Monoglycosylated polyisoprenol phosphates are essential sugar donors for prokaryotic and eukaryotic glycoconjugates (1). Eukaryotes use three such molecules, mannose-P-dolichol (MPD) (2), glucose-P-dolichol (GPD), and GlcNAc-P-P-dolichol (GnPPD). All three are required for the synthesis of 14-sugar dolichol-P-linked oligosaccharides, the precursors of asparagine-linked oligosaccharides, contributing 4, 3, and 1 residues, respectively (2). MPD is also required for synthesis of glycosylphosphatidylinositol (GPIs), the precursors of GPI anchors, and donates all 3 mannose residues in GPIs (3). An essential issue is the coordination and regulation of the enzymes that synthesize these three sugar donors, all of which compete for the same limited pool of dolichol-P (4). Isolation and characterization of recombinant DNAs encoding these enzymes represent important steps toward resolving this issue. cDNAs or genes encoding GlcNAc-1-P transferase, the enzyme responsible for GnPPD synthesis, have been cloned from Saccharomyces cerevisiae (ALG7) (5, 6). S. pombe (7), Leishmania (8), hamster (9, 10), and mouse (11). In contrast, the genes encoding the MPD (ALG1) (12) and GPD (ALGS) (13) synthases have been isolated only from S. cerevisiae. Thus, studies of the regulation of these enzymes in animal cells have been impeded.

To advance our understanding of MPD synthase (MPDS) regulation in animals, we designed an expression cloning strategy to isolate a hamster cDNA that could correct the defect in Lec15 Chinese hamster ovary (CHO) cell mutants. Cells of the Lec15 genotype lack MPDS activity in vitro (14) and, accordingly, accumulate Man5GlcNAc2-P-P-dolichol (15) and GlcNAc-acyl (16) in vivo. Thus, there is a good possibility that Lec15 cells lack MPDS or perhaps an accessory protein needed for MPDS activity. This report describes studies of a novel hamster suppressor of Lec15 cells, termed SL15 for suppressor of Lec15.

EXPERIMENTAL PROCEDURES

General Methods—Cell culture (17), microsomal membrane isolation (18), MPDS assays (18), thin layer chromatography (16), lipid-linked oligosaccharide preparation (18), high-pressure liquid chromatography (19), and all materials needed for these methods were described earlier. Preparation and Characterization of Polyoma Large-T antigen Transfected Lec15 Mutants—A MPDS synthase-deficient mutant line of the Lec15 genotype (termed Lec15.2) was isolated by selection of ethylmethanesulfonate-treated CHO-K1 cells with conA/swainsonine (16).

The MPDS deficiency was verified biochemically (16), and the complementation group was confirmed as Lec15 (20). CaPO4 (21) was used to co-transfect pSVE1-B1a encoding the polypoma virus large T-antigen (22) with pBl3 encoding a hygromycin resistance gene (20). Hygromycin-resistant colonies were picked (20) and screened for large T-antigen function by expression of G418 resistance after electrophoration of pcDNA/neo (Invitrogen Corp.), which contains the polypoma virus origin of replication. The colony with the highest transfection efficiency (termed pTLec15.2D6 and referred to as pTLec15 throughout this paper) was used for expression cloning. Lec35 cells (16) were transfected with polypoma large T-antigen and prepared as described for pTLec15 cells, yielding pTLec35 cells.

Expression Cloning of pLW1 in pTLec15 cells, Sequencing, and Biochemical Analysis—A unidirectional CHO-K1 cell cDNA library of 1.6 × 109 primary recombinants in the pcDNA vector was produced from Invitrogen Corp. (catalogue number A950-01, lot number 100930). The library (40 μg of DNA) was electroporated into 5 × 106 pTLec15 cells (600 V, 71 microfarads, 50 mA, 50 watts, infinite resistance) with an Invitrogen electroporator. The cells were selected in medium (see Fig. 1 legend) with 20 μg/ml G418 and 1 μg/ml conA (23). Surviving cells were re-screened with conA (no G418) to remove the Lec15/Lec1 double mutants (19), requiring 1 week. DNA was recovered from surviving cells by the Hirt procedure (23). The DNA preparation was enriched for a single plasmid at this point, and this was introduced into Escherichia coli strain MC1061/P3 by electroporation. Ten transformed...
FIG. 1. Selections used to study mannosylation mutants. 200 CHO-K1 cells of the indicated type (normal, ptLec15.2D6, Lec35/Lec1 (19)) were plated in wells of a 24-well plate with 1.5 ml of Ham's F-12 medium buffered at pH 7.2 with 20 mm Na-HEPES, serum (10% fetal bovine serum for conA and controls, 3% calf serum for PHA), 100 units/ml penicillin, and 100 µg/ml streptomycin plus, where indicated, 1 µg/ml swainsonine (Sw) and/or 10 µg/ml concanavalin A (conA) or 20 µg/ml phytohemagglutinin E (PHA). Colonies were stained after 7 days of growth at 37°C in a humidified 5% CO₂ atmosphere. Lec35/Lec1 cells were expected to behave the same as Lec15/Lec1 in these selections.

E. coli colonies picked at random all had the same plasmid (however, see “Results and Discussion”) which was termed pLW1.

Restriction fragments of the cloned insert of pLW1 were ligated into pUC18 and pUC19 and sequenced on both strands using specific primers with fluorescent terminators on an Applied Biosystems Model 373A Sequencer.

For biochemical experiments, pLW1 was transfected into ptLec15 or ptLec35 cells. After selection with PHA/Sw, there were many resistant colonies which were pooled for further use. No colonies were typically obtained with PHA/Sw selection if vector alone was transfected (data not shown), although in rare cases a presumed Lec15/Lec1 or Lec35/Lec1 double mutant was detected.

RESULTS AND DISCUSSION

Expression Cloning—The method employed was first suggested by Heffernan and Dennis (22) and subsequently used by others to clone cDNAs encoding several proteins involved in glycoconjugate synthesis (24–28). In this method, cells with recessive mutations are engineered to express polyoma virus large T-antigen. This allows plasmids containing the polyoma virus origin of DNA replication to replicate freely without incorporation into the genome. Upon transfection of a library of such plasmids and selection for correction of phenotype, freely replicating plasmids are easily recovered and enriched by re-transfection if necessary.

To obtain a Lec15 suppressor cDNA we used a Lec15 CHO-K1 mutant line, isolated previously in this laboratory (16), which lacked MPD activity in vitro and accumulated both Man₉GlcNAc₂-PP-dolichol and GlcNAc-(acyl)P1 in vivo. This line was stably transfected with the polyoma virus large T-antigen and used for expression cloning with a CHO-K1 cDNA library as described under “Experimental Procedures.” To isolate positive clones, the transfecants were selected with a mixture of PHA/Sw as described earlier (19). This selection relies upon the observation that loss of MPD products in truncated Man₉GlcNAc₂ dolichol-linked oligosaccharides. After transfer to protein, such oligosaccharides do not require the action of Golgi mannosidase II for processing from high mannose (conA binding) into complex (PHA-binding) oligosaccharides. Thus, cells lacking MPD are resistant to the effects of Sw, a specific inhibitor of Golgi mannosidase II. Cells can be selected for or against the ability to synthesize MPD depending on whether Sw, PHA, and/or conA are added (29). To aid the reader, these selections are demonstrated with normal CHO cells and Lec15 mutants in Fig. 1. These methods are also applicable to other mutations resulting in Man₉GlcNAc₂-PP-dolichol accumulation, such as Lec9 and Lec35 (20). To simulate the anticipated properties of Lec15/Lec1 double mutants, which were expected to interfere with expression cloning, Fig. 1 also includes a Lec35/Lec1 double mutant (19).

Colonies resistant to PHA/Sw were readily isolated after transfection of the CHO-K1 library into ptLec15. These could have been (i) positive transfecants, i.e. Lec15 cells carrying MPDS cDNA or a suppressor cDNA; (ii) genotypic revertants of the Lec15 mutation; or (iii) Lec15/Lec1 double mutants. Indeed, the PHA/Sw-resistant population included a large fraction of presumed Lec15/Lec1 double mutants which were routinely detected and removed by counter-selection with 10 µg/ml conA. Analysis of dolichol-linked oligosaccharides (data not shown) in surviving cells indicated a phenotypic correction, consistent with possibilities i or ii. Plasmids were recovered by the Hirt procedure (23) and analyzed after electroporation into Escherichia coli MC1061/P3 cells.

The Hirt DNA and plasmid preparations from individual E. coli colonies all contained the same cDNA species, eliminating the need for further rounds of enrichment or “sib” selection. The plasmid carrying the cDNA, termed pLW1, efficiently corrected the Lec15 phenotype (see below) whereas the vector did not, demonstrating that possibility i was correct. The functional insert cDNA of plW1 was 1.3 kb and designated SL15 for suppressor of Lec15. It was noted, however, that pLW1 had a tendency to rearrange in E. coli (data not shown). The transfected E. coli MC1061/P3 colonies had plasmids with inserts of 1.3 kb and 1.6 kb in various ratios. By isolating and reintroducing these plasmids into E. coli MC1061/P3 and ptLec15, it was determined that the 1.3-kb insert, but not the 1.6-kb insert, corrected the Lec15 phenotype and that the 1.3-kb insert rearranged irreversibly into the 1.6-kb species. Use of E. coli DH10B/P3 (Life Technologies, Inc.), a strain similar to MC1061/P3 but lacking various genes associated with DNA restriction and rearrangement, gave the same results as E. coli MC1061/P3 (data not shown).

Biochemical Effects of SL15 Expression in Lec15 Cells—pLW1 corrected the accumulation of Man₉GlcNAc₂-PP-dolichol in ptLec15 cells (Fig. 2, A–C). The predominant oligosaccharides (C) were all of the Glc₀–3Man₉GlcNAc₂ type. Expression of MPDS activity was examined in vitro with microsomal membranes as shown in Fig. 3. MPDS activities in pLW1-transfected ptLec15 cells were essentially normal and greatly in-
creased over untransfected ptLec15 cells. In addition, the principal lipid product made by membranes from pLW1-transfected ptLec15 cells (Fig. 4, lane 3) comigrated with MPD from normal membranes (Fig. 4, lane 1). None of the missing MPD activity of ptLec15 membranes was restored by mixing with T-ptLec15 membranes (Fig. 3), indicating that SL15 did not behave as a soluble trans-acting factor. When assayed under conditions similar to those in Fig. 3, the GPD and GnPPD synthases were not significantly altered in T-ptLec15 membranes compared with control or ptLec15 membranes (data not shown). This indicated that SL15 was not a general transferase activator or a promiscuous transferase.

Suppression of the Lec35 Mutation—Lec35 CHO-K1 mutants synthesize normal levels of MPD in vivo and in vitro, but fail to utilize it efficiently in vivo (29, 30). Thus, Lec35 cells accumulate both Man₉GlcNAc₂-PP-dolichol and GlcN-(acyl)PI (16). However, these molecules are readily mannosylated in vitro in Lec35 membranes. This has led to the idea that MPD may fail to be flipped across the ER membrane or be transported efficiently in Lec35 cells, such that the mechanical disruption associated with membrane isolation could weaken physical barriers and enhance mobility of MPD.

Lec35 cells transfected with SL15 survived selection in PHA/Sw (data not shown) and, as indicated in Fig. 2, D and E, synthesized Glc₀–3Man₉GlcNAc₂. To determine whether Man-GlcN-(acyl)PI synthesis was also restored, we took advantage of a previously described method (16) for examining the formation of [³H]Man-GlcN-(acyl)PI in Lec35 membranes from endogenous GlcN-(acyl)PI, with [³H]MPD generated with exogenous GDP-[³H]mannose. Lec35 cells failed to mannosylate GlcN-(acyl)PI in vivo and yielded membranes which made abundant amounts of [³H]Man-GlcN-(acyl)PI (Fig. 4, lane 4). Membranes from SL15-transfected Lec35 cells (lane 5) had the same properties as normal membranes (lane 1), indicating correction by SL15.

Sequence Analysis—The effects of SL15 in Lec15 and Lec35 cells could be explained if MPD synthesis was increased in vivo, correcting the Lec15 defect directly and overriding the defect in Lec35 by mass action. It was therefore of interest to determine whether the SL15 protein resembled the MPD synthase from S. cerevisiae encoded by the DPM1 gene. As shown in Fig. 5, the SL15 cDNA included 1285 nt without the poly(A) tail and encodes a protein of 248 residues. Possible transmembrane segments (underlined) are located from residues 130 to 157 and 211 to 236, and a potential ER retention signal (italics) was found at residues 245–248.

Leu35 membranes. Microsomal MPD activity was assayed in vitro with GDP-[²H]mannose at 37 °C as described (29), except that the volume was 0.2 ml, the incubation time was 10 min, and no greater than 10 μg of membrane protein was used per assay. The 10-min time point was in the linear range (data not shown). CHO-K1, ptLec15, and T-ptLec15 membranes were assayed alone or in combination in the quantities indicated. Each bar represents the mean of 6 independent determinations ± S.E. Assays were performed with either endogenous dolichol-P alone (upper panel) or supplemented with 0.4 μg/assay exogenous dolichol-P (lower panel), which was shown to be saturating (data not shown). A 0.7 mmol stock of dolichol-P (Sigma) was prepared in 0.35% (w/v) Nonidet P-40 and added to reaction mixtures containing microsomes. The resulting amount of Nonidet P-40 (0.001%) by itself did not affect MPD activity with endogenous dolichol-P (data not shown).
and a pl of 8.65. The amino-terminal sequence contains a segment of 8 apolar residues (residues 12–19) preceded by 2 positively charged residues (residues 10 and 11), a common feature of cleaved eukaryotic leader sequences (32). However, since the 8 residues following the apolar segment do not conform to the “-1,-3” rule (32), it is doubtful that this is a true cleaved leader sequence. There are two hydrophobic segments in the SL15 protein long enough to span the membrane, from residues 130 to 157 and residues 211 to 236. Applying the rules of Hartmann et al. (33), a speculative structure for the SL15 protein would have cytosolic amino and carboxyl termini with the two membrane spanning regions connected by a luminal loop (Fig. 6). In accord with this model, there are lysines at positions 245–246, in excellent agreement with the consensus for an elongated endoplasmic reticulum retention signal which are oriented toward the cytoplasm (34). An attractive feature of this model is that the bulk of the SL15 protein would face the cytosol, similar to the orientation proposed for S. cerevisiae MPDS (35) and other early reactions of oligosaccharide synthesis (36).

A BLAST search of combined nucleic acid data bases revealed a number of human EST sequences with apparent homology to the SL15 cDNA (accession numbers H27246, H51950, H77811, R04750, T16638, T81897, T81898) (32). However, since the 8 residues following the apolar segment do not conform to the “-1,-3” rule (32), it is doubtful that this is a true cleaved leader sequence. There are two hydrophobic segments in the SL15 protein long enough to span the membrane, from residues 130 to 157 and residues 211 to 236. Applying the rules of Hartmann et al. (33), a speculative structure for the SL15 protein would have cytosolic amino and carboxyl termini with the two membrane spanning regions connected by a luminal loop (Fig. 6). In accord with this model, there are lysines at positions 245–246, in excellent agreement with the consensus for an elongated endoplasmic reticulum retention signal which are oriented toward the cytoplasm (34). An attractive feature of this model is that the bulk of the SL15 protein would face the cytosol, similar to the orientation proposed for S. cerevisiae MPDS (35) and other early reactions of oligosaccharide synthesis (36).

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Expression cloning of a novel suppressor of the Lec15 and Lec35 glycosylation mutations of Chinese hamster ovary cells.

Felecia E. Ware and Mark A. Lehrman

This paper reported the cloning of a cDNA for a new gene, which we termed SL15. We reported that SL15 cDNA corrected two separate glycosylation-defective cell lines known as Lec15 and Lec35. In experiments to be reported in the future we have found that the Lec35 cells used in that study have a defect in the SL15 gene. This provides a clear explanation for correction of Lec35 cells by SL15 cDNA, a result that has now been obtained by three separate individuals in this laboratory as well as by another laboratory (T. Kinoshita, personal communication). However, similar attempts to repeat the correction of Lec15 cells by SL15 cDNA have consistently failed. Based upon further analysis of cell samples from that study, we now conclude that the Lec15 population was contaminated with Lec35 cells. Lec15 cells described in Figs. 2, 3, and 4 as being transfected with SL15 cDNA and having normal glycosylation phenotypes have now been shown to be Lec15-Lec35 hybrids, most likely formed during electroporation. It is likely that SL15 cDNA was carried by contaminating Lec35 cells during expression cloning and that the contaminating Lec35 cells accounted for the greater number of colonies that survived lectin selection after transfection with SL15 cDNA as compared with vector controls.

Since there is no evidence that SL15 cDNA can correct the Lec15 phenotype certain aspects of our paper must be rescinded, specifically Fig. 2, panel C; Fig. 3, all data with T-plLec15 microsomes; Fig. 4, lane 3; and all sections of the text indicating that SL15 can correct Lec15 cells. However, we remain confident that the correction of Lec35 cells by SL15 cDNA is a valid result. We sincerely apologize to the readers of this journal for any confusion or inconvenience this may have caused.

Specific substitutions at amino acid 256 of the sarcoplasmic/endoplasmic reticulum Ca\(^{2+}\) transport ATPase mediate resistance to thapsigargin in thapsigargin-resistant hamster cells.

Myounghee Yu, Lilin Zhong, Arun K. Rishi, Mohammed Khadeer, Giuseppe Inesi, and Arif Hussain

Dr. Zhong's name was misspelled. It is correct in the author line above.

Identification of the 170-kDa melanoma membrane-bound gelatinase (seprase) as a serine integral membrane protease.

Mayra L. Piñeiro-Sánchez, Leslie A. Goldstein, Johannes Dodt, Linda Howard, Yunyun Yeh, Huan Tran, W. Scott Argraves, and Wen-Tien Chen

Page 7595: Drs. Tran and Argraves were omitted. The corrected author line is shown above.

Pages 7597 and 7600: Due to an error in assembling Figs. 1F and 7C, the two D28 blot lanes in Fig. 1F were mounted upside down and Fig. 7C was mistaken as LOX melanoma seprase but it was actually derived from placental seprase data. The former had been shown previously (Monsky, W. L., Lin, C.-Y., Aoyama, A., Kelly, T., Mueller, S. C., Akiyama, S. K., and Chen, W.-T. (1994) Cancer Res. 54, 5702–5710), and the latter should be referenced with similar figures derived from two LOX seprase experiments done on July 9, 1992 and January 15, 1993. In all of these changes, there is no new scientific information added or changed, and there is no change in the conclusions of the paper.

We suggest that subscribers photocopy these corrections and insert the photocopies at the appropriate places where the article to be corrected originally appeared. Authors are urged to introduce these corrections into any reprints they distribute. Secondary (abstract) services are urged to carry notice of these corrections as prominently as they carried the original abstracts.