Cloning, Sequence, and Expression of a cDNA Encoding Hamster UDP-GlcNAc:dolichol Phosphate N-Acetylglucosamine-1-phosphate Transferase*

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UDP-GlcNAc:dolichol phosphate N-acetylglucosamine-1-phosphate transferase (GPT) catalyzes the initial reaction required for synthesis of dolichol-P-P-oligosaccharides. We report here on the sequence and expression of a full-length cDNA clone encoding hamster GPT. The cDNA predicts a protein of 408 amino acid residues including 10 hydrophobic segments. Several portions of the hamster GPT sequence constituting one-third of the protein have 60% or greater identity with yeast GPT, and one-half of the conserved sequence falls within the hydrophobic segments. In addition, hamster GPT has two copies of a putative dolichol recognition sequence recently identified in three yeast enzymes that interact with dolichol. The protein lacks KDEL or DEKKMP-type carboxyl-terminal ER sorting sequences.

When expressed in COS-1 cells, the cDNA causes a 5–7-fold increase of GPT activity in membrane fractions. The activity was completely inhibitable by tunicamycin, and the primary product was shown to be GlcNAc-pyrophosphoryldolichol.

This cDNA represents the first enzyme of the dolichol-oligosaccharide biosynthetic pathway to be cloned from a vertebrate source and demonstrates structural homology between the enzymes of the yeast and mammalian pathways.

Asparagine-linked glycosylation of nascent proteins within the lumen of the endoplasmic reticulum (ER) requires the enzymatic transfer of dolichol-linked oligosaccharides to asparagine residues. In almost all eukaryotes, the oligosaccharide consists of 2 N-acetylglucosamine, 9 mannose, and 3 glucose residues and are attached to dolichol by a pyrophosphoryl linkage (1). Although the structure of the oligosaccharide unit is well known, little has been established about the structure, regulation, or topology of the transferases required for its synthesis. This is due in part to the low abundance of these enzymes, their instability in detergent solution, and the lack of specific assays for many of these reactions.

The enzyme responsible for the first sugar addition of this pathway is N-acetylglucosamine-1-phosphate transferase (GPT) (2). This enzyme catalyzes the transfer of GlcNAc-1-P from UDP-GlcNAc to dolichol phosphate to form GlcNAc-P-P-dolichol. Thus, it is a likely control point for oligosaccharide assembly. The gene for this enzyme has been cloned from yeast (3), and the protein has been purified to apparent homogeneity from bovine mammary gland (4). A cDNA encoding a mammalian GPT would facilitate analysis of the enzyme at the molecular level and would provide primary sequence information which could be compared with the yeast enzyme to identify conserved regions that are likely to be important for function. Previous work from this laboratory (5) resulted in the cloning of a fragment of the hamster GPT gene from a tunicamycin-resistant CHO line that amplified the gene 40–50-fold. In this paper we describe the cloning of a full length cDNA encoding hamster GPT, structural features of the enzyme deduced from its sequence, and expression of the hamster cDNA in simian cells. A preliminary report of this work has been presented (6).

EXPERIMENTAL PROCEDURES

Materials—Oligo(dT)-cellulose and nonphosphorylated EcoRI linkers (octamer) were purchased from New England BioLabs. BioGel A-50m was from Bio-Rad. [α-32P]dCTP (3000 Ci/mmole), [α-32P]dATP (500 Ci/mmole), and [γ-32P]ATP (3000 Ci/mmole) were from Du Pont-New England Nuclear. A cDNA synthesis kit, T, DNA polymerase, kinase, and ligase, EcoRI methylase, and all restriction endonucleases were from Bethesda Research Laboratories. Escherichia coli C600 cells, C600-hfr cells, and λgt10 DNA were from Vector Cloning System. Gigapack Gold, PBS M13 (+/-) vectors, and Ecoli/mung bean nucleases were from Stratagene. DNA sequencing kits (Sequenase) were from United States Biochemical Corp. The pCMV-2 vector was kindly provided by Dr. David Russell of this institution. COS-1 cells were from the American Type Culture Collection. DEAE-dextran (M, = 5 x 105) was from Pharmacia LKB Biotechnology Inc. UDP-GlcNAc, GlcNAc₅₋₆, DR5-cellulose (fine mesh), chloroquine, and Dulbecco’s modified Eagle’s medium were from Sigma. SG-81 paper was from Whatman. Other materials have been described previously (5).

Construction of TnR-32 cDNA Library—Total RNA was prepared from TnR-32 cells, and Northern RNA analysis was performed with a TRC 5 DNA fragment to verify overexpression of GPT mRNA (5). Poly(A)+ RNA was further purified with an oligo(dT)-cellulose column. cDNA was synthesized with BRL cDNA synthesis system. Briefly, 2 μg of poly(A)+ RNA from TnR-32 cells was used to generate 0.64 μg of first strand cDNA with murine mammary tumor virus reverse transcriptase, and an equal amount of second strand cDNA was generated by E. coli DNA polymerase I in the presence of E. coli RNase H. In all cases, cDNA yield was determined by the incorpo-
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eration of a trace amount of \(\alpha-3^{2}P\)-dCTP. The cDNA was then treated with T. DNA polymerase to generate blunt ends and with EcoRI methylase to protect endogenous EcoRI sites. EcoRI linkers previously labeled with \(3^{2}P\) by T. kinase were ligated to the cDNA with T. DNA ligase. After extra linkers were digested away by EcoRI enzyme, the mixture of cDNA and free linkers was fractionated on a precolumnated 0.15 x 22-cm (1.5 ml) Bio-Gel A-50m column. Fractions containing the largest cDNA were pooled. 1.6 mg of Xgt10 EcoRI arms and 32 ng of cDNA were ligated together and packaged into viral particles (Gigapack Gold) to generate a library containing 3.6 x 10^6 recombinant phages. The library was then amplified before further use.

Screening the cDNA Library—Recombinant phages were screened with the TRG-3 DNA fragment (b). Positive plaques were obtained at a frequency of 10^-4 after the primary screening, reflecting the amplification of GPT in TnR-32 mRNA. Four positive clones were isolated and subcloned into the pBS M13- vector, with insert sizes ranging from 0.9 to 2.0 kilobases. Sequencing of the longest two, TRG12 and TRG14 (Fig. 1), indicated that neither contained a 

![Fig. 1. Physical map of GPT cDNA clones. The structure of the mRNA encoding GPT, deduced from the four cDNA clones shown, is described in the text. The translation initiation (AUG) and termination (UGA) sites, as well as two polyadenylation sites (A\(_1\) and A\(_2\)), are shown. A small gap in the 3'-untranslated region of TRG-12 was resistant to sequence analysis. The subclones TRG-5 and TRG-12-3 are described under "Experimental Procedures"; the portion of the cDNA corresponding to the exon in TRG-5 is shown, bp, base pairs.](image)

termination codon (TAG) precedes this methionine codon by 42 nucleotides. The next available methionine codon lies at residue 108 and is preceded by a poor initiation sequence (T-G-C-T-G-G-C).

In accord with the observations that GPT is tightly associated with microsomal membranes (11) and requires detergent for solubilization (4, 12), 48% of the amino acid residues of hamster GPT are apolar (Phe, Trp, Tyr, Ile, Leu, Met, Val). Hydrophobicity analysis by the algorithm of Kyte and Doolittle (13) (data not shown) revealed 10 hydrophobic segments (underlined in Fig. 2) ranging from 17 to 26 residues that could potentially interact with membrane bilayers. The first of these (residues 7-32) does not appear to be part of a typical cleavable leader sequence (14) due to its length (26 hydrophobic residues versus a consensus of roughly 10 residues) and because the amino-terminal flanking region is negatively charged, whereas the hydrophobic segments of cleavable leaders are usually preceded by positively charged residues. However, because there are two alanines 6 and 7 residues after this hydrophobic segment, and this type of sequence is favored by signal peptidase (15), further analysis will be required to verify that this segment is not cleaved. Based upon the guidelines for insertion of proteins into the ER membrane (16) and considering that the first hydrophobic segment is flanked by net charges of zero on the amino-terminal side and +2 within the first 15 residues on the carboxyl-terminal side, a working model illustrating a possible arrangement of the protein in the ER membrane is presented in Fig. 3. Although the prediction of a luminal amino-terminal residue for proteins with a net positive charge on the carboxyl-terminal side (such as GPT, net charge +2) is incorrect in a small number of cases (16), most exceptions to this rule have at least 5 charged residues on the amino-terminal side of the membrane-spanning region. Including the amino-terminal residue, GPT has a total of 2 charged residues on this side. Possible functions of the remaining hydrophobic segments will be discussed below.

GPT lacks carboxyl-terminal sorting sequences typical of soluble luminal ER proteins (Lys-Asp-Glu-Leu) (17) and monotopic ER proteins (Aps-Glu-Lys-Lys-Met-Pro) (18). There are four consensus sites for asparagine-linked glycosylation. However, purified bovine GPT does not appear to contain N-glycans (4), and the enzyme activity fails to adsorb to concanavalin A-agarose when solubilized from TnR-32 cells. Thus, there is no evidence to suggest that this protein is glycosylated at these consensus sites. The model in Fig. 3 predicts that only one consensus site faces the ER lumen, and this site may be sterically inaccessible due to an adjacent hydrophobic segment.

Possible Dolichol-recognition Sites—Previous results sug-

2 L. Camp and M. Lehrman, unpublished data.
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FIG. 2. Nucleotide sequence and translation of TRG-22. The nucleotide sequence corresponding to TRG-22 (Fig. 1) is shown with the appropriate translation below the sequence. Nucleotides 1064 and 1065, which are separated by an intron in the GPT gene, are denoted with asterisks, and the sequences that flank the initiator methionine and potentially form a stem-loop are denoted with crosses. Possible membrane-spanning regions are underlined, consensus dolichol-recognition sites are cross-hatched, and consensus asparagine glycosylation sites are in boldface type.

FIG. 3. Working model of the structure of hamster GPT. The model, with 10 possible membrane-spanning regions, is based on the sequence presented in Fig. 2. In the vertical dimension the model is drawn to scale. Diamonds indicate consensus N-linked glycosylation sites. Consensus dolichol-recognition sites are cross-hatched. Darkened areas represent sequences of 10 or more residues with at least 60% identity to yeast GPT, as identified with the "Homology" algorithm of Microgenie, Beckman Corp.

gest that GPT recognizes the saturated α-isopenyl unit of dolichol phosphate as well as other isoprenyl units in the molecule (19-22). A comparison of sequences of three yeast enzymes known to interact with dolichol phosphate or its derivatives (GPT, encoded by the ALG7 gene; the mannosyltransferase that synthesizes Man1GlcNAcz-P-P-dolichol, encoded by the ALG1 gene; and mannose-P-dolichol synthase, encoded by the DPM1 gene) revealed a 13-residue consensus sequence that was found in the putative membrane-spanning regions of these proteins (Fig. 4) (23). To determine whether this sequence was conserved between mammals and yeasts, the sequence of hamster GPT was searched. As shown in Fig. 4, two such sequences were found at residues 67-79 and 222-234. This not only demonstrated that the sequence was conserved among eukaryotes, but it also allowed a partial refinement of the consensus. Thus, position 3 may have isoleucine as well as valine, and positions 5 and 12 may have tyrosine as well as phenylalanine. Positions 2 and 10 favor phenylalanine, position 9 favors proline, and position 8 must be isoleucine. Moreover, positions 1 and 13 do not appear to be conserved.
Fig. 5. Alignment of hamster and yeast GPT. Hamster GPT (H) and yeast GPT (Y) were aligned with the algorithm GAP with default parameters (University of Wisconsin Genetics Computer Group Sequence Analysis Software Package). GAP introduces gaps (dotted lines) to create an alignment. Identical residues were found at a frequency of 42%. A vertical line indicates identity (value = 1.0), a pair of dots indicates strong similarity (0.5 or greater), and a single dot involves weak identity (0.1 or greater). Consensus dolichol-recognition sites in the hamster sequence are in boldface type and overlined.

Reducing the actual consensus to 11 residues. Note that the yeast sequence corresponding to the second peptide found in hamster GPT diverges from the consensus. The residues found at positions 5 (valine) and 12 (glycine) are phenylalanine or tyrosine in all other cases (Fig. 5), so the possible function of this portion of yeast GPT is unclear. Interestingly, the protein encoded by the yeast SECS9 gene, which is probably involved in assembly of dolichol-linked oligosaccharides and is likely to interact with a dolichol phosphate derivative (24), contains a peptide that matched the original consensus weakly (23) but matches the refined consensus strongly. Thus, at least one copy of this peptide can be found in all known sequences of eukaryotic enzymes that interact with dolichol phosphate or its derivatives.

Similarity to GPT from Other Sources—A search of the NBRF protein data base (release 21.0) revealed that only the yeast ALG7 gene product (25) had significant sequence similarity with hamster GPT. This finding was expected based on previous experiments with the ALG7 gene (5). An alignment of the two sequences is shown in Fig. 5, and Fig. 3 displays segments of hamster GPT of 10 or more residues that have at least 60% identity with corresponding segments of yeast GPT. It is clear that much of the identity is located in hydrophobic sequences 4, 5, 8, and 9. However, except for the possible dolichol-recognition sites, segments 2 and 7 have little similarity. The putative loops between hydrophobic segments 3/4, 5/6, 7/8, and much of the loop between segments 9/10 are also conserved.

A partial cDNA clone for GPT from rat liver has also been sequenced. The deduced amino acid sequence, which corresponds to residues 132-408 of the hamster sequence, has 94% identity with hamster GPT indicating high conservation among rodents.

Features of the mRNA—In addition to the open reading frame, the GPT clones examined (Fig. 1) have at least 147 nucleotides of 5'-untranslated sequence and either 0.46 or 0.75 kilobases of 3'-untranslated sequence. This variability of the 3'-untranslated region is in accord with the size heterogeneity of hamster GPT mRNA observed earlier (5). By comparison with sequence from a genomic DNA clone (5), we have identified one intron between nucleotides 1064 and 1065 (Fig. 2). No other unusual features have been identified in the mRNA, except for a possible stem-loop structure that includes the initiator methionine (Fig. 2). The stability of this hairpin, calculated to be −19.4 kcal/mol, was sufficient to cause considerable interference with T7 DNA polymerase during sequencing reactions (data not shown), and thus has the potential to affect translation of the GPT mRNA.

Expression of cDNA Encoding GPT in COS-1 Cells and Characterization of the Reaction Product—To confirm that the cDNA clone encoded the entire enzyme, the coding sequence was placed in a eukaryotic expression vector under the control of the cytomegalovirus promoter (8) as described under "Experimental Procedures." This vector contains the SV40 origin of replication and thus is suitable for transient expression in COS-1 cells. Table I shows the relative GPT activity in COS-1 membranes after sham transfection, after transfection with vector alone, or after transfection with vector containing GPT. Compared with controls, transfection with GPT cDNA routinely increased the specific activity by 5-7-fold, and in all cases the activity was inhibited by at least 96% by 200 ng/ml tunicamycin. Assuming that only about 10% of cells were actually transfected in the transient assays, this result indicates that the membranes from transfected cells may have actually had 50-70-fold higher GPT activity. The elevated activity was linear for up to 30 min of incubation and was proportional to the amount of membranes added, up to 40 μg of protein/assay (data not shown).

Upon addition of 1.0 μg/ml exogenous dolichol phosphate, the activities in both the vector-only and cDNA-transfected membranes doubled. However, while higher dolichol phosphate concentrations failed to give further enhancement with the vector-transfected COS-1 cells, the activity in cDNA-transfected membranes increased linearly up to the highest concentration tested (4.0 μg/ml dolichol phosphate, data not shown). This indicates that in the membranes derived from the fraction of cells that actually assimilated cDNA, the increased activity could be attributed to overexpression of the enzyme rather than an overabundance of endogenous dolichol phosphate. Since the transfection experiments yielded a heterogeneous mixture of membranes, the

| DNA transfected | Tunicamycin | GPT activity |
|-----------------|------------|-------------|
| None            | −          | cpm         |
| Vector (10 μg)  | −          | 327         |
| Vector (10 μg)  | +          | 152         |
| Vector (20 μg)  | +          | 382         |
| TRG-22 (5 μg)   | +          | 2640        |
| TRG-22 (5 μg)   | +          | 56          |
| TRG-22 (10 μg)  | +          | 3114        |
| TRG-22 (10 μg)  | +          | 51          |
| TRG-22 (20 μg)  | +          | 102         |

3 S. Khounlo and M. Lehrman, unpublished data.
bulk containing a normal amount of the COS-1 (simian) enzyme and smaller amounts of membranes containing the simian enzyme and elevated amounts of the hamster enzyme, more detailed kinetic analysis of the transfected enzyme was not performed. Stable overexpression in CHO cells will be required for such studies.

To verify that the increased activity was due to formation of GlcNAc-P-P-dolichol, the products of the in vitro reactions were subjected to three types of analyses. First, the products from membranes of both vector and cDNA-transfected COS cells co-eluted with GlcNAc-P-P-dolichol from DEAE-cellulose with 20 mM ammonium acetate, indicating the presence of a pyrophosphate bond. Second, after weak acid hydrolysis, 97% of the material comigrated with authentic GlcNAc and ahead of GlcNAc.

To verify that the increased activity was due to formation of GlcNAc-P-P-dolichol from DEAE-cellulose on descending paper chromatography, indicating that a single sugar residue was transferred (Fig. 6). The remaining peak of radioactivity migrating behind GlcNAc was not characterized further. Third, the major reaction products (83 and 72% of the radiolabel from cDNA and vector-transfected membranes, respectively) comigrated on silicic acid paper with $R_f = 0.50$, corresponding to GlcNAc-P-P-dolichol. For comparison, a mannose-P-dolichol standard migrated with $R_f = 0.47$. Several other minor products ($R_f = 0.10, 0.20,$ and 0.48) were detected at similar levels in both preparations but were not characterized further (data not shown). In contrast to GPT, the activities of the mannose-P-dolichol and glucose-P-dolichol synthases were found to be unchanged in membranes from control or cDNA-transfected COS-1 cells. However, these two assays must be interpreted with caution since the majority of the activity detected was probably derived from membranes of the fraction of cells that did not assimilate cDNA.

Evidence That the cDNA Encodes the Catalytic Moiety of GPT—Although overexpression of the cDNA or amplification of the gene (5) clearly result in enhanced GPT activity, this does not prove that the cDNA and gene encode the enzyme. It is possible, for example, that the cDNA could encode an allosteric subunit of the enzyme. However, several features of the cDNA support the conclusion that it encodes the catalytic moiety of GPT. First, the predicted molecular weight of the protein is very close to the size of the two major polypeptides of purified bovine GPT (46 and 50 kDa) (4). Second, the proteins encoded by the DPM1, ALG1, and ALG7 genes, which interact with dolichol phosphate or its derivatives, hamster GPT contains putative dolichol recognition sites. Third, overexpression of this protein in TnR-32 cells appeared to result in buffering of tunicamycin since the apparent $K_I$ of tunicamycin for GPT in vitro varied with the level of overexpression and the quantity of membranes in the assay (5). Since tunicamycin is a bi-substrate analog which is specific for GPT, this result suggests that the protein which was overexpressed in TnR-32 cells was the catalytic entity.

It remains to be established whether the catalytic site of GPT faces the ER lumen or the cytoplasm. If it faces the lumen, an additional possibility is that the cloned protein is not the enzyme but plays a role in transporting UDP-GlcNAc into the lumen. Thus, it could indirectly increase the activity of the endogenous enzyme by raising luminal substrate concentrations. If this were the case, the activity in cDNA-transfected membranes should be much more sensitive to membrane disruption than in the control membranes, and as the degree of disruption is increased the ratio of the activity in the cDNA transfected membranes to that in the control membranes should approach unity. However, membranes iso-
lated from transfected cells and then treated with Nonidet P-40, deoxycholate (Fig. 7), or toluene (data not shown) to disrupt membranes (26) maintained their enhanced activity relative to membranes from vector-transfected cells. This is consistent with the idea that the cloned protein does not require an intact permeability barrier to increase enzymatic activity, and that it does not act by concentrating UDP-GlcNAc into the lumen of microsomal vesicles as previously demonstrated for both ER and Golgi UDP-GlcNAc transporters (27, 28). The decrease of GPT activity observed with mannose-P-dolichol and GlcNAc-P-P-dolichol, respectively, and GPT catalyze similar reactions involving dolichol phosphate. Furthermore, if the catalytic site of GPT faces the lumen, this could also be due inhibition of net UDP-GlcNAc import by the endogenous transport apparatus, which would affect membranes with normal or high levels of enzyme to similar extents.

It is intriguing that mannose-P-dolichol synthase and GPT catalyze similar reactions involving dolichol phosphate and nucleotide diphosphosugars, i.e., the formation of mannose-P-dolichol and GlcNAc-P-P-dolichol, respectively, their structures are quite different. First, it is not clear why hamster GPT should need two dolichol recognition sequences, since GPT and mannose-P-dolichol synthase from Saccharomyces cerevisiae have only one (23, 31). Perhaps one of these recognition sequences is responsible for interaction of hamster GPT with mannose P-dolichol, which activates the mammalian enzyme in vitro (4, 12, 32, 33) and possibly in vivo (34). Second, while the sequences of yeast and hamster GPT indicate that they may span the membrane several times, this does not appear to be the case for yeast mannose-P-dolichol synthase (31). The conservation of several of these putative membrane-spanning regions indicates an important biochemical function. Further studies will be required to determine whether these portions of the protein play a direct role in the GPT reaction or whether GPT could be a protein with two separate functions.

In summary, we report here on the cloning and expression of a full length cDNA encoding hamster N-acetylgalactosamine-1-phosphate transferase, which catalyzes the committed reaction for dolichol-P-Oligosaccharide biosynthesis. To our knowledge, this is the first enzyme of dolichol-oligosaccharide biosynthesis to be cloned from a vertebrate source. The isolation of a functional cDNA clone will make possible a direct analysis of the topological orientation of the catalytic site, to define the location of the early steps of oligosaccharide assembly, as well as analysis of the interactions of the enzyme with dolichol phosphate, UDP-GlcNAc, and the putative regulator mannose-P-dolichol.

Acknowledgments—We thank Dr. David Russell for supplying the pCMV-2 vector and advising us on its use, Dr. Joel Goodman for preparing Fig. 3, and Dr. Yucheng Zeng and Laura Camp for their helpful discussions and critical reading of the manuscript. The manuscript was prepared with the skillful assistance of Diane Cali.

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