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Both Potential Dolichol Recognition Sequences Of Hamster GlcNAc-1-phosphate Transferase Are Necessary For Normal Enzyme Function*

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A potential dolichol recognition sequence (PDRS) with the 11-residue consensus F-I/V-*X*-F/Y-*X*-*X*-I-P-F-*X*-F/Y can be found in each of five microsomal enzymes that interact with dolichol or a dolichol derivative. However, no direct evidence demonstrating a role for the PDRSs has been reported. Hamster UDP-GlcNAc:dolichol phosphate *N*-acetylglucosamine-1-phosphate transferase (GPT) differs from the other enzymes by having two PDRSs.

Stable CHO-K1 transfectants were created that expressed elevated amounts of normal GPT, GPT with a scramble mutation at the first PDRS (nearest the amino terminus), or GPT with a triple alanine-replacement mutation at the second PDRS. The mutant enzymes had no detectable catalytic activity *in vivo*, but were fully capable of conferring cellular resistance to the GPT inhibitor tunicamycin. *In vitro* studies with membrane preparations confirmed that the mutant enzymes were catalytically inactive and also showed that their recovery in microsomes was diminished compared with normal enzyme.

These data demonstrate that each PDRS of hamster GPT is necessary for normal enzyme function. The implications of these data for possible roles of the PDRSs are discussed.

The polyisoprenoid lipid dolichol plays important roles as both a coenzyme and a carrier in many eukaryotic glycosyltransferase reactions (1). In its monophosphorylated form, dolichol can act as a coenzyme, accepting a sugar residue from a nucleotide sugar and then donating the sugar to an appropriate acceptor with concomitant recovery of dolichol monophosphate (Dol-P).[1] This includes the synthesis of dolichol-linked oligosaccharides and glycosylphosphatidylinositol anchor precursors in most eukaryotes (1, 2), and *O*-linked polymannose chains in yeast (3). In the formation of dolichol-linked oligosaccharides, dolichol pyrophosphate acts as a carrier during the assembly of the glycoconjugate prior to its transfer to acceptor polypeptide. In all dolichols the double bond closest to the "business end" of the lipid, *i.e.* the α isoprene unit, is reduced, and this reduction is necessary for efficient coenzyme activity (4, 5).

A potential advantage of dolichol coenzymes and carriers is that the polyisoprene chain may play an active role in promoting interactions with enzymes. Along these lines, Albright and co-workers (6) have described a potential dolichol recognition sequence (PDRS) found in potential membrane-spanning regions of four yeast enzymes, encoded by the genes *ALG1* (mannosyltransferase I), *ALG7* (GlcNAc-1-phosphate transferase), *DPM1* (mannose-P-dolichol synthase), and *SEC59* (dolichol kinase) (6, 7), that interact with dolichol or a dolichol derivative. Their observation was extended to mammalian enzymes upon the cloning and sequencing of the hamster UDP-GlcNAc:dolichol phosphate GlcNAc-1-phosphate transferase (GPT), which reacts with UDP-GlcNAc and Dol-P to form GlcNAc-P-P-dolichol (8–10). Surprisingly, GPT had two copies of the PDRS, whereas the four yeast enzymes each had a single copy (8). Upon the availability of the mammalian GPT sequence (8, 11), a modification of the original consensus (6) was suggested as shown here.

F I/V *X* F/Y *X* *X* I P F *X* F/Y

1 2 3 4 5 6 7 8 9 10 11

SEQUENCE 1

The residues specified by "*X*" are not specific, but are limited to those that are permissible in transmembrane regions. These positions tend to be void of glycine and alanine residues (12). All PDRSs match at least 5 of the 7 specified residues, and all match at residues 4, 7, and 11 (11). It should be noted that the I/V and F/Y substitutions are among the most conservative amino acid replacements that have been described (13).

No direct evidence, however, has been presented demonstrating a functional role for the PDRSs. In addition to dolichol recognition, it is possible that they could be involved in some other common feature of these enzymes, such as retention in the endoplasmic reticulum or perhaps enzyme complex formation. Alternatively, the PDRSs may be nonfunctional evolutionary remnants found in members of this enzyme family. In this report, we examined both PDRSs of hamster GPT (termed PDRS-1 and PDRS-2) by site-directed mutagenesis. The data indicate that these sequences are important and that each mutation diminishes the enzyme's catalytic activity, but not its ability to confer cellular resistance to an inhibitory substrate analogue, tunicamycin.

EXPERIMENTAL PROCEDURES

Reagents

UDP-[^{3}H]*N*-acetylglucosamine (16.9 Ci/mmol) and [2-^{3}H]mannose (23.0 Ci/mmol) were from Du Pont. All reagents used for routine

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[1] The abbreviations used are: Dol-P, dolichol monophosphate; PDRS, potential dolichol recognition sequence; Tn, tunicamycin; GPT, UDP-GlcNAc:dolichol phosphate *N*-acetylglucosamine-1-phosphate transferase; CHO, Chinese hamster ovary; HPLC, high pressure liquid chromatography.

cell culture, including Ham's F-12 medium, sera, antibiotics, and G418 sulfate (Geneticin) were from GIBCO/BRL. Tunicamycin (catalog no. T7765), concanavalin A, and dolichol monophosphate grade III were from Sigma. Protein-grade Nonidet P-40 (catalog no. 492017) was from Calbiochem. Swainsonine was from Toronto Research Chemicals. Enhanced chemiluminescence (ECL) kits were from Amersham Corp. All other reagents were from reliable commercial sources.

Plasmids and Mutagenesis

Mutant PDRS-1/Sc—Mutagenesis was performed with the Muta-Gene kit, version 2, from Bio-Rad. A 1.6-kilobase pair *Eco*RI-*Pst*I fragment that included the entire coding region of hamster GlcNAc-1-phosphate transferase (GenBank/EMBL accession no. J05590) (8) was subcloned into the mutagenesis phagemid vector pTZ18U. Sequence analysis of this construct indicated a spurious mutation, differing from the sequence reported earlier (8), changing the nucleotide at position 944 from C to G and amino acid residue 266 from Gly to Ala. The "normal" unmutagenized cDNA used in this study also had this change, and separate studies demonstrated that this change had no apparent effect on the activity of GPT (data not shown). After conversion to single-stranded phage DNA in the sense orientation (cDNA in the anti-sense orientation appeared unstable) with M13K07 helper phage, mutagenesis was performed with the oligonucleotide 5′-ACAAAGCAGTTCAGGGGGAAGGGGAAGAAGATGAAGCA-GATGAGGATAAGGAAAACAGC-3′, corresponding to nucleotides 337–395 of the published sequence (8) under the conditions recommended by the manufacturer, to create the mutation termed PDRS-1/Sc. The nucleotide sequence of the entire coding region of this cDNA was confirmed by standard sequencing methods (Sequenase version 2.0, U. S. Biochemical Corp.).

Mutant PDRS-2/4,7,11A—We took advantage of unique restriction sites (*Bcl*I at nucleotides 804–809 and *Sty*I at nucleotides 851–856) that conveniently flanked PDRS-2 (nucleotides 814–847) in hamster GPT. The plasmid was propagated in a Dam-negative strain of *Escherichia coli* (GM2163; New England Biolabs) to eliminate inhibitory methylation at the *Bcl*I site. A pair of complementary oligonucleotides: 5′-GATCATGTCTTTTCCCTCGCGTTCATGG-CACCATTTTTTGCTACCAC-3′, corresponding to nucleotides 805–851; and 5′-CAAGGTGGTAGCAAAAAATGGTGCCATGAACGC-GAGGGAAAAGACAT-3′, corresponding to nucleotides 855–809, containing cohesive ends for these restriction sites and the desired amino acid changes were synthesized. The double-stranded oligomer pair was ligated into GPT at these sites by standard methods (14). The mutagenized region was confirmed by DNA sequencing.

For all transfection experiments, 1.6-kilobase pair cDNA fragments (*Eco*RI-*Hin*dIII, the latter site acquired from the polylinker of the pTZ18U vector) were subcloned into the expression vector pJB20 (10), which contains a selectable marker for resistance to G418 sulfate. The part of the 3′-untranslated region lost in these constructions (nucleotides 1618–1837) was not necessary for GPT expression (data not shown).

Cell Culture and Transfection Methods

COS-1 and CHO-K1 cells were obtained from ATCC and grown at 37 °C in a humidified 5% CO_2 incubator. COS-1 cells were routinely cultured in Dulbecco's modified Eagle's medium buffered at pH 7.5 with 20 mM Na-HEPES, and containing 10% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin. CHO-K1 cells were routinely cultured in Ham's F-12 medium buffered at pH 7.3 with 20 mM Na-HEPES, and containing 2% fetal bovine serum, 8% calf serum, 100 units/ml penicillin, and 100 μg/ml streptomycin. However, 10% fetal bovine serum with no calf serum was used for tunicamycin and concanavalin A/swainsonine toxicity experiments (Figs. 1 and 3).

Transient transfection with COS-1 cells was performed by plating 1–2 × 10^6 cells/100-mm dish and culturing for 16–24 h before transfection by the DEAE-dextran method (14) using 2.2 μg of plasmid/dish. Cells were cultured for 48 h before harvesting.

Stable transfection with CHO-K1 cells was performed by plating 0.5–1.0 × 10^6 cells/100-mm dish and culturing for 20–24 h before transfection by the calcium phosphate method (14) using 2.0 μg of plasmid/dish. During transfection, a 1:1 mixture of Dulbecco's modified Eagle's medium and Ham's F-12 medium was used. Stable transfectants were selected with 1 mg/ml G418 sulfate in the medium for at least 2 weeks. Where indicated, colonies of transfectants were either pooled or isolated and subcloned by limiting dilution. Five subcloned transfectants were used extensively in this study: CHO-v (vector), CHO-n.1 and CHO-n.2 (normal GPT), CHO-mo1.3 (PDRS-1/Sc), and CHO-mo4.9 (PDRS-2/4, 7, 11A). The last four were resistant to 2–4 μg/ml Tn, 16–32-fold higher than CHO-v.

Tn-resistant Transfectants

Tn was suspended at 1 mg/ml in 20 mM Tris-Cl, pH 9.5, and 1 N NaOH was added dropwise until the Tn dissolved. Tn-resistant CHO-K1 transfectants were obtained by adding 1 mg/ml G418 plus 1 μg/ml Tn to dishes 24 h after transfection. Identically transfected dishes were selected with 1 mg/ml G418 sulfate alone to determine transfection efficiency (approximately 200 colonies/transfection) and the percentage of transfectants that were resistant to Tn. Whereas no colonies resistant to 1 μg/ml Tn were obtained with the vector alone, the normal, PDRS-1/Sc, and PDRS-2/4,7,11A constructs yielded Tn-resistant colonies at frequencies of 15, 3, and 6%, respectively.

Cellular resistance to Tn was estimated as described earlier by plating cells in media containing different concentrations of Tn and staining colonies after 7 days of growth (15). The four Tn-resistant subcloned transfectants were periodically rechecked to ensure that they retained their resistance to Tn and G418. Resistance to Tn in *in vitro* assays was estimated as an IC_{50} value as described previously (10).

Detection of GPT Activity, Protein, and mRNA

Preparation of microsomal membranes from transfected cells (15), GPT enzyme assays (15), and immunoblotting of GPT with an antibody against a synthetic peptide representing amino acid residues 42–56 of GPT (10) was performed essentially as described earlier. GPT mRNA was detected by Northern blot analysis as described earlier (15), except that Pall A membranes were used and total RNA was prepared by acid-phenol extraction (16).

Evaluation of GPT Activity in Vivo by Concanavalin A/Swainsonine Toxicity

As described in the text, GPT activity in cells was evaluated by determining the effects of various constructs on the swainsonine-dependent change of concanavalin A toxicity (10, 17). Briefly, 100 cells were plated in multiple wells of a 48-well plate in Ham's F-12 medium containing 10% fetal bovine serum, and various concentrations of concanavalin A in the absence or presence of 2 μg/ml swainsonine. Colonies were fixed and stained after 7 days of growth. In cells with no alteration of GPT activity, addition of swainsonine decreased by 4-fold the concentration of concanavalin A necessary to prevent growth; cells that had elevated GPT activity exhibited no change.

High Pressure Liquid Chromatography

Preparation of dolichol-linked oligosaccharides and HPLC with a silica column was performed exactly as described earlier (10, 18).

RESULTS

Design of PDRS Mutants—PDRS-1 and PDRS-2 of hamster GPT, found at residues 68–78 and 223–233, respectively, fall within possible membrane-spanning segments and have the expected high content of apolar residues (8). PDRS-1 corresponds to the single PDRS found in the *ALG7* gene, which encodes GPT from *Saccharomyces cerevisiae*, and the sequences flanking these two PDRSs are poorly conserved (11).

By standard methods, either a "scramble" mutation termed PDRS-1/Sc or a triple alanine replacement termed PDRS-2/4,7,11A was introduced into hamster GPT cDNA (Table I). PDRS-1/Sc was designed by moving amino acid residues by one position upstream or downstream so as to destroy agreement with the consensus, with minimal effects on composition, hydrophobicity, and structure as judged by computer-assisted analysis (Microgenie, Beckman Corp.) of the mutant sequence. The PDRS-2/4,7,11A mutation was designed by simultaneously replacing the 3 most highly conserved residues of the PDRS consensus (tyrosine at position 4, isoleucine at position 7, and phenylalanine at position 11) with alanine.

TABLE I

The PDRS mutations and their effects on GPT activity in microsomes from transient and stable transfectants

The sequences of PDRS-1 and PDRS-2 in normal GPT (with consensus positions underlined) and in the PDRS mutants are shown with corresponding GPT activities (averages of duplicates) from microsomes of either transiently transfected COS-1 cells or pooled (approximately 200 colonies) stably transfected CHO-K1 cells. Assays included 20 μg of membrane protein and 5 μg of Dol-P, added as described for Fig. 5. ND, not determined.

Construct	Sequence of residues		GPT activity of transfectants[a]	
	68–78	223–233	COS-1	CHO-K1, pooled[b]
Vector			422 (± 27)	492 (± 27)
Normal GPT	IILFCFIPFPF	FSLYFMIPFFF	1280 (± 99)	5633 (± 32)
PDRS-1/Sc	ILICFIFFPFP	Normal	487 (± 67)	699 (± 37)
PDRS-2/4,7,11A	Normal	FSLAFMAPFFA	ND	785 (± 5)

[a] cpm per 20 μg of membrane protein in standard assay.
[b] All pooled after selection with 1 mg/ml G418 sulfate and, except for vector, 1 μg/ml tunicamycin.

Preliminary Analysis of PDRS Mutants in Transient COS-1 and Pooled, Stable CHO-K1 Transfectants, and Isolation of Tunicamycin-resistant CHO-K1 Transfectants—For an initial assessment, the normal and PDRS-1 mutant forms of GPT were ligated into the expression vector pJB20 (10) and used for transient expression with COS-1 cells. Assay of GPT activity in microsomes revealed that normal GPT cDNA caused a 2-fold stimulation of activity over the endogenous activity, but that no increase was observed with the PDRS-1 mutant or the vector alone (Table I). The normal GPT, PDRS-1/Sc, and PDRS-2/4,7,11A constructs were then used to create stable transfectants in CHO-K1 cells by ligating into pJB20, transfecting, and selecting for expression of the G418 resistance marker with 1 mg/ml G418 sulfate.

For the following reason, 1 μg/ml Tn (an inhibitory substrate analogue for GPT) was also included as a selective agent for some of the transfections. When overexpressed in CHO cells, GPT is thought to confer resistance to Tn by a compensatory increase of activity (10, 15). However, every 4-fold increase of cellular resistance is accompanied by only a 1-fold increase of microsomal enzyme activity (10, 15), and indirect immunofluorescence microscopy of overexpressing transfectants reveals that GPT can be found in the nuclear envelope as well as the endoplasmic reticulum (Ref. 10 and data not shown). Thus, a large fraction of the transfected enzyme probably does not contribute to cellular *N*-acetylglucosamine-P-P-dolichol biosynthesis, although it may contribute to Tn resistance by binding to the inhibitor. Since Tn acts primarily as an analogue of the nucleotide sugar donor, UDP-GlcNAc, it was hypothesized that a mutation that prevented the interaction between Dol-P and GPT would not prevent the mutated enzyme from binding to Tn and conferring resistance.

As described under "Experimental Procedures," Tn-resistant transfectants could be obtained with normal GPT, PDRS-1/Sc, and PDRS-2/4,7,11A, but not with the vector itself or with sham-transfected CHO cells. Initially, pooled CHO transfectants selected with 1 μg/ml Tn were used and gave results qualitatively similar to those obtained with COS-1 cells; compared with normal cDNA there was no significant activity associated with either the PDRS-1 or PDRS-2 mutation (Table I).

Since the experiments with COS-1 and pooled CHO-K1 cells did not permit a detailed analysis of the PDRS defects, normal and PDRS mutant transfectants that were resistant to 2–4 μg/ml Tn (16–32-fold higher than vector-transfected controls) were identified (Fig. 1) and subcloned by limiting dilution. Northern blot analysis indicated that these PDRS mutants expressed roughly twice as much mRNA as normal

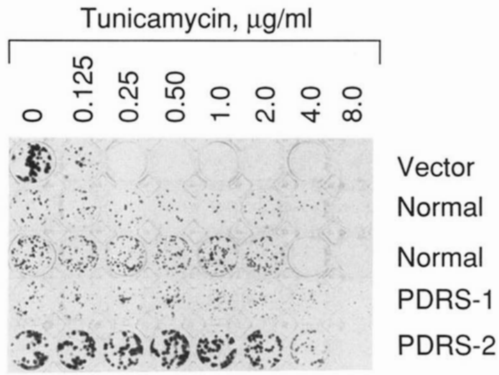

FIG. 1. **Resistance of GPT transfectants to tunicamycin.** After subcloning by limiting dilution, transfectants were plated (100 cells/well) in media containing varying concentrations of tunicamycin and stained after 7 days of growth as described under "Experimental Procedures." Shown in order are a vector transfectant (CHO-v), two normal GPT transfectants (CHO-n.1 and CHO-n.2), a PDRS-1/Sc transfectant (CHO-mo1.3), and a PDRS-2/4,7,11A transfectant (CHO-mo4.9).

GPT transfectants having similar resistance to Tn (data not shown).

The PDRS-1/Sc and PDRS-2/4,7,11A Mutations Decrease GPT Enzymatic Activity in Vivo—In preliminary experiments, we were unable to directly measure the synthesis of *N*-acetylglucosamine-P-P-dolichol in the CHO transfectants by metabolic labeling with 0.25 mCi/ml [^{3}H]glucosamine. As an alternative, we took advantage of the disruption of dolichol-linked oligosaccharide biosynthesis that occurs when GPT is overexpressed in Tn-resistant transfectants. This has been attributed to depletion of the Dol-P pool causing inhibition of mannose-P-dolichol biosynthesis (10, 19). The accumulation of truncated dolichol-linked oligosaccharides was determined by metabolic labeling with [^{3}H]mannose followed by HPLC analysis. Each of the transfectants shown in Fig. 1 was examined (Fig. 2). Vector control (*panel A*), PDRS-1/Sc (*panel D*), and PDRS-2/4,7,11A (*panel E*) transfectants gave ordinary patterns exhibiting primarily $Glc_3Man_9GlcNAc_2$ oligosaccharides, whereas two normal GPT transfectants (*panels B* and *C*) gave patterns with mostly $Man_5GlcNAc_2$ oligosaccharides. Thus, the PDRS mutants had no detectable activity by this criterion. To determine the sensitivity of this method, a similar analysis was performed with a normal transfectant isolated in an earlier study, Tn-5 (10), that had 4-fold lower Tn resistance than the transfectants of *panels B–E* (data not shown). Tn-5 gave an abnormal oligosaccharide pattern (*panel F*) with an unusually high proportion of oligosaccharides eluting as $Man_9GlcNAc_2$. Thus, the limit of

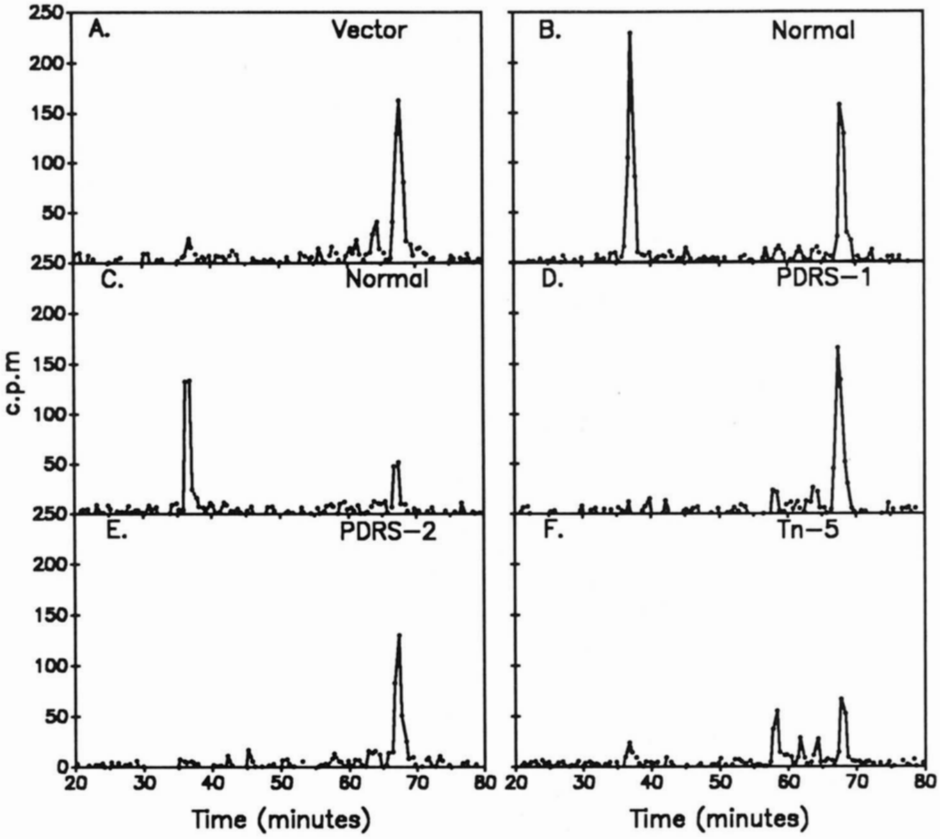

FIG. 2. **HPLC of dolichol-linked oligosaccharides.** *Panels A–E* show, in order, the same transfectants as described for Fig. 1. *Panel F* shows Tn-5, a normal transfectant isolated previously (10) with 4-fold lower Tn resistance than those in *panels B–E*. Labeling of cells with 1 mCi/ml [^{3}H]mannose and preparation of dolichol-linked oligosaccharides was performed as described previously (10). Using an ISCO/Chemresearch dual pump system and a Radiomatic A140 flow detector, HPLC was performed at a flow rate of 1 ml/min using an Alltech silica column (18) with slight modifications (25). Based on comparisons with oligosaccharides produced by wild-type and mutant CHO cells with known defects (17, 26), the peaks eluting at 37, 58, and 68 min were $Man_5GlcNAc_2$, $Man_9GlcNAc_2$, and $Glc_3Man_9GlcNAc_2$, respectively. Between 3,000 and 9,000 cpm of each sample was loaded. The flow cell contained 0.33 ml of eluate, and radioactivity was measured for every 10-s interval. Every third interval (2/min) was plotted. Integration with Chemresearch software revealed that the ratios of $Man_5GlcNAc_2$ to $Glc_3Man_9GlcNAc_2$ in *panels A–E* were 0.21, 1.30, 2.10, 0.06, and 0.05, respectively.

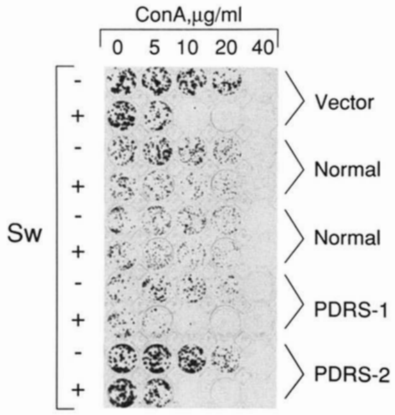

FIG. 3. **Resistance of GPT transfectants to swainsonine/concanavalin A toxicity.** This analysis was performed as described under "Experimental Procedures." Shown in order are the same transfectants as described for Fig. 1.

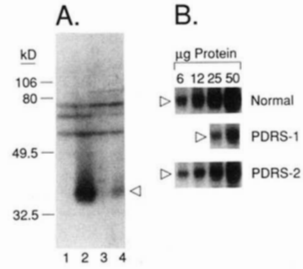

FIG. 4. **Immunoblotting of membranes from transfected CHO cells expressing normal GPT or PDRS mutant GPT.** *Panel A*, 50-μg aliquots of microsomal membranes from CHO cells transfected with vector (*lane 1*; CHO-v), normal GPT (*lane 2*; CHO-n.1), the PDRS-1/Sc mutant (*lane 3*; CHO-mo1.3), or the PDRS-2/4,7,11A mutant (*lane 4*; CHO-mo4.9) were analyzed by SDS-polyacrylamide gel electrophoresis, immunoblotting with an anti-peptide antibody specific for residues 42–56 of GPT (10), and detection by the enhanced chemiluminescence (ECL) method (Amersham). The *open arrowheads* in both panels indicate GPT. The positions of prestained standards (Bio-Rad) with their effective molecular weights are shown (phosphorylase *b*, 106 kDa; bovine serum albumin, 80 kDa; ovalbumin, 49.5 kDa; carbonic anhydrase, 32.5 kDa). *Panel B*, lanes containing varying amounts of normal or PDRS mutant samples are shown to allow estimation of the relative abundance of GPT protein.

sensitivity of this method is no higher than 25% of normal activity.

Since the accumulation of $Man_5GlcNAc_2$ oligosaccharides during metabolic labeling experiments can arise artifactually (20), we sought to confirm this result by another means. These truncated species lack the 2 mannose residues normally removed by Golgi mannosidase II. Their presence promotes a bypass that prevents the mannosidase II inhibitor swainsonine from enhancing the toxic effects of concanavalin A; cells with mature oligosaccharides are sensitive to the effects of swainsonine (10, 17). As shown in Fig. 3, the two independent normal GPT transfectants exhibited complete resistance to swainsonine, whereas the two PDRS mutants and the vector control transfectants were equally sensitive to this alkaloid.

Properties of the PDRS Mutant Enzymes in Vitro—As shown in Fig. 4, GPT enzyme was detected by immunoblotting in microsomes from the various Tn-resistant transfectants (*panel A*). By comparison of lanes containing different amounts of protein loaded onto the gel, we estimate that microsomes from the PDRS-1 and PDRS-2 mutants contained roughly 25 and 50%, respectively, of the amount of GPT protein found in microsomes from normal GPT transfectants (*panel B*). Since cellular Tn resistance is proportional to GPT expression (10), it is not clear how the PDRS mutants with less GPT protein could have Tn resistances

similar to those in the normal transfectants (however, see "Discussion").

As shown in Fig. 5*A*, GPT activity in microsomes from the various transfectants was determined in the presence of increasing concentrations of UDP-[^{3}H]GlcNAc. The PDRS-1/Sc and PDRS-2/4,7,11A mutant subclones exhibited essentially no activity compared with that found in the vector-transfected control, whereas the normal GPT subclone had increased expression of 10–20-fold over the vector control. Even with the reduced recovery of PDRS mutant protein in microsomes, activities of 2–10-fold over background should have been observed if these enzymes were fully active. In some experiments the PDRS mutant subclones had a low level of GPT activity, up to 50% over background. We have not been able to determine whether this represented true activity of the mutant enzymes or clonal variation with respect to endogenous GPT. When this experiment was repeated at a fixed concentration of UDP-[^{3}H]GlcNAc in the presence of increasing concentrations of Dol-P (*panel B*), the result was qualitatively similar.

As shown previously (10), the apparent IC_{50} of Tn *in vitro* is shifted to higher values when high concentrations of normal GPT enzyme are present, presumably due to its ability to bind Tn. Similar analyses were performed with the PDRS mutants. In these experiments, the enzyme activities with the vector and mutant transfectants were due to the endogenous enzyme in microsomes, whereas the activities with the normal transfectants reflected the combined endogenous and transfected enzyme activities and were 10–20-fold higher. Measurements of IC_{50} values with the Tn-resistant transfectants revealed a 200-fold shift for normal GPT with respect to the vector control, but only a 2–4-fold shift for the PDRS-2/4,7,11A mutant and a 2-fold shift for the PDRS-1/Sc mutant (data not shown). Although these results with the PDRS mutant membranes were unexpected based upon the *in vivo* data (Fig. 1), they could be explained in part by the lower quantities of the mutant enzymes.

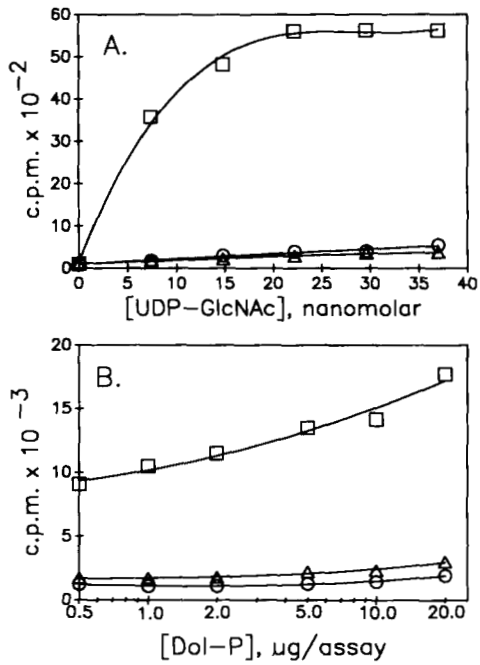

FIG. 5. **GPT enzyme activities of microsomes from stable CHO transfectants expressing normal or PDRS mutant GPT.** Aliquots (20 μg) of microsomal membranes from transfectants were assayed for GPT activity. In both panels, averaged duplicate values for vector control (CHO-v; *circles*), normal (CHO-n.1; *squares*), and the PDRS-1 mutant (CHO-mo1.3; *triangles*) are shown. For clarity, data for the PDRS-2 mutant (CHO-mo4.9), which were superimposable with the PDRS-1 data, were not plotted. *Panel A*, only the endogenous Dol-P of the microsomes was used, and the concentration of UDP-[^{3}H]GlcNAc was varied as shown. *Panel B*, the concentration of UDP-[^{3}H]GlcNAc was fixed at 37 nM, and the concentration of exogenously added Dol-P was varied as shown. Dol-P was added to reaction mixtures as a 1 mg/ml solution in 20 mM Tris-Cl, pH 7.0, containing 0.15 M NaCl and 0.1% Nonidet P-40. The final concentration of Nonidet P-40 in each assay was adjusted to be 0.005%.

DISCUSSION

The aim of this study was to determine whether there was a functional role for the potential dolichol recognition sequences of hamster GPT. These sequences have been found in five enzymes thought to interact with dolichol or a dolichol derivative. The PDRSs were originally described in four yeast enzymes (encoded by the genes *ALG1*, *ALG7*, *SEC59*, and *DPM1*), each having a single copy of the PDRS (6), and the consensus was later modified (8) upon the identification of two copies that were found in hamster GPT. A similar sequence has also been described in the ribophorin I subunit of canine oligosaccharyl transferase (12). Although a reasonable case was presented for a role in the recognition of the polyisoprene chain of dolichol (6), there are a number of other plausible roles for the PDRS. For example, this sequence could be involved in the retention of these enzymes in the endoplasmic reticulum. Another role could involve the formation of multi-enzyme complexes, therefore facilitating the assembly of lipid-linked oligosaccharides and other glycoconjugates that require dolichol derivatives. Alternatively, this sequence could simply be a nonfunctional evolutionary remnant in an enzyme family. In an earlier report (8) we suggested that PDRS-2 could be involved in activation of GPT by mannose-P-dolichol. This now appears unlikely because the GPT activity in CHO Lec15 mutants (mannose-P-dolichol synthase-defective) is unchanged from parental cells (data not shown); according to this hypothesis, the PDRS-2 mutant described here should have remained active.

The results of this study show that both PDRSs of hamster GPT are necessary for full expression of enzyme activity *in vitro* and *in vivo* (Table II). Since the PDRS mutant enzymes were still capable of conferring resistance to Tn *in vivo*, it appears that the mutations did not grossly affect the structure of GPT, although a selective localized change cannot be ruled out. The simplest explanation for these data is that the PDRSs are necessary for binding of Dol-P. Thus, even when the assays were supplemented with exogenous Dol-P, there

TABLE II

Comparison of properties of normal and PDRS-mutant GPT transfectants

Relative values for parameters obtained with subcloned transfectants (as described for Fig. 1) are listed. Values were determined for whole cells or for 100,000 × *g* membrane fractions (memb.) as indicated. Tn resistance and enzyme activity are listed with respect to the value for "vector." mRNA and protein levels are reported with respect to the normal cDNA because the vector levels were too low to determine accurately (ND, not determined).

	mRNA (cells)	Protein (memb.)	Tn resistance		Enzyme activity	
			Cells	Memb.	Cells	Memb.
	-fold	*-fold*	*-fold*		*-fold*	
Transfectant						
Vector	ND	ND	1	1	1	1
Normal	1	1	16	200	>4	10
PDRS-1/Sc	2	0.25	16	2	1	1
PDRS-2/4,7,11A	2	0.5	16	4	1	1

was no activity associated with the PDRS mutants. Since the mutants still bound Tn, their binding sites for nucleotide sugar are most likely intact.

Such an explanation, however, is not completely satisfying because it does not explain the lower recovery of GPT protein from the mutant transfectants that we routinely observed by immunoblotting (Fig. 4), although when compared with the normal transfectants the mutant transfectants had equivalent Tn resistance and slightly higher mRNA levels. One possibility is that in addition to inhibiting catalysis, the PDRS mutations create fragile proteins that are stable inside the cell (and thus able to confer resistance to Tn) but are sensitive to denaturation or degradation upon cell fractionation. Such proteins might also be unable to alter the IC_{50} for Tn *in vitro* and be poorly detected under the specialized denaturation conditions required to detect GPT by immunoblotting (10). Addition of the protease inhibitors phenylmethylsulfonyl fluoride, leupeptin, and aprotinin during membrane fractionation did not improve the immunoblotting (data not shown). Another explanation could involve inhibition of catalysis coupled with mislocalization of the PDRS mutants. Thus, some of the mutant protein would be lost during preparation of microsomes used for immunoblotting. Since Tn would be expected to partition into cellular membranes and continuously be redistributed with the membrane flow, the mislocalized PDRS mutants could still have access to the cellular pool of Tn and protect the endogenous GPT residing in the endoplasmic reticulum. Studies are now in progress to examine these possibilities.

Sequence comparisons for GPTs from different species also make it unclear as to whether the PDRSs are required for binding to Dol-P. Whereas hamster GPT has two PDRSs, GPT from *S. cerevisiae* has one PDRS (6, 8), corresponding to hamster GPT PDRS-1, and computer-assisted analysis of the recently reported sequence of GPT from *Leishmania amazonesis* (21) yielded no apparent PDRS. However, each enzyme is responsible for formation of *N*-acetylglucosamine-P-P-dolichol from UDP-GlcNAc and Dol-P, and each is inhibited by tunicamycin. Although the chain length of *L. amazonesis* dolichol has not yet been reported, the chain length in a related kinetoplast parasite, *Trypanosoma brucei*, is 11–12 isoprene units (22). Since the dolichol chains in *S. cerevisiae* and in rodents have been reported to be 14–16 and 17–19 isoprene units, respectively (23), there appears to be a mild correlation between the length of dolichol in a given species and the number of PDRSs in its enzymes. However, if the PDRSs were involved in binding to the polyisoprene chain, it might be expected that enzymes dealing with shorter chains would require greater numbers of PDRSs to achieve the necessary substrate affinity; Dol-Ps with progressively shorter chain lengths are poorer substrates for yeast GPT (24). In order to clarify this issue, we are currently attempting to isolate less disruptive PDRS mutants that retain a fraction of their normal enzyme activity, to determine whether such mutants have elevated K_m values for Dol-P.

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