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Requirement of the Lec35 Gene for All Known Classes of Monosaccharide-P-Dolichol-dependent Glycosyltransferase Reactions in Mammals

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The Lec35 gene product (Lec35p) is required for utilization of the mannose donor mannose-P-dolichol (MPD) in synthesis of both lipid-linked oligosaccharides (LLOs) and glycosylphosphatidylinositols, which are important for functions such as protein folding and membrane anchoring, respectively. The hamster Lec35 gene is shown to encode the previously identified cDNA SL15, which corrects the Lec35 mutant phenotype and predicts a novel endoplasmic reticulum membrane protein. The mutant hamster alleles Lec35.1 and Lec35.2 are characterized, and the human Lec35 gene (mannose-P-dolichol utilization defect 1) was mapped to 17p12-13. To determine whether Lec35p was required only for MPD-dependent mannosylation of LLO and glycosylphosphatidylinositol intermediates, two additional lipid-mediated reactions were investigated: MPD-dependent C-mannosylation of tryptophanyl residues, and glucose-P-dolichol (GPD)-dependent glucosylation of LLO. Both were found to require Lec35p. In addition, the SL15-encoded protein was selective for MPD compared with GPD, suggesting that an additional GPD-selective Lec35 gene product remains to be identified. The predicted amino acid sequence of Lec35p does not suggest an obvious function or mechanism. By testing the water-soluble MPD analog mannose-β-1-P-citronellol in an in vitro system in which the MPD utilization defect was preserved by permeabilization with streptolysin-O, it was determined that Lec35p is not directly required for the enzymatic transfer of mannose from the donor to the acceptor substrate. These results show that Lec35p has an essential role for all known classes of monosaccharide-P-dolichol-dependent reactions in mammals. The in vitro data suggest that Lec35p controls an aspect of MPD orientation in the endoplasmic reticulum membrane that is crucial for its activity as a donor substrate.

INTRODUCTION

Glycosyltransferases catalyze the transfer of single monosaccharides to a variety of acceptor substrates, and can be divided into two main classes based upon the type of sugar donor. Most glycosyltransferases use nucleotide-sugar donors (Kornfeld and Kornfeld, 1985). Others use polyisoprenol-P-monosaccharide donors (Waechter and Lennarz, 1976). Although such reactions can often be reconstituted in vitro by inclusion of the transferase, the donor, and an appropriate acceptor, these reactions often have additional requirements in vivo due to compartmentalization of the enzymes and substrates. For example, lumenal Golgi apparatus glycosyltransferase reactions can be blocked by the absence of transporters that import specific nucleotide-sugars from the cytoplasm. Similarly, endoplasmic reticulum (ER) reactions that use mannose-P-dolichol (MPD) or glucose-P-dolichol (GPD) are believed to require additional factors to deal with the problem of substrate compartmentalization (Hirschberg and Snider, 1987; Lennarz, 1987).

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Abbreviations used: ER, endoplasmic reticulum; GPD, glucose-P-dolichol; GPI; glycosylphosphatidylinositol; HPLC, high pressure liquid chromatography; LLO, lipid-linked oligosaccharide; MPC, mannose-P-citronellol; MPD, mannose-P-dolichol; MPN, mannose-P-nerol; SLO, streptolysin-O.

These donors are embedded in the ER membrane, and are synthesized from dolichol-P by cytoplasmically oriented ER membrane-associated enzymes that transfer sugar from cytoplasmic GDP-mannose or UDP-glucose. However, the glycosyltransferases that use these sugar-P-dolichol donors, as well as the respective acceptors, are thought to face the ER lumen. Thus, it is likely that additional factors, such as "flippases," are required to reorient the sugar-P-dolichol from the cytoplasmic side to the lumenal side of the ER membrane.

Chinese hamster ovary (CHO-K1) mutants with the recessive Lec35 genotype (originally designated "PIR") can synthesize MPD, but have defects in two MPD-dependent transferase reactions. These mutants were originally isolated due to their inability to transfer mannose from MPD to the lipid-linked oligosaccharide (LLO) $Man_5GlcNAc_2$-P-P-dolichol (Lehrman and Zeng, 1989). Thus, Lec35 mutants were blocked in the first MPD-dependent step in the LLO pathway, because $Man_5GlcNAc_2$-P-P-dolichol synthesis requires only cytoplasmic nucleotide-sugars. The Lec35 mutation prevents the synthesis of $Man_6GlcNAc_2$-P-P-dolichol, all other downstream LLO intermediates, and the mature LLO $Glc_3Man_9GlcNAc_2$-P-P-dolichol. However, MPD, $Man_5GlcNAc_2$-P-P-dolichol, and the mannosyltransferase activities that add the sixth through ninth mannosyl residues are all present in Lec35 mutant cells (Zeng and Lehrman, 1990). The initial MPD-dependent step in glycosylphosphatidylinositol (GPI) synthesis, i.e., the transfer of mannose from MPD to GlcN-(acyl)PI, is also blocked by the Lec35 mutation (Camp *et al.*, 1993). As with the LLO pathway, GPI mannosyltransferase I activity and the acceptor substrate are present in Lec35 cells (DeLuca *et al.*, 1994). Because two distinct pathways (LLO and GPI synthesis) are blocked in Lec35 mutants, a defect in a specific transferase or acceptor is unlikely. Thus, the Lec35 gene may be needed for an aspect of MPD utilization involved in all MPD-dependent reactions. In this report, we examine the effect of the Lec35 mutation on C-mannosylation of tryptophanyl residues, which requires MPD (Doucey *et al.*, 1998), and LLO glucosylation, which uses GPD, to determine whether the Lec35 gene has a general role in sugar-P-dolichol–dependent pathways in mammalian cells in vivo.

To perform these studies it was necessary to identify the Lec35 gene. Data are presented to show that the Lec35 gene corresponds to the cDNA SL15, reported previously to encode a novel ER membrane protein of 247 amino acid residues and to correct the Lec35 phenotype (Ware *et al.*, 1996). Although apparent Lec35 homologs exist in mice, humans, *Drosophila melanogaster*, and *Caenorhabditis elegans*, the predicted amino acid sequence has no similarity to any other protein of known function, and therefore provides no clue as to the mechanism by which Lec35p promotes utilization of MPD. The MPD usage defect in Lec35 mutants is partially corrected in vitro after different methods of physical perturbation, as well as chemical perturbation with detergent or pH = 10 treatment (Zeng and Lehrman, 1990). This phenomenon has hindered development of an in vitro system that preserves the Lec35 phenotype. In this report, we demonstrate that streptolysin-O (SLO) can be used to permeabilize Lec35 cells while preserving the MPD utilization defect. With this in vitro system, a water-soluble analog of MPD (mannose-β-P-citronellol; MPC) was used to examine possible mechanisms of the Lec35 gene product.

MATERIALS AND METHODS

Cell Culture, Transfection, and Recombinant DNA Methods

CHO-K1 cells, Lec35.1 (SwR-100) and Lec35.15 (CsR-1000) cells, which arose spontaneously under gradual selective pressure (Lehrman and Zeng, 1989), and Lec35.2 (Cs-1) cells, which were obtained by chemical mutagenesis (Camp *et al.*, 1993), were cultured in Ham's F-12 with 2% fetal bovine serum/8% calf serum (Atlanta Biologicals, Norcross, GA) as described (Camp *et al.*, 1993) unless indicated otherwise. Lec35.1 cells were cotransfected by the calcium phosphate procedure (Sambrook *et al.*, 1989) with pTet-Off (Clonetech, Palo Alto, CA; to provide a doxycyclin-repressible transcription factor for pTRE) and either the "empty" vector pTRE (Clonetech) or the plasmid pTRE-SL15. To construct pTRE-SL15, pTRE was digested with *Bam*HI and *Xba*I, and ligated with the SL15 (Lec35) insert of pLW4 (homologous to pLW1 [Ware *et al.*, 1996], except that the 5′ UT region was 70 nucleotides (nt) (pLW4) instead of 15 nt [pLW1]), which was excised with *Bam*HI and *Xba*I. Transfectants were selected with medium containing 1 mg/ml G418 for pTet-Off, and PHA-E/swainsonine (Ware *et al.*, 1996) for SL15. Approximately 10 colonies were subcloned by limiting dilution, and then screened for SL15 mRNA expression under repressed conditions (4 d in medium with 10 ng/ml doxycyclin) or induced conditions (medium without doxycyclin), with 10% tetracyline-free fetal bovine serum (Clonetech).

All other molecular biology methods, including Southern and Northern blotting, isolation of genomic DNA and total RNA, and preparation of ^{32}P probes, were standard, such as described (Lehrman *et al.*, 1985; Sambrook *et al.*, 1989). When appropriate, details are given in the text or figure legends.

Fluorescence In Situ Hybridization of the Human Lec35 Gene

Fluorescence in situ hybridization (FISH) analysis with human lymphocytes was performed commercially (SeeDNA Biotech, North York, Ontario, Canada) with IMAGE consortium clone 163003, a human expressed sequence tag homologous to hamster Lec35, as described (Heng *et al.*, 1992). The probe was 1.3 kbp and labeled with biotinylated dATP by using a BioNick kit (Life Technologies, Gaithersburg, MD). FISH images were superimposed over 4,6-diamino-2-phenylindole images for chromosomal assignment. Of 100 mitotic images, 82 showed FISH signals, and all were over the p12-p13 region of chromosome 17.

Streptolysin-O (SLO) Treatment

SLO-PBS was reconstituted with water as instructed by the supplier (Murex, Norcross, GA). Adherent cells were treated with SLO-phosphate-buffered saline (PBS) on ice for 4 min, rinsed, and allowed to warm at 37°C for 4 min in transport buffer as done previously (Martys *et al.*, 1995). Transport buffer (2 ml) was then used for glycosylation reactions. Alternatively, cells were dislodged from dishes with PBS-1 mM Na_3EDTA, washed with PBS, suspended in 0.2 ml of SLO-PBS on ice for 4 min, rinsed, and then incubated in 0.2 ml of transport buffer at 37°C for 4 min. The permeabilized cells were then collected and suspended in 0.2 ml of transport buffer for glycosylation reactions.

Analysis of C-Mannosylation of Tryptophan in Recombinant RNase 2.4

CHO-K1 and Lec35.1 cells were transiently transfected (Doucey *et al.*, 1998) with a plasmid encoding the hybrid RNase 2.4 (Krieg *et al.*,

1998), which was secreted into the medium and collected from the pooled conditioned media of eight 10-cm dishes after 4 d. Approximately 2.5 μg of RNase 2.4 was purified by immunoaffinity chromatography and C8 reversed phase high pressure liquid chromatography (HPLC) as previously described (Krieg *et al.*, 1998), digested with thermolysin, and the presence or absence of C-mannosylated tryptophan on the resulting peptides was measured quantitatively by reversed phase C18 HPLC (Krieg *et al.*, 1997). It has previously been established that in this system peak "b" contains the C-mannosylated peptide [FT(C^2-Man-)WAQW], whereas peaks "a" and "c" contain two forms of the unmodified peptide, TWAQW and FTWAQW (Krieg *et al.*, 1997).

In Vitro C-Mannosyltransferase Assay

Total microsomal membranes were isolated and assayed for C-mannosyltransferase activity as described (Doucey *et al.*, 1998), except that the final Triton X-100 concentration was 0.05% (wt/vol). In brief, 75 μg of membrane protein was incubated with 0.9 mM Ac-WAKW-NH_2 acceptor peptide (Doucey *et al.*, 1999) and 45 pmol [^{3}H]mannose-P-dolichol (5.61 Ci/mmol) for 30 min at 37°C. The reaction was terminated by the addition of chloroform/methanol 3:2 (vol/vol), and radioactive peptide in the aqueous phase was determined by scintillation counting. Background incorporation determined in the absence of peptide was subtracted out.

Labeling, Isolation, and Analysis of LLO

Labeling of Intact Cells. Cells were suspended in 50 μl of F-12 medium with 0.5 mM glucose and labeled for 15 min with 1 mCi/ml of either [2-^{3}H]mannose (18.0 Ci/mmol; Amersham, Arlington Heights, IL) or [1-^{3}H]galactose (20.0 Ci/mmol; ARC, St. Louis, MO) as described (Zeng and Lehrman, 1990), followed by a 5-min chase period with medium containing 10 mM unlabeled mannose.

Labeling of Cells Permeabilized with SLO. SLO-permeabilized cells (CHO-K1, Lec35.1, or Lec35.1 transfected with a doxycyclin-repressible Lec35 cDNA) were incubated for 10 min in transport buffer (Martys *et al.*, 1995) containing 0.3 μM GDP-[2-^{3}H]mannose (15.0 Ci/mmol; ARC), 1 μM unlabeled UDP-GlcNAc, and 0.2 mM 5′ AMP, followed by a 5-min chase period with buffer containing 0.1 mM unlabeled GDP-mannose. Various concentrations of unlabeled UDP-glucose were also included during incubation.

The LLO fractions of intact and permeablized cells were recovered, treated with mild acid to hydrolyze the pyrophosphate linkages between the dolichol and oligosaccharide moieties, and analyzed by HPLC as described (Turco, 1981; Zeng and Lehrman, 1991), except that final cleanup of reduced oligosaccharides was achieved by treatments with Dowex 50WX8-200 (hydrogen form) followed by Dowex AG1-X8 (formate form). In figures showing HPLC profiles, the positions of the following tritium-labeled standards [obtained in the general manner described (Zeng and Lehrman, 1991)] are indicated where appropriate: M_5Gn_2, $Man_5GlcNAc_2$; $G_3M_5Gn_2$, $Glc_3Man_5GlcNAc_2$; M_9Gn_2, $Man_9GlcNAc_2$; $G_3M_9Gn_2$, $Glc_3Man_9GlcNAc_2$. $Glc_3Man_9GlcNAc_2$ was obtained from CHO-K1 cells labeled with [^{3}H]mannose. $Man_9GlcNAc_2$ was obtained from CHO-K1 microsomes labeled with GDP-[^{3}H]mannose. $Glc_3Man_5GlcNAc_2$ was obtained from Lec15 cells incubated with [^{3}H]galactose. $Man_5GlcNAc_2$ was obtained from Lec15 or Lec35 cells incubated with [^{3}H]mannose. In figures displaying multiple HPLC chromatograms, all of the LLO samples were prepared from dishes labeled and processed at the same time. However, changes in retention times of a few minutes were sometimes noted when HPLC solvents were replenished in the course of analyzing a series of samples. The peaks on affected chromatograms were labeled based upon oligosaccharide retention times that were reestablished with subsequent standard runs.

In Vitro Glycosylation Reactions

MPC, [^{3}H]MPC, and mannose-β-1-P-nerol (MPN) were prepared as described (Rush *et al.*, 1993) and stored as solutions in 50% ethanol. GDP-[^{3}H]mannose (15 Ci/mmol) was from American Radiolabeled Chemicals, St. Louis, MO. Glycosylation reactions were initiated by addition of these compounds as indicated in the figure legends. When SLO-permeabilized Lec15 and Lec35 cells preincubated with [^{3}H]-mannose in vivo were used, MPC incubations were preceded by 10-min incubations with 100 μM unlabeled GDP-mannose to ensure extension of [^{3}H]$Man_{1-4}GlcNAc_2$-P-P-dolichol to [^{3}H]$Man_5GlcNAc_2$-P-P-dolichol.

Quantitation of LLO Glucosylation

The following approach, which was internally controlled and not subject to variations in sample recovery or load, was used to assess relative glucosylation of [^{3}H]mannose-labeled $Glc_{0-3}Man_9GlcNAc_2$ oligosaccharides. Because each oligosaccharide had the same [^{3}H]mannose content, their specific activities were identical. HPLC baselines were inferred from the detector signal obtained after the elution time for $Glc_3Man_9GlcNAc_2$, and for each oligosaccharide the HPLC peak height above baseline was measured. These heights were summed to give a value "A" for the total $Glc_{0-3}Man_9GlcNAc_2$ recovered from the column. For each oligosaccharide the peak height was multiplied by 0, 1, 2, or 3 to reflect the content of glucose residues, and these adjusted heights were summed to give a value "B" for the amount of glucosylation. For each sample, a glucosylation index could then be calculated by dividing B/A. For example, a sample with only unglucosylated $Man_9GlcNAc_2$ would have an index of 0.0. A sample in which all oligosaccharides were $Glc_3Man_9GlcNAc_2$ would have an index of 3.0.

Quantitation of LLO Mannosylation

Figure 8 reports HPLC data from MPC incubations of cells containing prelabeled [^{3}H]$Man_5GlcNAc_2$-P-P-dolichol. Therefore, in these experiments the specific radioactivity was the same for all MPC products. To obtain a normalized measurement of the amount of MPC-derived mannose that was transferred to the LLO pool, these data were analyzed after HPLC in the following way. 1) The peak heights for $Glc_{0-3}Man_{5-9}GlcNAc_2$ were measured, and the baseline backgrounds were subtracted out to give a peak height value for each oligosaccharide species. These values were then summed to give the total yield of LLO ("A") on the chromatogram. 2) The values for $Man_6GlcNAc_2$, $Man_7GlcNAc_2$, $Man_8GlcNAc_2$, and $Man_9GlcNAc_2$ species (regardless of glucose contents) were multiplied by factors of 1, 2, 3, and 4, respectively, resulting in weighted values reflecting the amounts of MPC-derived mannose in each. 3) The weighted values for $Glc_{0-3}Man_{6-9}GlcNAc_2$ were summed to give a value for total MPC-dependent products ("B"), and this was divided by the value for total LLO ("A"). This quotient ("B/A") reflected the amount of MPC-derived mannose transferred in each experiment, and was not affected by variations in the amounts of total LLO recovered or applied to the HPLC column. For example, an experiment in which no $Man_5GlcNAc_2$ was mannosylated by MPC would give a value of 0. If half of the $Man_5GlcNAc_2$ was converted to $Man_6GlcNAc_2$ the result would be 0.5. If all the $Man_5GlcNAc_2$ was converted to $Man_9GlcNAc_2$ the result would be 4.0.

Thin-layer Chromatography (TLC)

TLC was used to assess [^{3}H]glucose-P-dolichol synthesis in SLO-permeabilized cells incubated with 0.1 μM UDP-[6-^{3}H]glucose (60 Ci/mmol; ARC). After incubation, chloroform/methanol (2:1) extracts were back-washed with chloroform/methanol/water (3:48:47), applied to silica gel 60A TLC plates (Whatman, Tewksbury, MA), separated in chloroform/methanol/0.25% KCl (55:45:10), treated with fluor (Camp *et al.*, 1993), and exposed to x-ray film. [^{3}H]GPD was identified by comparison to a standard. Because the

plate was loaded with an excess of cellular lipid that might have affected the separation, the [^{3}H]GPD was recovered from the plate and TLC was repeated to verify its identity. TLC (DeLuca *et al.*, 1994) was used similarly to identify [^{3}H]MPD from SLO-treated cells incubated with GDP-[^{3}H]mannose.

RESULTS

Exon Organization of the Hamster Gene Encoding SL15

Plasmid pLW1 carries a novel cDNA designated SL15 that corrects the phenotype of Lec35 mutants (Ware *et al.*, 1996), and encodes a predicted protein of 247 amino acids with a molecular weight of 26,546 and a pI of 8.55. Although SL15 was originally reported to correct the Lec15 phenotype, this result was later found to be invalid (Ware and Lehrman, 1998). Lec15 cells have now been shown to result from mutations in the DPM2 subunit of MPD synthase (Maeda *et al.*, 1998). A genomic clone corresponding to SL15 was isolated as a LA-PCR fragment by using primers from the 5′ untranslated region (UTR) and 3′ UTR regions, and sequenced with other primers from SL15 cDNA. The entire sequence of the fragment plus flanking untranslated sequences deduced from additional cDNA clones homologous to SL15 has been deposited in GenBank with annotations (accession AF250376, 5361 nt), and the exon organization is summarized in Figure 1. Seven coding exons were identified, with all introns having canonical GT-AG splice junctions. Numerous additional cDNAs with identical splicing patterns were isolated by rescreening the original cDNA library (Ware *et al.*, 1996), although one splice variant was identified that included an additional exon between exons 3 and 4 (designated exon X). Exon X encoded an altered reading frame and a termination codon after exon 3, and the splice variant did not correct the Lec35 phenotype when transfected (unpublished results), so this splice variant is unlikely to be functional. Taking exon X into account the introns between exons 2 and 7 were all short, the longest being intron 5 (155 nt), and not likely to harbor additional cryptic exons. However, further experiments will be required to determine whether intron 1 (3198 nt) contains additional embedded exons. Various computer-assisted analyses of intron 1 identified several potential open reading frames, but none had high probabilities of being true exons (unpublished results). Intron 1 had an unusually high proportion of repetitive gene sequences (Figure 1 and Table 1) that were all of the same 5′ to 3′ orientation. This includes an RSINE1 element near the 3′ end of intron 1. A similar RSINE1 element was found in intron 3, partially overlapping exon X (Table 1).

The Lec35 Gene Encodes SL15

To determine whether SL15 was encoded directly by the Lec35 gene, or merely suppressed Lec35 defects while being derived from an independent gene, nucleic acids from two independent Lec35 isolates were tested. Lec35.1 arose spontaneously under gradual selective pressure (Lehrman and Zeng, 1989), whereas Lec35.2 was obtained after chemical mutagenesis and stringent selection (Camp *et al.*, 1993). As shown by Southern blots with *Bam*HI- or *Hin*dIII-digested genomic DNA (Figure 2A), a probe prepared from the entire SL15 cDNA coding region detected a series of highly abnormal DNA fragments for Lec35.1 cells compared with parental CHO-K1 cells. Only normal fragments were detected for Lec35.2 cells. The Lec35.1 pattern was due to a disruption rather than a polymorphism because abnormal patterns were also obtained with *Eco*RI, *Bss*HI and *Pvu*II (unpublished results). This shows that the gene that encodes SL15 also gives rise to the Lec35 genotype when mutated. Thus, SL15 and related cDNA clones will be designated Lec35.

No gross gene disruption was apparent in the Lec35.2 allele, which most likely resulted from a point mutation. In

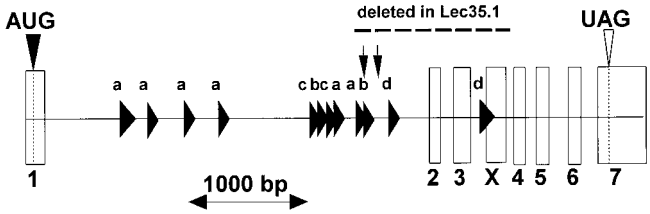
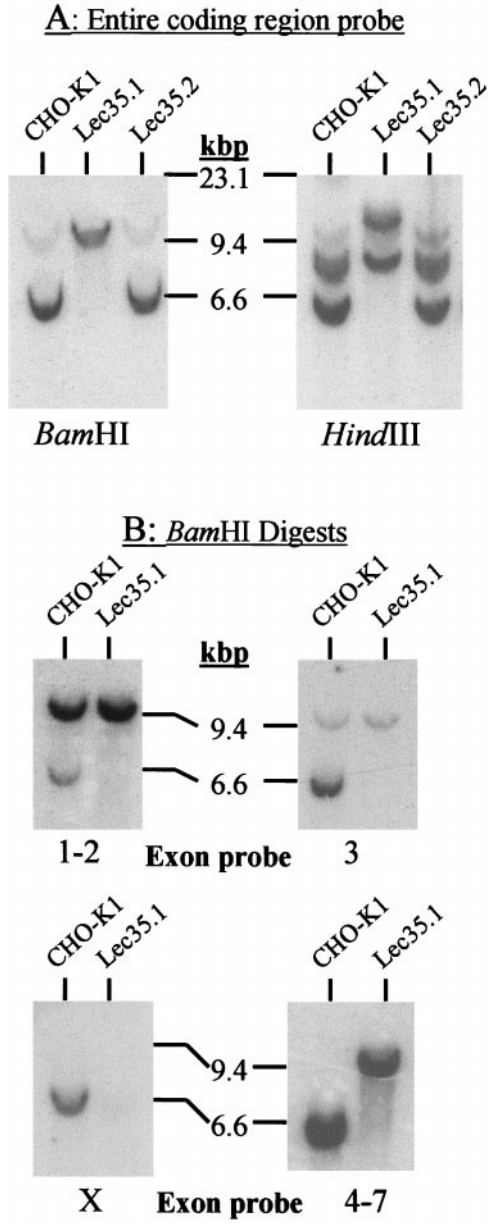


Figure 1. Exon-intron organization of the hamster Lec35 gene. A fragment of normal CHO-K1 genomic DNA, ~5 kbp, was amplified by LA-PCR (Takara Shuzo, Tokyo, Japan) with 5′ UTR and 3′ UTR primers from SL15. The fragment was gel-purified and used directly for automated DNA sequence analysis on both strands by using various sequencing primers suggested by SL15 cDNA sequence (GenBank accession U55387) as well as sequence obtained from introns. Exon-intron boundaries were deduced by directly comparing the genomic and cDNA sequences. Exon X was identified in a rare nonfunctional clone from the original cDNA library (Ware *et al.*, 1996). Inclusion of exon X altered the 5′ splice junction of exon 4. Rodent genomic repetitive elements (Table 1; Jurka *et al.*, 1996) are indicated by horizontal arrowheads: a, B1 class; b, B2 class; c, B3 class; d, RSINE1 class. Two *Bam*HI sites identified by sequencing and used for restriction enzyme digests are noted by the downward vertical arrows. Translation initiation (AUG) and termination (UAG) sites are shown by vertical arrowheads. The entire annotated sequence has been deposited with GenBank (accession AF250376). A region extending from within intron 1 through exon X that appears deleted in the Lec35.1 allele (see text) is indicated. Because the Lec35.1 mutation is recessive, it is highly likely that the parental cell that gave rise to the Lec35.1 mutant had a single copy of a normal allele at the Lec35 locus. It is highly unlikely that the exact same deletion occurred twice in a single cell starting with two normal Lec35 alleles.

Table 1. Repetitive elements in the hamster Lec35 gene (GenBank AF250376)

Nucleotide position	Length (nt)	Location	Class	Portion of consensus
848	135	intron 1	B1	1–135
977	93	intron 1	B1	21–114
1308	102	intron 1	B1	23–125
1462	96	intron 1	B1	35–131
2336	33	intron 1	B3	97–130
2369	60	intron 1	B2	34–92
2497	96	intron 1	B3	1–96
2539	150	intron 1	B1F	1–150
2668	55	intron 1	B1F	95–150
2776	165	intron 1	B2	1–165
2986	97	intron 1	RSINE1	48–145
3738	126	intron 3/exon X	RSINE1	9–135



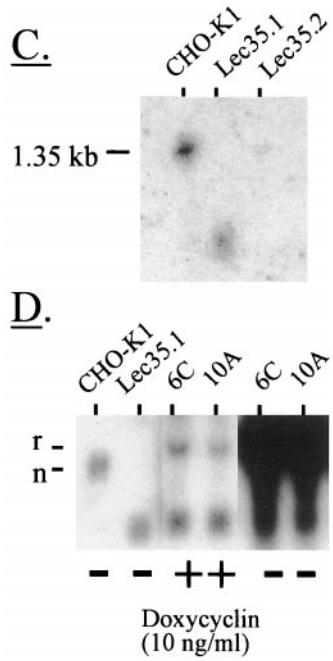


Figure 2. Analysis of Lec35 nucleic acids. Southern blot analysis of genomic DNA. ^{32}P probes were generated with specific PCR primers and covered either the complete coding sequence of SL15 cDNA (A), or exons 1 and 2, exon 3, exon X, or exons 4–7 (B). Genomic DNA (Easy-DNA; Invitrogen, San Diego, CA) from parental CHO-K1, Lec35.1, or Lec35.2 cells (10 μg for all probes except exon X [50 μg]) was digested with either *Bam*HI or *Hin*dIII as indicated and analyzed by Southern blots according to standard methods (Sambrook *et al.*, 1989). The positions of λ phage-*Hin*dIII markers are shown. In addition to a strongly hybridizing fragment of ~6 kbp, a faint *Bam*HI fragment of ~10 kbp was reproducibly detected with the exon 3 probe in CHO-K1 DNA. Because exon 3 lacks a *Bam*HI site, this is likely to be an irrelevant cross-reacting fragment. Northern blot analysis of RNA. Total RNA was isolated from the CHO-K1, Lec35.1, or Lec35.2 cells lines, as well as the 6C and 10A transfectants of Lec35.1 grown in the absence or presence of 10 ng/ml doxycyclin for 4 d, and 10 μg of total RNA was analyzed by gel electrophoresis and blotting (C and D) as described (Sambrook *et al.*, 1989). A ^{32}P probe prepared from the complete coding sequence of SL15 cDNA was used. Relative intensities of the Lec35 mRNA bands, as described in the text, were measured with a phosphorimager (Fuji, Stamford, CT). The migrations of native (n) and recombinant (r) Lec35 transcripts (D) were reproducible. The position of a 1.35-kb RNA marker (Life Technologies) is shown (C).

contrast, the Lec35.1 allele was clearly caused by a disruption, and a preliminary map was obtained with smaller probes encompassing exons 1–2, exon 3, exon X, or exons 4–7 (Figure 2B). All four probes detected abnormal patterns of *Bam*HI fragments in Lec35.1 DNA. In conjunction with the exon/intron organization and sequence of this gene (Figure 1), it was deduced[1] that a deletion began within intron 1 upstream of the *Bam*HI sites, and extended downstream through exon X but not beyond exon 7. LA-PCR of genomic

[1] The Lec35.1 deletion indicated in Figure 1 was deduced from Southern blot data (Figure 2). The major normal hybridizing bands detected with exon 3 and exon X probes were absent in Lec35.1, with no corresponding abnormal bands of similar intensity, indicating that these exons were deleted. Because a probe for exons 1 and 2 (on opposite sides of the two *Bam*HI sites in normal intron 1) gave a single Lec35.1 *Bam*HI fragment, the most likely 5′ end of the deletion is between exon 1 and the *Bam*HI sites. A probe encompassing exons 4–7 detected a single abnormally large *Bam*HI fragment in Lec35.1, of the same size detected with the exon 1–2 probe. Thus, the 3′ end of the deletion is between exon X and exon 7. Fortuitously, the normal *Bam*HI fragment containing exon 1 (upper fragment detected by the exon 1–2 probe) is similar in size to the abnormal Lec35.1 *Bam*HI fragment containing (minimally) exons 1 and 7.

DNA with various combinations of primers readily generated the expected products from normal genomic DNA, but LA-PCR products from Lec35.1 DNA were never observed (data not shown), preventing a detailed analysis of the deletion joint. Similar RSINE1 repetitive elements (labeled "d" in Figure 1) were found downstream of the *Bam*HI sites in intron 1 and exon X/intron3, and are within the proposed deleted region. This raises the possibility that recombination between these two elements initiated the rearrangement in the Lec35.1 allele (see DISCUSSION).

Ermonval and coworkers recently reported chemical mutagenesis and isolation of a CHO-K1 mutant, MadIA214, with secretory pathway defects and a glycosylation phenotype highly similar to Lec35 (Ermonval *et al.*, 1997, 2000). Interestingly, genomic DNAs from MadIA214 and the parental cell Cl42 had equivalent restriction patterns with genomic Southern blots probed with Lec35 cDNA, consisting of a set of normal hybridizing fragments and a set of abnormal fragments (our unpublished results). Thus, it appears that the MadIA214line has two defective Lec35 alleles, one disrupted spontaneously and one inactivated by chemical mutagenesis.

Compared with normal mRNA of 1.35 kb, RNA blots probed with SL15 revealed a truncated transcript (~0.5 kb) in Lec35.1 cells (Figure 2C). Little or no transcript was detected from Lec35.2 cells, suggesting a point mutation that affected RNA synthesis or stability. A series of reverse transcription-PCR experiments with various pairs of SL15-specific primers detected products with CHO-K1 RNA, but not Lec35.1 RNA (unpublished results).

Chromosomal Location of MPDU1

The human gene homologous to Lec35 has been designated MPDU1 (MPD utilization defect 1) and its DNA sequence has been determined (GenBank accession AC007421). The chromosomal location was determined by two independent approaches. Multiple images obtained by fluorescence in situ hybridization with a 1.3-kb cDNA probe from the MPDU1 gene revealed signals only at 17p12-p13 (our unpublished results). MPDU1 mapping to human 17p12-p13.1 was also reported by radiation-induced gene segregation.[2] Due to defective glycosylation, a hereditary loss of MPDU1 might be expected to result in symptoms similar to those in various forms of congenital deficiency of glycosylation (Jaeken *et al.*, 1993; First International Workshop on CDGS, 2000). However, a search of the Online Mendelian Inheritance in Man (OMIM listing 604041) database did not reveal any obvious candidate diseases in the region of MPDU1.

MPD-dependent C-Mannosylation Is Defective in Lec35.1 Mutant Cells

The data presented in Figure 2 showed that the Lec35.1 allele is functionally null. Hence, Lec35.1 cells could be used to determine whether the Lec35 gene was required for sugar-P-dolichol–dependent reactions other than mannosylation of LLOs and GPIs.

To determine whether the Lec35 mutation altered C-mannosylation of tryptophanyl residues, which is known to require MPD (Doucey *et al.*, 1998), RNase 2.4 was expressed in both the parental CHO-K1 and Lec35.1 cells. This hybrid enzyme consists of residues 1–13 of RNase 2, containing the C-mannosylation site WAQW, and residues 11–119 of RNase 4. C-Mannosylation of this protein has been characterized (Krieg *et al.*, 1998). C-Mannosylation of CHO-K1-expressed (Figure 3A) or Lec35.1-expressed (Figure 3B) RNase 2.4 was determined by quantitative peptide mapping. From the data in Figure 3 it was calculated that Trp7 in RNase 2.4 from CHO- K1 cells was 67% C-mannosylated, whereas that from Lec35.1 was only 9% modified. The residual C-mannosylation in Lec35.1 cells (14% of CHO-K1) is not surprising because the Lec35.1 mannosylation defect was also somewhat leaky for GPI anchors (Slonina *et al.*, 1993).

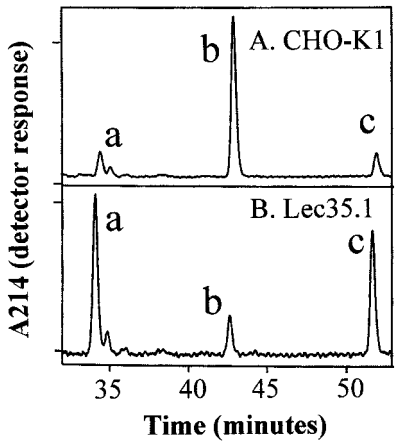


Figure 3. C-mannosylation of RNase 2.4. RNase 2.4 was expressed in either CHO-K1 (A) or Lec35.1 (B) cells, purified from the conditioned medium, digested with thermolysin at 75°C, and the peptides were fractionated by reversed phase C18 HPLC. Only the portion of the chromatogram where peptides from the N terminus of the protein eluted has been shown. Peak b contains the C-mannosylated peptide [FT(C^2-Man-)WAQW], whereas peaks a and c contain two forms of the unmodified peptide, TWAQW and FTWAQW.

To examine whether the 7.4-fold decrease in C-mannosylation of RNase 2.4 in Lec35.1 cells was due to a lower activity of the C-mannosyltransferase, enzyme activity in Triton X-100–treated membrane fractions of the two cell lines was examined in vitro. Incubation of the membranes with exogenous [^{3}H]MPD and the general acceptor peptide Ac-WAKW-NH_2 (Doucey *et al.*, 1999) showed that the specific activities of the transferases in the CHO-K1 and Lec35.1 membranes were nearly the same, 8799 and 8905 cpm/75 μg of membrane protein/30 min, respectively (average value of two independent determinations). Analysis of the incubation mixture by C18 reversed phase HPLC demonstrated that the radioactivity was covalently associated with the peptide (our unpublished results). Thus, the differences in Figure 3 were not due to variations in transferase activity. Furthermore, the Lec35 phenotype with respect to C-mannosylation is corrected in isolated microsomes, as it is for LLO and GPI synthesis.

These results demonstrate a role for the Lec35 gene in all glycoconjugate pathways known to require MPD in animal cells, i.e., those that produce LLOs, GPIs, and C-mannosylated tryptophan.

[2] www.ncbi.nlm.nih.gov/UniGene/clust.cgi?ORG=Hs&CID = 6710.

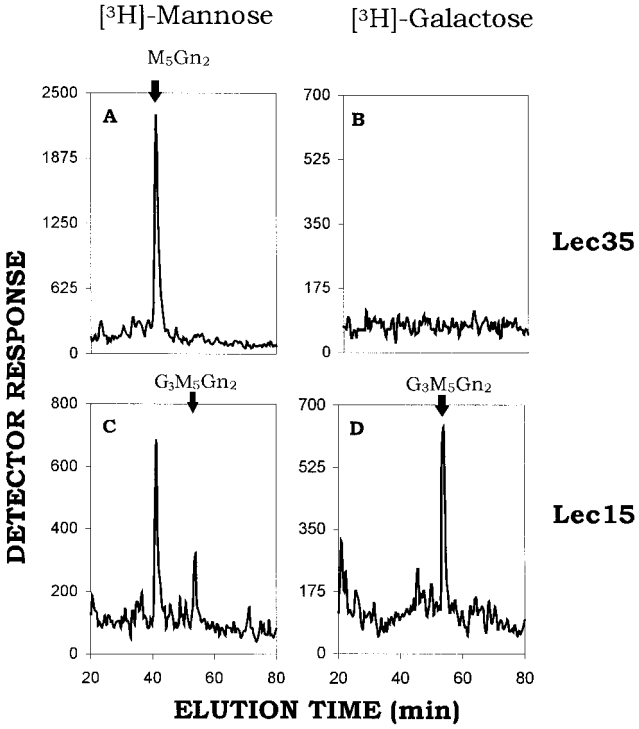


Figure 4. Absence of $Glc_3Man_5GlcNAc_2$-P-P-dolichol in Lec35 mutant cells. Lec35.1 cells (A and B) and Lec15.2 cells (Camp *et al.*, 1993) (C and D) were labeled with 1 mCi/ml [2-^{3}H]mannose (A and C) or [1-^{3}H]galactose (B and D) for 15 min as described under MATERIALS AND METHODS. Lipid-linked oligosaccharides were extracted, and the labeled oligosaccharides were released by mild acid hydrolysis and analyzed by HPLC.

GPD-dependent Glucosylation of LLO Is Defective in Lec35 Mutant Cells: Evidence for MPD-Selectivity by Lec35 Protein

To determine whether Lec35 function was limited to MPD-dependent reactions, we examined GPD-dependent glucosylation of $Man_5GlcNAc_2$-P-P-dolichol, which accumulates in Lec35 mutants. Approximately 10–30% of $Man_5GlcNAc_2$-P-P-dolichol is converted to $Glc_3Man_5GlcNAc_2$-P-P-dolichol in a GPD-dependent manner in the MPD synthase-deficient mutants Thy-1-E and Lec15 (Chapman *et al.*, 1979, 1980; Stoll *et al.*, 1992), which have defects in the DPM1 (Tomita *et al.*, 1998) and DPM2 (Maeda *et al.*, 1998) genes, respectively. In contrast, Lec35.1 accumulated $Man_5GlcNAc_2$-P-P-dolichol with no detectable $Glc_3Man_5GlcNAc_2$-P-P-dolichol, whether [^{3}H]mannose or [^{3}H]galactose (which results in labeling of glucosyl residues) was used (Figure 4). LLO analyses of Lec35.15, Lec35.2 (our unpublished results), and MadIA214 (Ermonval *et al.*, 1997) cells also revealed accumulation of $Man_5GlcNAc_2$-P-P-dolichol with no detectable $Glc_3Man_5GlcNAc_2$-P-P-dolichol, demonstrating that the glucosylation defect was a phenotype consistently associated with the Lec35 genotype. However, $Man_5GlcNAc_2$-P-P-dolichol is a relatively poor glucosylation substrate (Chapman *et al.*, 1979; Burda *et al.*, 1999; Cipollo and Trimble, 2000). The extent to which the Lec35 gene was necessary for glucosylation of the preferred natural substrate, $Man_9GlcNAc_2$-P-P-dolichol, was not clear.

To address this problem we took advantage of an unexpected observation. Lec35 cDNA was ligated into pTRE and cotransfected with pTet-Off into Lec35.1 cells. By using selection methods described previously (Ware *et al.*, 1996), stable transfectants were selected that had restored synthesis of $Glc_{0-3}Man_9GlcNAc_2$-P-P-dolichol. Colonies were propagated in the absence or presence of 10 ng/ml doxycyclin (a tetracycline analog that, in conjunction with pTet-Off, suppresses transcription of inserts cloned into pTRE), and screened by Northern blot analysis to determine the basal (with doxycyclin) and induced (without doxycyclin) levels of recombinant Lec35 mRNA. Similar results were obtained with four independent subclones, two of which (6C and 10A) were chosen for further study. Compared with parental CHO-K1 cells, 6C and 10A generally had 0.5–1× basal expression and 100–200× induced expression (our unpublished results). Typical results are shown in Figure 2D. To reach basal mRNA levels it was necessary to treat the cells with 10 ng/ml doxycyclin for 4 d (our unpublished results).

Figure 5 shows the LLO profiles for parental CHO-K1, untransfected Lec35.1, and the 6C and 10A subclones grown in the absence or presence of 10 ng/ml doxycyclin. The drug had no effect on the CHO-K1 (Figure 5, A and B) or Lec35.1 profiles (Figure 5, C and D). Under both basal and induced conditions, expression of Lec35 cDNA in Lec35.1 cells restored MPD-dependent mannosylation, yielding $Man_9GlcNAc_2$ (Figure 5, E–H). However, glucosylation of $Man_9GlcNAc_2$ was highly dependent upon the level of Lec35 cDNA expression. To quantitatively assess the glucosylation differences, a normalized glucosylation index was calculated (see MATERIALS AND METHODS) on a scale of 0.0 (no glucosylation) to 3.0 (complete glucosylation). With basal expression, the glucosylation indices were 0.33 for 6C and 0.62 for 10A, indicating that LLO glucosylation was markedly diminished even though these cells had expressed amounts of Lec35 transcript that were similar to those in CHO-K1 cells (glucosylation index = 2.15). However, when Lec35 mRNA was overexpressed, the glucosylation indices reached 1.79 for 6C and 1.55 for 10A.

From these results it can be concluded that 1) glucosylation of LLO requires the Lec35 gene; 2) the cloned Lec35 cDNA appears to encode protein that is selective for MPD compared with GPD; and 3) when overexpressed, the cloned Lec35 cDNA can restore GPD-dependent glucosylation of LLO to nearly normal levels.

The Lec35 Gene Is Required for LLO Glucosylation In Vitro

To rule out the possibility that the results of Figure 5 with intact cells were due to differences in the intracellular concentrations of nucleotide-sugars or dolichol-P-sugars in various transfectants, an independent in vitro system was developed (described in the following section) that takes advantage of the ability of SLO to gently permeabilize Lec35 cells without affecting the Lec35 phenotype. In all experiments, a labeled pool of [^{3}H]$Man_{5-9}GlcNAc_2$-P-P-dolichol was synthesized by incubation of SLO-permeabilized cells (grown in the absence or presence of 10 ng/ml doxycyclin) with GDP-[^{3}H]mannose. To increase GPD-dependent glucosylation, some incubations also included a chase with 0.5 mM unlabeled UDP-Glc, a concentration determined in sep-

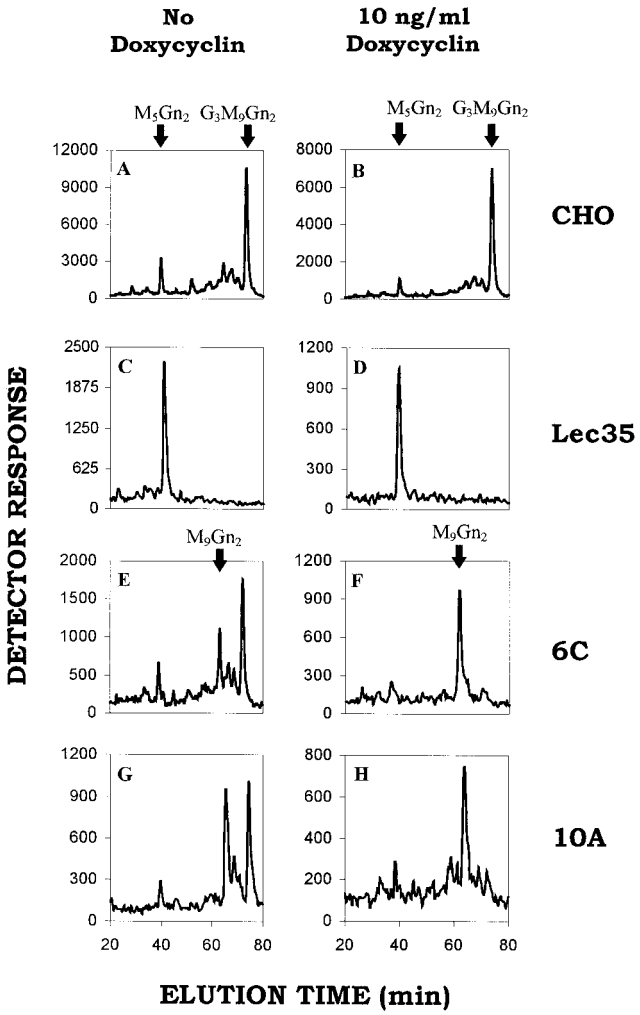


Figure 5. Glucosylation of $Man_9GlcNAc_2$-P-P-dolichol in vivo depends upon the level of expression of Lec35 mRNA. CHO-K1 (A and B), Lec 35.1 (C and D), 6C (E and F), or 10A cells (G and H) were grown in the absence (A, C, E, and G) or presence (B, D, F, and H) of 10 ng/ml doxycyclin for 4 d, and labeled with 1 mCi/ml [2-^{3}H]mannose for 15 min. Oligosaccharides were obtained from the LLO fraction by mild acid hydrolysis and analyzed by HPLC.

arate controls (unpublished results) to give optimal results with SLO-CHO-K1 cells. As indicated in Table 2, even in the absence of UDP-Glc some glucosylation of $Man_9GlcNAc_2$-P-P-dolichol occurred in CHO-K1 cells (average index = 0.89), presumably due to endogenous GPD. Furthermore, the amount of $Man_9GlcNAc_2$-P-P-dolichol glucosylated in SLO-CHO-K1 cells with the optimal concentration of UDP-Glc (average index = 1.63) was lower than that seen with intact cells. However, this range of glucosylation was sufficiently large to test the effects of Lec35 expression.

As listed in Table 2, regardless of growth in the presence of doxycyclin or the use of a UDP-glucose chase, glucosylation of $Man_9GlcNAc_2$-P-P-dolichol formed in vitro was lower in 6C cells than in similarly treated CHO-K1 cells. Further, omission of doxycyclin did not significantly affect

Table 2. Glucosylation of $Man_9GlcNAc_2$-P-P-dolichol in vitro depends upon Lec35 expression

Cell line	Doxycyclin (ng/ml)	UDP-glucose	Glucosylation index Exp. 1	Glucosylation index Exp. 2	Glucosylation in presence of doxycyclin (%)
CHO-K1	0	−	0.86	0.92	
	10	−	0.85	0.91	99
6C	0	−	0.75	0.69	
	10	−	0.51	0.33	58
CHO-K1	0	+	1.51	1.74	
	10	+	1.66	1.88	109
6C	0	+	1.29	1.62	
	10	+	0.71	0.48	41

As described under MATERIALS AND METHODS, CHO-K1 and 6C cells were treated with SLO, and incubated with GDP-[^{3}H]mannose. Some experiments included a chase with 0.5 mM unlabeled UDP-glucose, as indicated. The LLO pool was recovered, and oligosaccharides released by mild acid hydrolysis were fractionated by HPLC. The HPLC data were used to calculate glucosylation indices. Results with two independent series of culture dishes are presented. The effect of doxycyclin treatment (final column) was calculated by dividing the average of the glucosylation indices obtained with doxycylin by the average of the indices obtained without doxycyclin, and is expressed as a percentage.

glucosylation in CHO-K1 cells, whereas glucosylation in 6C cells was enhanced due to higher expression of the Lec35 cDNA. Inclusion of a UDP-glucose chase increased glucosylation in all permeabilized cells, but the relative effects of prior doxycyclin treatment on CHO-K1 cells compared with 6C cells were the same as without a chase. This confirmed the in vivo observations (Figure 5) that the cloned Lec35 cDNA was more effective for MPD, and that glucosylation was increased by high expression of Lec35 cDNA. Both in vivo and in vitro, doxycyclin did not inhibit glucosylation in CHO-K1 cells. No glucosylation of $Man_5GlcNAc_2$-P-P-dolichol was observed in SLO-Lec35.1 cells, even with inclusion of 0.5 mM UDP-glucose (our unpublished results). This was consistent with the ability of the SLO treatment to preserve the Lec35 phenotype, and the limited ability of $Man_5GlcNAc_2$-P-P-dolichol to act as a glucosylation substrate (Chapman *et al.*, 1979; Burda *et al.*, 1999; Cipollo and Trimble, 2000). Control experiments showed that there were no significant differences in the abilities of the various SLO-cells to synthesize [^{3}H]GPD in vitro (our unpublished results).

Development of a Streptolysin-O Permeabilized Cell System That Preserves the MPD-Utilization Phenotype

In our hands, all previous attempts to permeabilize Lec35 cells by detergent treatment or physical breakage restored MPD utilization and resulted in synthesis of $Man_{6-9}GlcNAc_2$-P-P-dolichol (Zeng and Lehrman, 1990) and ManGlcN-(acyl)PI (Camp *et al.*, 1993) upon addition of GDP-[^{3}H]mannose. However, because such experiments did not determine the fraction of the preexisting acceptor that was

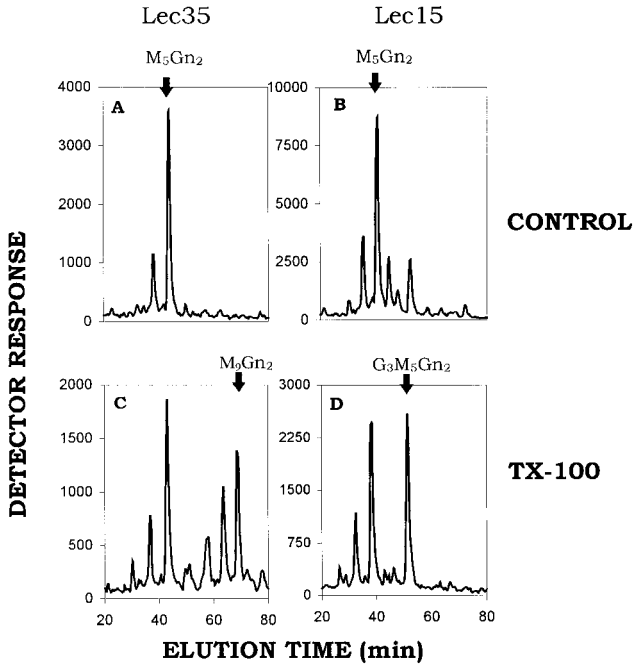


Figure 6. Mild perturbation efficiently corrects defective glycosylation in Lec35 cells. Lec35.1 (A and C) and Lec15.2 (B and D) cells were incubated with [^{3}H]mannose to label endogenous $Man_5GlcNAc_2$-P-P-dolichol. Cells were treated in the absence (A and B; control) or presence (C and D; TX-100) of 0.05% (wt/vol) Triton X-100, and then incubated with 0.1 mM unlabeled GDP-mannose. The oligosaccharides were recovered and compared with known standards, as indicated, by HPLC. Note that $Man_5GlcNAc_2$-P-P-dolichol in treated Lec15 cells (D) is converted to $Glc_3Man_5GlcNAc_2$-P-P-dolichol, but additional mannose residues are not added.

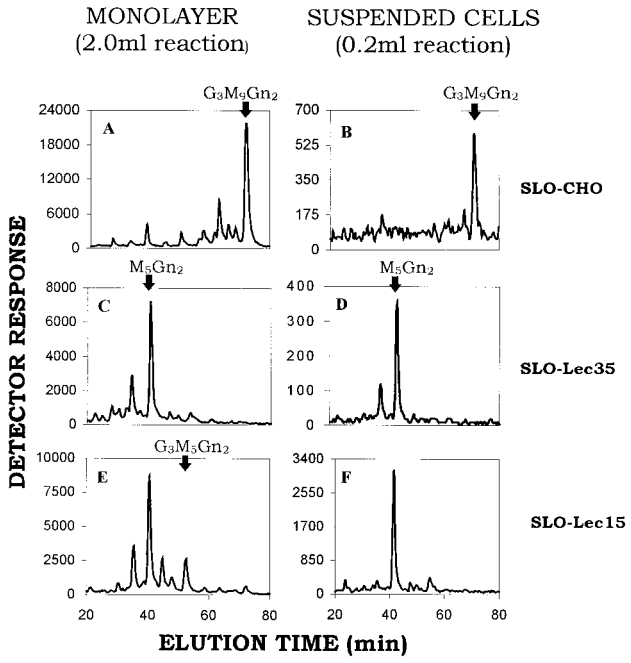


Figure 7. Streptolysin-O permeabilizes Lec35 cells and preserves the mannosylation defect. CHO-K1 (A and B), Lec35.1 (C and D), and Lec15.2 (E and F) cells were treated with SLO either when attached to dishes (Monolayer, A, C, and E) or after suspension (Suspended Cells, B, D, and F). The permeabilized cells were incubated with either 2.0 ml (A, C, and E) or 0.2 ml (B, D, and F) of transport buffer (Martys *et al.*, 1995) at 37°C for 10 min containing 1 μM unlabeled UDP-GlcNAc to initiate LLO synthesis, 0.1 μCi GDP-[^{3}H]mannose to label and extend LLOs, and 0.2 mM 5′ AMP to inhibit breakdown of nucleotide sugars. LLO were recovered and characterized by HPLC.

extended, the efficiencies of such treatments were not clear. Figure 6 shows an experiment in which intact cells were prelabeled with [^{3}H]mannose, treated with 0.05% (wt/vol) Triton X-100, and incubated with 0.1 mM unlabeled GDP-mannose. The Lec35.1 cells extended ~60% of the preexisting $Man_5GlcNAc_2$-P-P-dolichol to $Glc_{0-3}Man_{6-9}GlcNAc_2$-P-P-dolichol. Thus, correction of the defect by disruption is efficient. Lec15.2 cells have no detectable MPD synthase activity because they lack the membrane-associated DPM2 subunit (Maeda *et al.*, 1998; Tomita *et al.*, 1998). Much of the Lec15.2 $Man_5GlcNAc_2$-P-P-dolichol was extended to $Glc_3Man_5GlcNAc_2$-P-P-dolichol (presumably requiring endogenous GPD), but no $Glc_{0-3}Man_{6-9}GlcNAc_2$-P-P-dolichol was detected.

Fragile intervesicular transport processes in CHO-K1 cells can be maintained after selective permeabilization of the plasma membrane with SLO (Martys *et al.*, 1995). Intact CHO-K1 and Lec35 cells did not stain with trypan blue, whereas in each case >99% stained with trypan blue after SLO treatment (our unpublished results). After treatment with SLO and incubation with GDP-[^{3}H]mannose, permeabilized CHO-K1 (SLO-CHO) and Lec15.2 (SLO-Lec15) cells synthesized $Glc_{0-3}Man_{6-9}GlcNAc_2$-P-P-dolichol and $Glc_{0-3}Man_5GlcNAc_2$-P-P-dolichol, respectively, as anticipated (Figure 7). Importantly, SLO-Lec35.1 cells synthesized $Man_5GlcNAc_2$-P-P-dolichol but no appreciable $Glc_{0-3}Man_{6-9}GlcNAc_2$-P-P-dolichol (Figure 7). The results were SLO-dependent because no labeling of oligosaccharides was detected when intact cells were incubated with GDP-[^{3}H]mannose (our unpublished results). Similarly, $Man_5GlcNAc_2$-P-P-dolichol but no larger LLOs were observed when intact Lec35 cells were preincubated with [^{3}H]mannose, treated with SLO, and then incubated with unlabeled GDP-mannose (Figure 8, upper left). In summary, when GDP-mannose was present $Man_5GlcNAc_2$-P-P-dolichol was extended in SLO-CHO cells, but not in adherent (Figure 7) or suspended (Figures 7 and 8) SLO-Lec35 cells. Therefore, SLO permeabilized the Lec35 cells without alteration of the MPD utilization defect. Control experiments confirmed that MPD was synthesized by SLO-Lec35 and SLO-CHO cells, but not in SLO-Lec15 cells. Furthermore, Man-GlcN-(acyl)PI was synthesized from endogenously accumulated GlcN-(acyl)PI in Lec35 microsomal membranes as reported previously (Camp *et al.*, 1993), but not in SLO-Lec35 cells (our unpublished results) as expected if the MPD utilization phenotype was preserved.

Mannose-P-Citronellol Is Used Efficiently in SLO-treated Lec15 and Lec35 Cells

MPC has two isoprene units, and is a water-soluble analog of MPD that typically has a dolichol chain of 18–19 isoprene units. MPC is also a mannosyl donor in vitro for the four

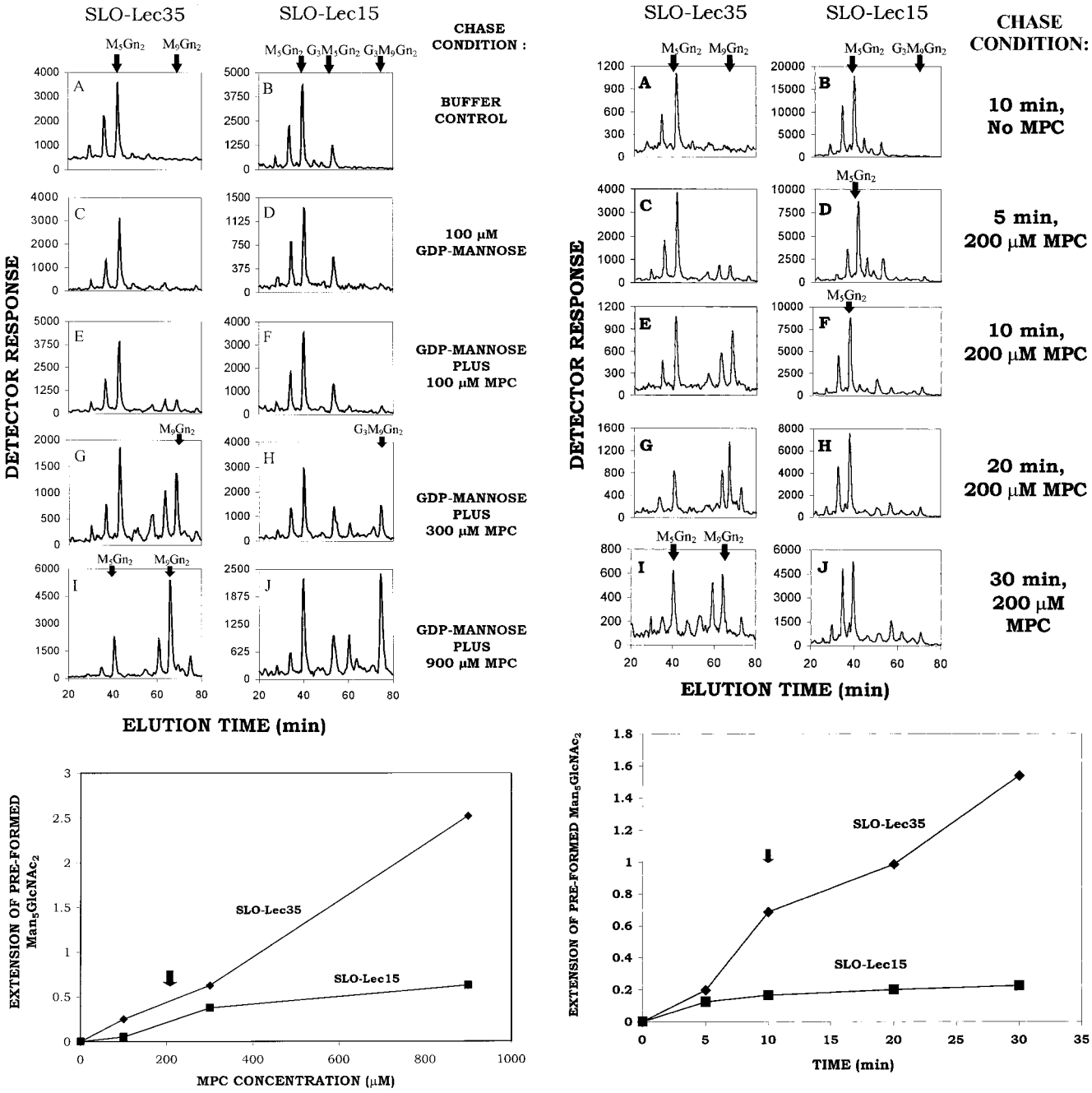


Figure 8. Mannose-P-citronellol is an efficient mannose donor in SLO-treated Lec35 cells. (Upper left) Lec35.1 (A, C, E, G, and I) and Lec15.2 (B, D, F, H, and J) cells were labeled with [^{3}H]mannose. They were treated with SLO, and then incubated for 10 min with transport buffer (A and B), or buffer plus 100 μM unlabeled GDP-mannose (to convert any [^{3}H]$Man_{1-4}GlcNAc_2$-P-P-dolichol to [^{3}H]$Man_5GlcNAc_2$-P-P-dolichol) and either 0 μM (C and D), 100 μM (E and F), 300 μM (G and H), or 900 μM (I and J) unlabeled MPC. LLO were recovered and analyzed by HPLC. Note that $Man_9GlcNAc_2$-P-P-dolichol is glucosylated by endogenous GPD more effectively in SLO-Lec15 cells than SLO-Lec35 cells, as expected. (Lower left) HPLC data from the upper left figure were processed (see MATERIALS AND METHODS) to give a normalized, quantitative value for the amount of MPC-dependent mannosylation that occurred at each concentration. The maximum possible value is 4.0. The downward arrow indicates the MPC concentration used in the upper right figure. It is not known why MPC-treated SLO-Lec35 cells had higher values than SLO-Lec15 cells. (Upper right) Lec35.1 (A, C, E, G, and I) and Lec15.2 (B, D, F, H, and J) cells were labeled with [^{3}H]mannose and treated with SLO in a manner comparable to that shown in the upper left figure. The cells were then incubated in buffer alone for 10 min (A and B), or buffer with unlabeled 100 μM GDP-mannose and 200 μM MPC for either 5 min (C and D), 10 min (E and F), 20 min (G and H), or 30 min (I and J). LLO were analyzed by HPLC. (Lower right) The data from the upper right figure were processed to gives values for the extent of MPC-dependent mannosylation, as done for the lower left figure. The downward arrow shows the time used for incubations in the upper left figure.

Table 3. Enzymatic transfer of mannose from C_{10} donors

	[^{3}H]mannose transferred to acceptor (pmol/min/mg membrane protein)	
Mannosyl donor	$Man_5GlcNAc_2$-P-P-dolichol (Lec15)	mannan (M. luteus)
[^{3}H]MPC	1.60	21.0
[^{3}H]MPN	0.04 (2.5%)	18.2 (86.7%)

To assay LLO synthesis, enzymatic reactions included microsomes (0.4 mg of protein) from Lec15 cells (which accumulate acceptors for MPD-dependent mannosyltransferases), 40 mM Tris-HCl (pH 7.4), 4 mM $CaCl_2$, and either [^{3}H]MPC (100 cpm/pmol) or [^{3}H]MPN (22 cpm/pmol) in a total volume of 0.025 ml. Following incubation at 37°C for 5 min, the amount of labeled mannose transferred into LLO was determined (Rush *et al.*, 1993). To assay the Micrococcus luteus mannosyltransferases, reaction mixtures contained micrococcal membranes (0.22 mg of protein), 20 mM Na-PIPES (pH 6.6), 10 mM $MnCl_2$, and either [^{3}H]MPC or [^{3}H]MPN (25 cpm/pmol) in a total volume of 0.02 ml. Following incubation for 10 min at 21°C, the amount of radiolabeled mannose incorporated into mannan was determined (Rush *et al.*, 1993). The results for both donors are expressed as picomoles of mannose transferred per minute per milligram of membrane protein, and the results for MPN are expressed as a percentage of the MPC activity.

MPD-dependent LLO pathway mannosyltransferases (Rush *et al.*, 1993). Thus, MPC was evaluted for extension of $Man_5GlcNAc_2$-P-P-dolichol to $Man_{6-9}GlcNAc_2$-P-P-dolichol in SLO-Lec35 cells, and for comparison in SLO-Lec15 cells. Two methods were used: extension of prelabeled $Man_5GlcNAc_2$-P-P-dolichol with unlabeled MPC; or extension of preexisting unlabeled $Man_5GlcNAc_2$-P-P-dolichol with [^{3}H]MPC. The former method was used in most of the experiments.

Lec15 and Lec35 cells were preincubated with [^{3}H]mannose to generate an endogenous pool of [^{3}H]$Man_5GlcNAc_2$-P-P-dolichol, and then suspended and permeabilized with SLO in a manner similar to that in Figure 7. Parental CHO-K1 cells could not be used in this experiment because it was impossible to limit LLO synthesis to $Man_5GlcNAc_2$-P-P-dolichol. Before MPC treatment a brief preincubation with unlabeled GDP-mannose was used to chase any [^{3}H]$Man_{1-4}GlcNAc_2$-P-P-dolichol intermediates in the SLO-Lec15 and SLO-Lec35 cells to [^{3}H]$Man_5GlcNAc_2$-P-P-dolichol. This created a stable pool of [^{3}H]$Man_5GlcNAc_2$-P-P-dolichol available for the subsequent MPC incubation.

Cells were incubated with different concentrations of MPC for 10 min, and the LLO were recovered and analyzed by HPLC. As shown in Figure 8, upper left, in both SLO-Lec15 and SLO-Lec35 cells incubated with MPC, $Man_5GlcNAc_2$-P-P-dolichol was extended to $Man_{6-9}GlcNAc_2$-P-P-dolichol. The results are displayed graphically in Figure 8, lower left. More [^{3}H]$Man_{6-9}GlcNAc_2$ was recovered from MPC-treated SLO-Lec35 cells than similarly treated SLO-Lec15 cells, although the significance of the difference is unclear. At a fixed MPC concentration of 200 μM, similar incubations were conducted for various times. In Figure 8, the HPLC chromatograms (upper right) and processed data (lower right) are presented. Again, more [^{3}H]$Man_{6-9}GlcNAc_2$ was recovered from MPC-treated SLO-Lec35 cells. These results show that the Lec35 defect does not directly interfere with the enzymatic transfer of mannose from the donor to the acceptor substrate.

SLO-treated Lec35 Cells Do Not Use MPN

Although the critical micellar concentration of MPC has been estimated to be >2.5 mM (Rush and Waechter, 1995), well above the range used in Figure 8, it was still necessary to demonstrate that the effects of MPC were not due to trivial detergent properties that promoted the use of endogenous MPD. It is well known that mammalian dolichol pathway transferases are selective for substrates containing the physiological polyisoprene dolichol, rather than polyprenol, which lacks the saturated α-isoprene unit of dolichol (Rush *et al.*, 1993; D'Souza-Schorey *et al.*, 1994). MPN, a C10 analog of mannose-P-polyprenol (Rush *et al.*, 1993), was found to be an ineffective donor for mammalian LLO mannosyltransferases, whereas it was used efficiently by micrococcal mannan mannosyltransferases (Table 3). Therefore, the C10 derivatives displayed the expected enzymatic stereoselectivity. Because MPC and MPN differ only by the presence or absence of a saturated α-isoprene unit, they should have very similar physical properties. Thus, they were compared to determine whether the effects of MPC were nonspecific. As shown in Figure 9, there was no extension of $Man_5GlcNAc_2$-P-P-dolichol by MPN in SLO-Lec35 cells. When mixed with MPC, MPN had no significant inhibitory effects.

To corroborate that MPC did not act by nonspecific detergent effects, it was reasoned that trace amounts of [^{3}H]MPC, well below the MPC concentrations used in Figure 8, should still result in synthesis of [^{3}H]$Man_{6-9}GlcNAc_2$-P-P-dolichol. Indeed, incubation of unlabeled SLO-Lec15 and SLO-Lec35 cells with 50 pM [^{3}H]MPC yielded [^{3}H]$Man_{6-9}GlcNAc_2$-P-P-dolichol (our unpublished results), although the fraction of endogenous $Man_5GlcNAc_2$-P-P-dolichol that was extended could not be determined. Incubation of SLO-CHO cells also yielded [^{3}H]$Man_{6-9}GlcNAc_2$-P-P-dolichol.

DISCUSSION

This report demonstrates that the Lec35 gene product is required for all four classes of sugar-P-dolichol–dependent reactions known in mammalian cells: GPD-dependent reactions in LLO synthesis, and MPD-dependent reactions in LLO, GPI, and C-mannosyl tryptophan synthesis. In LLO and GPI synthesis only the monosaccharide-P-dolichol requirements in the first reaction of each class could be examined, because completion of the first reaction also promoted the subsequent reactions. Thus, although it is likely that most or all of these individual reactions require the Lec35 gene product(s), this point remains to be proven. The biological consequence of a Lec35 defect would be complex because many proteins normally modified with GPI-anchors (Takeda and Kinoshita, 1995) or C-mannosylation of tryptophan (Hofsteenge *et al.*, 1999; Hartmann and Hofsteenge, 2000) would be affected. Similarly, the synthesis of lipid-linked oligosaccharides, which give rise to asparagine-linked oligosaccharides, would be defective. In the case of N-linked $Glc_3Man_9GlcNAc_2$, both mannose and glucose residues that require Lec35 are involved in ER quality control.

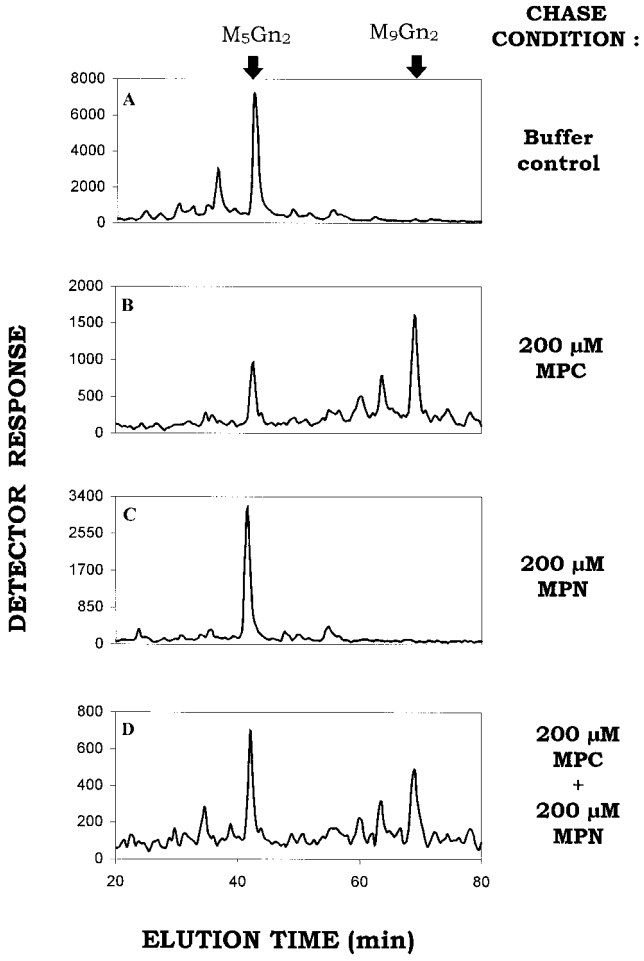


Figure 9. Mannose-P-nerol is not a mannose donor with SLO-treated Lec35 cells. Lec35.1 cells were incubated with [^{3}H]mannose and treated with SLO as in Figure 8. The cells were then incubated in buffer containing 100 μM unlabeled GDP-mannose alone (A), with MPC (B), with MPN (C), or with MPC plus MPN (D), each at a concentration of 200 μM. LLO were then analyzed by HPLC.

For example, on glycoproteins the glucose residue linked α1,3 to mannose is a critical determinant for recognition by the lectin-chaperones calnexin and calreticulin (Ware *et al.*, 1995; Spiro *et al.*, 1996). During LLO synthesis this residue is derived from GPD. The four mannose residues derived from MPD contribute to the efficiency by which this glucose residue is added, both on LLO by the GPD-dependent glycosyltransferase (Chapman *et al.*, 1979; Burda *et al.*, 1999; Cipollo and Trimble, 2000) and on unfolded glycoproteins by the UDP-glucose–dependent glucosyltransferase (Sousa *et al.*, 1992). In addition, the MPD-derived α1,2 linked mannose residue on $Man_9GlcNAc_2$, which is the substrate of the ER mannosidase (Lipari and Herscovics, 1994), is now believed to play a critical role in ER-associated degradation of malfolded proteins (Jakob *et al.*, 1998). Thus, it is not surprising that protein-folding defects were reported in the MadIA214 mutant (Ermonval *et al.*, 2000). As described above, this mutant carries a Lec35 defect. $Glc_3Man_9GlcNAc_2$ is transferred to appropriate asparagine residues on nascent proteins more efficiently than $Man_9GlcNAc_2$ (Turco *et al.*, 1977) and $Glc_3Man_5GlcNAc_2$ is transferred more effectively than $Man_5GlcNAc_2$ (Chapman *et al.*, 1979). Therefore, due to the inability to utilize MPD and GPD, Lec35 mutations might also result in underglycosylation of glycoproteins.

An unexpected outcome was the observation that, under conditions where recombinant Lec35 mRNA was expressed at levels close to those of normal Lec35 mRNA, synthesis of $Man_9GlcNAc_2$-P-P-dolichol was restored but GPD-dependent glucosylation was defective. However, glucosylation was almost completely restored by overexpression of the Lec35 cDNA. There are at least three plausible explanations, all of which postulate that the original SL15 cDNA clone used in these studies encodes a protein that is more effective with MPD than GPD. In one case, although the basal amount of recombinant Lec35 mRNA expressed in the 6C and 10A transfectants was similar to the level that naturally occurs in CHO-K1 cells (Figure 2D), it is possible that the natural transcript might be translated more effectively than the recombinant transcript. Even if the resulting Lec35 protein was selective for MPD, this enhanced translation could account for GPD-dependent reactions observed in normal cells. Unfortunately, the physical properties of Lec35 protein have prevented the development of a sensitive method of measuring its concentration in cells (Anand and Lehrman, unpublished data), so the relative amounts of the natural and recombinant Lec35 proteins could not be compared. A second explanation is that the Lec35 gene might produce two transcripts, perhaps due to alternative processing, encoding proteins selective for either MPD or GPD. Neither transcript would be efficiently produced by the Lec35.1 allele, which has a gross disruption, or the Lec35.2 allele, which produces little or no detectable mRNA. The original SL15 cDNA clone would therefore represent the MPD-selective form, resulting in underglucosylated $Man_9GlcNAc_2$-P-P-dolichol when expressed at basal levels. Yet, when overexpressed this cDNA might also result in some activity with GPD. As described above, no functional alternative mRNAs were detected by either rescreening of the CHO-K1 library or by reverse transcription-PCR, and no high-probability cryptic exons were found in Lec35 introns, so at this time there are no candidates for the proposed GPD-selective form. A third possibility is that overexpression of Lec35p disrupts the ER, restoring glucosylation nonspecifically.

In our hands the swainsonine-concanavalin A selection for $Man_5GlcNAc_2$-P-P-dolichol mutants detected only spontaneous Lec35 mutants (Lehrman and Zeng, 1989), and after chemical mutagenesis (Camp *et al.*, 1993) Lec35 mutants were isolated ~10 times as frequently as Lec9 and Lec15 mutants (our unpublished results). The reason for this bias has remained unclear, but the Southern blot data with Lec35.1 cells suggest an explanation. The repetitive elements in intron 1 and intron 3 would be expected to cause gene disruptions and deletions spontaneously, as they do in familial hypercholesterolemia and β-thalassemia (Lehrman *et al.*, 1987). Thus, although most of the cells in the parental CHO-K1 population should be diploid for Lec35, such gene instability might cause a high percentage of cells to have only a single functional copy of Lec35. After mutagenesis, this would result in a higher fraction of recessive Lec35 mutants than other recessive $Man_5GlcNAc_2$-P-P-dolichol

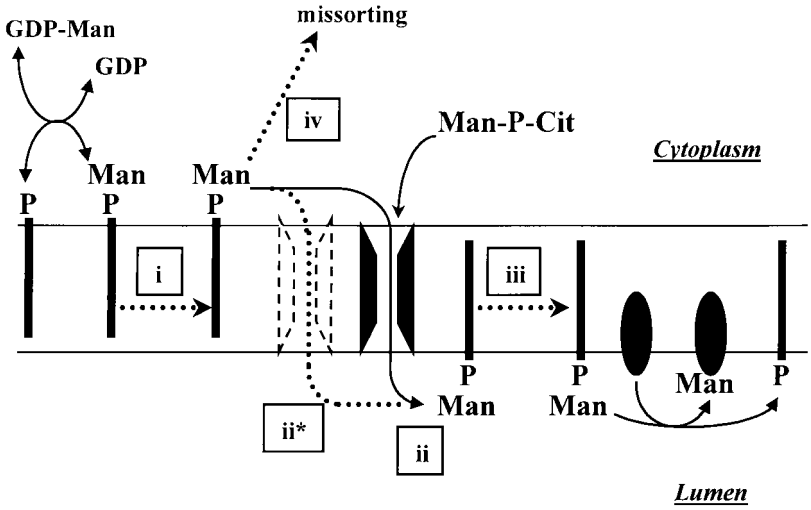


Figure 10. Possible sites of action of Lec35 protein. A schematic representation of MPD-dependent mannosylation reactions in the ER membrane is shown. Lec35 protein (Lec35p) is required for mannosylation of at least three classes of acceptor substrate (LLO, GPI, tryptophan residues of specific proteins) represented as a black oval oriented at the lumenal side of the ER membrane. Reactions that do not require Lec35p directly are shown by solid arrows, whereas reactions that might involve Lec35p are indicated by dotted arrows. The experiments reported here show that the catalytic transfer of mannose from MPD to acceptor does not directly involve Lec35p, and previous work established that MPD synthesis did not require Lec35p. As explained in the INTRODUCTION, any proposed mechanism must have the potential to account for correction of the Lec35 phenotype by physical or chemical perturbation. (i) Lec35p might be involved on the cytoplasmic leaflet in movement or exchange of MPD from MPD synthase to the MPD flippase (indicated by paired trapezoids). (ii) Lec35p might be involved in MPD flippase function, perhaps as the flippase itself, a regulatory subunit, or a regulator of its expression. As discussed in the text, it appears unlikely that Lec35p is involved in the function of the flippase-like activity assayed by MPC transport. However, there still remains a possibility of other uncharacterized MPD flippase activities (ii*) that require Lec35p. (iii) Lec35p might be involved on the lumenal leaflet in movement or exchange of MPD from the flippase to the various mannosyltransferases. (iv) Lec35p might be necessary to prevent missorting of MPD to an enzymatically inactive domain of the ER membrane, or to another organelle.

mutants. Comparison of the hamster Lec35 gene with the homologous human MPDU1 gene sequence reveals that the latter has a very similar exon/intron organization, including similarly aligned *Alu-Sq* repetitive elements in intron 1 and intron 3.[3] Thus, the propensity for gene rearrangements in Lec35 appears to be evolutionarily conserved.

A related issue is the apparent lack of an obvious Lec35 protein homolog in *Saccharomyces cerevisiae* databases, although highly homologous proteins can be found in *C. elegans* (GenBank AAA83473), mouse (GenBank BAA78781), and human (GenBank AAC39875) protein databases, as well as by translation of DNA sequence (GenBank AE003608) from the *D. melanogaster* genome. The extensive efforts of the Robbins, Orlean, and Aebi laboratories have uncovered *S. cerevisiae* mutant strains with defects corresponding to many steps in mammalian LLO synthesis, but no *S. cerevisiae* mutant has yet been identified with a Lec35 phenotype. However, all of these eukaryotes produce $Glc_3Man_9GlcNAc_2$-P-P-dolichol and GPI-anchored proteins. It may be that *S. cerevisiae* has a Lec35 homolog that has gone undetected, or a separate gene that serves a similar function. Because the requirement for Lec35p can be efficiently supplanted by alteration of the membrane environment, *S. cerevisiae* might not require Lec35p because of the size or composition of its ER membrane.

The permeabilized cell studies showed that the inability to utilize MPD in Lec35 mutants was not directly due to an inability to catalyze the transfer of mannose from donor to acceptor. Thus, Lec35 cells do not accumulate a general inhibitor of MPD-dependent reactions. Similarly, these data exclude the possibility that Lec35 mutants have an activity that degrades donors such as MPD and MPC. It was highly unlikely that the Lec35 defect was due to a mutant mannosyltransferase with a sorting or a structural abnormality that could be partially corrected by cellular perturbation, because the LLO, GPI, and C-mannosyl tryptophan reactions were affected. The data presented here formally rule out this possibility. Rather, the in vitro data are most consistent with a defect in the orientation or localization of MPD. Possibilities include 1) restriction from subdomains of the cytoplasmic leaflet containing the MPD flippase; 2) inability to achieve a lumenal orientation (i.e., transverse flipping); 3) restriction from subdomains of the lumenal leaflet that contain the appropriate enzymes and acceptors; or 4) transport of MPD to an inappropriate subdomain or organelle (Figure 10). Such defects might be corrected by perturbation of Lec35 membranes by physical or chemical methods, whereas gentle permeabilization with SLO would have no effect. Although not yet examined directly, it is likely that similar possibilities apply to GPD because GPD utilization is also defective in Lec35 mutants.

Of these possibilities, the inability to achieve a lumenal orientation is perhaps the most provocative as this would suggest a lack of MPD flippase activity. Evidence for the existence of this flippase comes from several sources, as reviewed (Hirschberg and Snider, 1987; Lennarz, 1987). We have demonstrated in mouse liver microsomes a transporter of MPC that has the expected properties of a MPD flippase (Rush and Waechter, 1995), including saturability, stereoselectivity, sensitivity to proteolytic digestion, enrichment in the ER, and dependence upon an intact permeability barrier. A corresponding activity was demonstrated for glucose-P-citronellol, supporting the existence of a separate glucose-P-

[3] Coding regions of the human MPDU1 gene (counterpart of the hamster Lec35 gene) can be identified at these nucleotides in human chromosome 17 genomic sequence AC007421 (GenBank): exon 1, 6293–6189; exon 2, 4418–4359; exon 3, 4207–4074; exon 4, 3463–3374; exon 5, 3223–3139; exon 6, 2988–2880; exon 7, 2729–2601. Repetitive elements are as follows: in intron 1, *Alu-Sq*, 5382–5095 and *Alu-Sx*, 4838–4548; in intron 3, *Alu-Sq*, 3928–3645.

dolichol flippase (Rush *et al*., 1998). However, despite several attempts it was not possible to directly assess the MPD flippase status in Lec35 mutants. For example, direct attempts to measure MPC transport in SLO-permeabilized cells were complicated by high background binding of [^{3}H]MPC, as demonstrated with nonpermeabilized cells. Similar rates of [^{3}H]MPC uptake were measured with isolated CHO-K1, Lec15, and Lec35 microsomal membranes (our unpublished results). However, interpretation of this result was complicated by the fact that methods used to prepare the microsomes also correct the Lec35 phenotype. MPC is unable to cross a nonbiogenic membrane at appreciable rates (Rush and Waechter, 1995). Yet, as judged by formation of LLO products (Figure 8), a significant amount of MPC was able to enter the ER of SLO-Lec35 cells in a concentration range (100–900 μM) that is comparable to the estimated K_d of the MPC transporter (660 μM). This raises the possibility that the MPD flippase-like activity defined by MPC transport is unaffected by the Lec35 mutation (step ii, Figure 10), although it cannot be ruled out that the Lec35 defect might alter some other unknown MPD flippase (step ii*). Current efforts are directed at recombinant expression of Lec35p, with the goal of incorporating Lec35p into synthetic vesicles for MPC transport studies. Preliminary studies (Anand and Lehrman, unpublished data) suggest that Lec35p is extremely hydrophobic, and is refractory to detection by conventional immunological and electrophoretic methods. Although this prevented further analyses of Lec35p, these observations are consistent with a role for Lec35p that involves intimate contact with other hydrophobic components of the ER membrane.

In summary, the results with SLO-treated cells and MPC demonstrate that the Lec35 mutation inhibits utilization of MPD, but has no direct effect on the enzymatic transfer of mannose from the donor substrate to the acceptor substrate. This indicates that Lec35p has a role in MPD orientation or localization that is essential for its activity as a donor substrate. It is likely that a product of the Lec35 gene has a similar function in the utilization of GPD, and perhaps other sugar-P-dolichols that remain to be discovered.

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