown).

Although native GPT (46 kDa calculated and 36 kDa experimental) and the loop 9/10 Factor Xa fragment (38 kDa calculated and 30 kDa experimental) both deviated significantly from their calculated sizes, the loop 6/7 Factor Xa fragment (24 kDa calculated and 25 kDa experimental) did not. This indicates that the anomalous behavior of GPT noted earlier (27) may be due to an element between residues 213 and 336, perhaps due to formation of a compact SDS-resistant structure or excessive binding of SBS.

In Vitro Translation of Hamster GPT—To test for N-glycosylation and other covalent posttranslational modifications, in vitro transcription and translation were performed. Hamster GPT has one potential N-linked glycosylation site in loop 4/5 and three in loop 9/10 (8). However, the enzyme is not retained by a column of immobilized concanavalin A. Purified bovine mammary GPT was also judged to be free of N-glycans (7), although it is not known whether the bovine enzyme contains N-glycosylation sequons.

Synthetic GPT mRNA was translated in vitro with a rabbit reticulocyte lysate in the absence or presence of canine pancreatic microsomes. Control experiments (not shown) indicated that 5'-capping of the mRNA did not improve translation, so uncapped mRNA was used in all experiments. A single predominant polypeptide was observed with an apparent size of 35 kDa (Fig. 5A, lane 2). As discussed above, this anomalous behavior of GPT (predicted size, 46 kDa) may be due to the sequence between residues 213 and 336. A series of smaller polypeptides of approximately 20 kDa or less, possibly due to proteolytic degradation or premature translational termina-

![Fig. 3. Topological analysis of GPT.](image-url)

| Table I Effects of mutations in cytosolic loop 9/10 on GPT activity |
|-----------------|----------------|------------------|------------------|------------------|------------------|
| Vector         | 1   | 1   | 0.5  | 0.65 |
| Normal GPT     | 5.0 ± 1.0 | 11.0 ± 1.0 | 16   |
| Region 1 mutations | Arg301-His302-Arg303 |
| 1               | 1.1 ± 0.2 | 1.1 ± 0.2 | 2   (0.07) |
| 2               | 1.2 ± 0.2 | 1.0 ± 0.1 | 2   (0.00) |
| 3               | 1.2 ± 0.1 | 1.2 ± 0.2 | 4   (0.06) |
| 4               | 2.0 ± 0.1 | 1.1 ± 0.1 | 6   (0.02) |
| 5               | 1.1 ± 0.2 | 1.1 ± 0.2 | 12  (0.01) |
| Region 2 mutations | Leu365-Ile364-Asn365-Leu366 |
| 6               | 1.0 ± 0.2 | 1.1 ± 0.1 | 4   (0.03) |
| 7               | 3.2 ± 0.7 | 3.5 ± 0.5 | 8   (0.33) |
| 8               | 2.3 ± 0.3 | 2.4 ± 0.2 | 6   (0.25) |

* Values are average & S.E. of multiple determinations done with at least two membrane preparations.
* Values are average & S.E. of multiple determinations done with at least two membrane preparations. Parentheses indicate that the GPT activity value used for calculation of the ratio was not significantly above 0.
Carbonate extraction of material shown in lane 3 subjected to carbonate extraction to yield supernatant (absence (with analysis with an antibody specific for loop 1/2. Each lane had 5 g of microsomal protein. Closed and open arrowheads, loop 6/7 and loop 9/10 Factor Xa, respectively. Microsomes from cells transfected with vector, loop 6/7 Factor Xa, loop 9/10 Factor Xa, or wild-type (WT) GPT were analyzed. A, as described under “Experimental Procedures,” microsomes were treated in the absence (lanes 1, 3, 5, and 7) or presence (lanes 2, 4, 6, and 8) of Factor Xa without (upper) or with (lower) 0.2% Nonidet P-40, and GPT was analyzed by immunoblot analysis with an antibody specific for loop 1/2. Each lane had 5 μg of microsomal protein. Closed and open arrowheads, loop 6/7 and loop 9/10 Factor Xa fragments, respectively. B, in parallel with the experiment shown in A, the same batch of microsomes from lane 9/10 Factor Xa transfecant were treated without or with trypsin in the absence or presence of Nonidet P-40 as described under “Experimental Procedures” and analyzed by immunoblot analysis with an antibody directed against the last 7 amino acids of BiP. Each lane had 15 μg of microsomal protein.

Fig. 4. Effects of Factor Xa digestion. Microsomes from cells transfected with vector, loop 6/7 Factor Xa, loop 9/10 Factor Xa, or wild-type (WT) GPT were analyzed. A, as described under “Experimental Procedures,” microsomes were treated in the absence (lanes 1, 3, 5, and 7) or presence (lanes 2, 4, 6, and 8) of Factor Xa without (upper) or with (lower) 0.2% Nonidet P-40, and GPT was analyzed by immunoblot analysis with an antibody specific for loop 1/2. Each lane had 5 μg of microsomal protein. Closed and open arrowheads, loop 6/7 and loop 9/10 Factor Xa fragments, respectively. B, in parallel with the experiment shown in A, the same batch of microsomes from lane 9/10 Factor Xa transfecant were treated without or with trypsin in the absence or presence of Nonidet P-40 as described under “Experimental Procedures” and analyzed by immunoblot analysis with an antibody directed against the last 7 amino acids of BiP. Each lane had 15 μg of microsomal protein.

Fig. 5. Characterization of GlcNAc-1-P transferase synthesized in vitro. In vitro transcription and translation and various analyses were performed as described under “Experimental Procedures.” Open and closed arrowheads, full-length translation products for hamster GPT and yeast a-mating factor, respectively. A, identification of GPT polypeptide. Lane 1, 14C-labeled standards. Lane 2, translation of GPT mRNA with microsomes. Lane 3, carbonate-resistant products of material shown in lane 2. Carbonate extraction (see C below) was used to ensure that the subsequent immunoprecipitation steps (lanes 4 and 5) involved only microsomal-associated translation products. Lanes 4 and 5, immunoprecipitation of material shown in lane 3 with antibodies directed against amino acids 42–56 (lane 4) or 398–408 (lane 5) of hamster GPT. B, evidence of lack of N-glycosylation. Translations of GPT (lanes 1–4) or yeast a-mating factor (lanes 5–8) were performed in the absence (lanes 1 and 6) or presence (lanes 2–5, 7, and 8) of microsomes. Lanes 3, 4, 7, and 8 represent material subjected to the endoglycosidase H (Endo H) protocol in the absence (lanes 3 and 7) or presence (lanes 4 and 8) of endoglycosidase H. Note that sample differences due to the endoglycosidase H protocol had a small effect on the mobility of GPT (compare lanes 2 and 3). C, effects of carbonate extraction. GPT (lanes 1–5) and a-mating factor (lanes 4–6) were translated in the presence of microsomes, and total translation products were either analyzed directly by SDS-PAGE (lanes 1 and 4, 7) or subjected to carbonate extraction to yield supernatant (lanes 2 and 5, 8) and pellet (lanes 3 and 6, 7) fractions and then analyzed by SDS-PAGE.
Specific residues in loop 9/10 were altered by site-directed mutagenesis in two highly conserved 6–7-residue segments (Fig. 6): Pro298-Arg303 (region 1) and Asn360-Leu366 (region 2). Each mutant was characterized after transient expression in COS-6 cells or stable expression in CHO-K1 cells. As summarized in Table I, multiple substitutions in both regions (mutations 1, 2, and 6) resulted in essentially complete loss of activity in both expression systems. However, the mutated enzymes were readily detected on immunoblots when expressed transiently or stably and conferred resistance to Tn when expressed stably. In addition, one multiple substitution in a conserved tripeptide segment of loop 9/10 (Ile333-Leu334-Lys335) also inactivated GPT in transient assays (data not shown).

Further analysis of region 2 by altering residues 363–364 (mutation 7) or only residue 366 (mutation 8) yielded enzymes with activities roughly one-half of normal. This indicated that the enzyme can reasonably tolerate some mutations in region 2. In contrast, it was found that GPT could not tolerate any mutation of Arg303 in region 1. This region was originally chosen for more detailed analysis, since it contained a cluster of positively charged residues that could potentially interact with the phosphorylated substrates. Conservative replacement of Arg303 with Lys (mutation 5) resulted in a loss of essentially all enzymatic activity, both in transient and stable transfections, although the mutant protein could be expressed at levels comparable with those of the normal enzyme. As expected, less conservative replacement of Arg303 to Asn or His (mutations 3 and 4) also inactivated GPT.

Membranes from stable CHO-K1 transfectants overexpressing either normal GPT or Arg303 Lys (mutation 5) were also assayed after detergent solubilization and addition of saturating dolichol-P in the presence of variable concentrations of UDP-GlcNAc. After subtracting out the activities corresponding to endogenous GPT (i.e., vector-transfected CHO-K1 cells), the mutant appeared to have a small amount of activity (approximately 10% of the normal enzyme), although it was not possible to obtain accurate enzymatic measurements because of the background of endogenous enzyme. Although the precise function of Arg303 cannot be deduced from these data, the profound effect of the conservative Arg303 Lys mutation and the effects of the other mutations in loop 9/10 demonstrate that this cytosolic loop has an essential function.

**DISCUSSION**

This article reports new information regarding the topological organization of hamster GPT. Both loop 6/7 and the car-
boxyl terminus have a luminal orientation, whereas loops 1/2 and 9/10 are cytosolic. Loop 9/10 has highly conserved elements that are essential for enzyme function. This work provides a clear example of a eukaryotic glycosyltransferase with multiple transmembrane spans, in contrast to the well known type II model for Golgi apparatus transferases. This is also the first direct evidence that an enzyme involved in the initial steps of dolichol-P-oligosaccharide synthesis has critical elements in the cytoplasm.

The results of this and prior studies are summarized as a schematic model of GPT (Fig. 7). The topological orientations of four regions have been mapped (large letters C and L), including essential loop 9/10. One residue in loop 9/10, Arg\textsuperscript{392}, fails to tolerate even the most conservative replacements (Table I). In addition, GPT has important elements in spans 2, 7, and 10. The sequence Phe\textsuperscript{395}-Ser\textsuperscript{396}-Ile\textsuperscript{397} at the carboxyl-terminal end of the lumenal orientation, whereas loops 1/2 and 9/10 are cytosolic. Loop 9/10 have a luminal orientation, whereas loops 1/2 and 9/10 are cytosolic. Loop 9/10 has highly conserved elements that are essential for enzyme function. This work provides a clear example of a eukaryotic glycosyltransferase with multiple transmembrane spans, in contrast to the well known type II model for Golgi apparatus transferases. This is also the first direct evidence that an enzyme involved in the initial steps of dolichol-P-oligosaccharide synthesis has critical elements in the cytoplasm.

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