SRD5A3 Is Required for Converting Polyprenol to Dolichol and Is Mutated in a Congenital Glycosylation Disorder

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SUMMARY

N-linked glycosylation is the most frequent modification of secreted and membrane-bound proteins in eukaryotic cells, disruption of which is the basis of the congenital disorders of glycosylation (CDGs). We describe a new type of CDG caused by mutations in the steroid 5α-reductase type 3 (SRD5A3) gene. Patients have mental retardation and ophthalmologic and cerebellar defects. We found that SRD5A3 is necessary for the reduction of the alpha-isoprene unit of polyprenols to form dolichols, required for synthesis of dolichol-linked monosaccharides, and the oligosaccharide precursor used for N-glycosylation. The presence of residual dolichol in cells depleted for this enzyme suggests the existence of an unexpected alternative pathway for dolichol de novo biosynthesis. Our results thus suggest that SRD5A3 is likely to be the long-sought polyprenol reductase and reveal the genetic basis of one of the earliest steps in protein N-linked glycosylation.

INTRODUCTION

N-glycosylation occurs on certain asparagine residues present on nascent polypeptides in all eukaryotic cells. The glycan structures resulting from this process show an incredible variability depending on the protein, cell type, and species. This essential posttranslational modification occurs on most secreted and plasma membrane proteins and is involved in protein folding and trafficking with implications for cell-cell and cell-matrix interactions and intracellular signaling (Freeze, 2006; Helenius and Aebi, 2001). The process of N-linked protein glycosylation is localized in the endoplasmic reticulum (ER) and the Golgi
compartment. Three separate phases can be distinguished: First, an oligosaccharide precursor, a block of 14 monosaccharides (Glc3Man9GlcNAc2), is assembled on the lipid carrier dolichol-phosphate (Dol-P) in the ER membrane. Second, this glycan is transferred cotranslationally or posttranslationally to dedicated asparagine residues of nascent glycoproteins (Ruiz-Canada and Danikiewicz, 2009). In this reaction, oligosaccharyltransferase (OST) recognizes the acceptor sequence NX[S/T] (where X can be any amino acid except proline) on nascent polypeptides and catalyzes the transfer of the glycan precursor en bloc from its lipid carrier to the protein (Chavan and Lennarz, 2006). Third, the N-linked glycan is further modified by a series of trimming and elongation reactions beginning in the ER and ending in the late Golgi compartment.

The early steps of this pathway are present not only in eukaryotic cells but also in archae and bacteria, all relying on a lipid to build an oligosaccharide precursor (Jones et al., 2009). This carrier lipid, a polyisoprenoid, is assembled from a variable number of isoprene units, linked head to tail. The length of the carrier polyisoprenoid varies across evolution: bacteria posses a single predominant polyisoprenol, usually undecaprenol (composed of 11 isoprene units), but in eukaryotic cells these lipids typically occur as mixtures of different lengths, depending upon the species. In mammalian cells, dolichols are predominantly 18–21 isoprene units in length.

A requirement in eukaryotic organisms is the reduction of the precursor polyisoprenol to dolichol on the terminal isoprene unit (alpha) (Swiezewska and Danikiewicz, 2005), followed by phosphorylation to generate Dol-P. The identification of Dol-P as a glycosyl carrier lipid in glycosylation was described 40 years ago (Behrens and Leloir, 1970), but the role of the free lipid, as glycosyl carrier lipids in glycosylation was described 40 years ago (Behrens and Leloir, 1970), but the role of the free lipid, as glycosyl carrier lipids in glycosylation was described 40 years ago (Behrens and Leloir, 1970), but the role of the free lipid, as glycosyl carrier lipids in glycosylation was described 40 years ago (Behrens and Leloir, 1970), but the role of the free lipid, as glycosyl carrier lipids in glycosylation was described 40 years ago (Behrens and Leloir, 1970), but the role of the free lipid, as glycosyl carrier lipids in glycosylation was described 40 years ago (Behrens and Leloir, 1970), but the role of the free lipid, as glycosyl carrier lipids in glycosylation was described 40 years ago (Behrens and Leloir, 1970), but the role of the free lipid, as glycosyl carrier lipids in glycosylation was 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Consequently, we screened 38 patients with CDG type I-x (CDG type I negative for known gene mutations), and we identified five other independent homozygous or compound heterozygous mutations and one genomic rearrangement (Figure 1F, Table 1, and Figure S1). Among the mutations identified were a 2 bp deletion and four single-base substitutions resulting in stop codons. Additionally, patient AK0295 carried a homozygous truncation of the gene encompassing within exon 5, in the 3' part of SRD5A3 open reading frame (ORF). Further expression analysis in patients' fibroblasts showed partial nonsense-mediated messenger RNA (mRNA) decay of SRD5A3 transcript in some patients (Figure 1G).

Based on the phenotype of 11 children from seven families, the most striking features observed were the presence of congenital eye malformations with variable degree of visual loss, nystagmus, muscle hypotonia, motor delay, mental retardation, and facial dysmorphism. Microcytic anemia, elevated levels of liver enzyme activities, coagulation abnormalities, and decreased antithrombin III levels were detected in nine evaluated cases. Most children presented with ocular coloboma or hypoplasia of the optic disc (unique features in the CDGs) (Morava et al., 2009), with striking cerebellar atrophy or vermis malformation. Ichthyosiform erythroderma or dry skin and congenital heart malformations were sporadically present. Midline malformations and endocrine anomalies were only present in the index patients (Al-Gazali et al., 2008) (Table 1). The relative uniformity in the biochemical and clinical phenotype associated with frequent early truncating mutations suggests loss-of-function of SRD5A3 as the genetic mechanism.

**Patients with Mutation in SRD5A3 Show an Early Defect in Lipid-Linked Oligosaccharide Synthesis**

The absence of whole glycan chains on proteins indicated that the metabolic block occurred early in the N-glycosylation pathway, altering synthesis or transfer of the glycan part of lipid linked oligosaccharide (LLO), to recipient proteins. Epitope tagged SRD5A3 localized predominantly to the ER (Figure 2B), where LLO synthesis occurs (Aebi and Hennet, 2001).

An abnormal composition of the glycan precursor impairs its transfer to acceptor proteins. Accordingly, we investigated the size of the LLO glycols using HPLC after [2-3H]-mannose metabolic labeling with fibroblasts from index patients CVH-385-IV-11 and CVH-385-IV-13. Since no major structural abnormalities in LLO were detected (Figure S2E), we also determined the amount of radiolabeled LLO. We detected a severe reduction in the amount of newly synthesized LLO in four of the five patients tested compared to three control cell lines (Figure 2C), suggesting that the N-glycosylation block occurs prior the glycan transfer step.

The reduced levels of LLO could be explained by a limited availability of Dol-P. To test this hypothesis, we used an in vitro assay to assess the production of Dol-PP-GlcNAc1 and Dol-PP-GlcNAc2, the first two reactions of LLO synthesis. With fibroblast homogenates used as a source of enzyme and UDP-[14C]GlcNAc as glycosyl donor, all SRD5A3 deficient patient samples showed a reduced synthesis of Dol-PP-GlcNAc1/2 without addition of exogenous Dol-P, compared to controls (Figure 2D). However, when exogenous Dol-P was added to the incubation mixture, formation of Dol-PP-GlcNAc1/2 was increased to levels comparable with or even higher than controls. Fibroblasts from patients with other known CDG-I defects (CDG-ik or CDG-lo) behaved comparable to controls, showing no evidence of Dol-P mediated rescue (Figure 2D). Elongation of Dol-PP-GlcNAc2 to Dol-PP-GlcNAc2-Man9 was unremarkable. Similarly, OST activity was normal (data not shown). Altogether, the rescue of the enzymatic GlcNAc transferases deficiencies by exogenous Dol-P indicates that the amount of Dol-P is limiting in the patients’ fibroblasts and suggests a defect in Dol or Dol-P biosynthesis.

**SRD5A3 Is the Human Ortholog of the Yeast DFG10/YIL049W Gene**

A yeast mutant for the DFG10 gene, called dfg10-100, was previously isolated by a genetic screen for mutant strains defective for filamentous growth (dfg), using insertional mutagenesis (Mösch and Fink, 1997). The product of this gene shows 25% amino acid identity and 43% similarity with the human SRD5A3 protein (Blastp, NCBI). To determine whether DFG10 is the yeast ortholog of SRD5A3 (Figure 3A), we first asked whether the dfg10-100 mutant displays a lack of N-glycan modifications. Carboxypeptidase Y (CPY) is a secreted enzyme with a mature form that contains four N-glycan sites, all of which are occupied under optimal growth conditions (Hasilik and Tanner, 1978) and all of which can be removed with PNGase F treatment. In contrast with the WT strain (L5366), the dfg10-100 mutants (diplot and homogygous at the DFG10 locus) produced hypoglycosylated CPY, with the detection of the tri- and monoglycosylated CPY (Figure 3B). Because the dfg10-100 mutant is a result of a transposon insertion into the DFG10 promoter, it was possible that still some protein was expressed, thus accounting for the nonlethal phenotype. This possibility was excluded by engineering a deletion of the whole DFG10 ORF, which produced an identical growth delay and CPY phenotype (Figure S3). The identification of a similar biochemical defect in yeast and human suggests a conserved function for SRD5A3 across evolution.

In human, five partially homologous genes compose the steroid 5x-reductase family, including the well-characterized SRD5A1, SRD5A2 involved in testosterone reduction (Russell and Wilson, 1994), encoding for proteins targeted in treatments against prostatic hyperplasia and male pattern hair loss (Aggarwal et al., 2010), and also SRD5A2L2, GPSN2, SRD5A3 that are less characterized. Bioinformatics comparison showed that the DFG10 sequence shares most identity with SRD5A3 (BLASTP, E value = 2e-13) whereas SRD5A1, SRD5A2, SRD5A2L2 and GPSN2 show E values of, respectively 6e-04, 3e-04, 3e-04 and 5e-05 (Figure 3A). To test for functional conservations we expressed each mammalian ORF under the control of a strong constitutive yeast promoter (Alber and Kawasaki, 1982) in the dfg10-100 mutant. The mutant transformed with yeast DFG10 showed a full correction of the CPY underglycosylation. Furthermore, SRD5A3 was the only homolog able to rescue the phenotype (Figure 3C). This experiment shows that SRD5A3 is the diverged human ortholog of the yeast DFG10 gene and suggests a specific role for SRD5A3 in protein glycosylation compared with other family members.
Figure 1. Identification of Mutations in the SRD5A3 Gene in Patients with Multisystemic Syndrome Including Cerebellar Hypoplasia

(A) Pedigree of family CVH-385 showing several levels of consanguinity with cousin marriages. The two branches each produced two affected offspring represented by filled symbols in generation IV.

(B) Whole-genome analysis of linkage results with chromosomal position (x axis) and multipoint LOD score (y axis) showing a peak LOD score of 4.2 on chromosome 4 (arrowhead).

(C) Expanded view of the candidate interval on chromosome 4q12, containing 42 candidate genes including SRD5A3 (red), spanning 25.5 kb of genomic DNA with 5 exons. A mutation in exon 2 was identified in family CVH-385.
Phylogenetic analysis of proteins with a steroid 5α-reductase domain from multiple species indicates that this steroid reductase family can be separated in three main groups consisting of (1) the SRD5A1-SRD5A2 group, (2) the SRD5A3 group containing DFG10, and (3) the GPSN2-SRD5A2L2 group (Figure S3), supporting the idea that different classes of lipids can be substrates for these enzymes and suggesting that the substrate of the enzyme coded by the common ancestral gene was potentially not a steroid.

Table 1. Clinical Phenotype Associated with SRD5A3 Mutations

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<th>08-0904</th>
<th>07-0153</th>
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NA, data not available.

(©) DNA sequence of exon 2 of SRD5A3 from a control individual, an obligate carrier, and an affected family member from CVH-385. The mutation consists of a 3 bp deletion associated with a 10 bp insertion, resulting in a frame shift and premature termination at amino acid 96 of 318, within the second of six transmembrane domains.

(E) Brain MRI midline sagittal view showing cerebellar vermis hypoplasia (red arrowhead) in SRD5A3 mutated patients.

(F) Topology model of SRD5A3 with mutations indicated and six transmembrane domains. Mutations were scattered throughout the ORF, all leading to predicted protein termination before the steroid reductase domain (in red).

(G) Amplification of the SRD5A3 transcript by RT-PCR with RNA extracted from controls and patient fibroblasts. The expression level of the gene is lower in almost all patients CVH-385-IV-13, CVH-385-IV-11, 07-0153 compared to control, suggesting nonsense-mediated mRNA decay. No expression was detected in patient AK0295, as a result of a homozygous genomic rearrangement. RT−, no reverse transcriptase; water, no cDNA.

See also Figure S1.
Figure 2. SRD5A3 Mutated Patients Have a Congenital Disorder of Glycosylation Type I Caused by a Defect in LLO Synthesis, Rescued In Vitro with Exogenous Dolichol Phosphate

(A) Mass spectra of transferrin, normally N-glycosylated on two sites, Asn-432 and Asn-630 (control). Transferrin containing a single N-glycan in SRD5A3 patient samples was increased, indicating a CDG type I disorder. An example of transferrin profile from CDG type II patient, with two glycan chains but abnormal structure (depicted by lack of certain sugar moieties), is shown for comparison.

(B) Intracellular localization of SRD5A3 containing a N terminus DsRed tag (center panel) in COS7 cells costained with antibody against ER-specific marker, calreticulin, ERGIC-specific marker ERGIC53, and Golgi-specific marker Giantin. DsRed-SRD5A3 colocalized with most of the ER whereas Giantin staining did not colocalize. The scale bar represents 10 μm.

(C) Incorporation of [3H]-mannose into LLO after labeling of human fibroblasts. The results indicate severely reduced levels of LLO in four out of five patient samples. Error bars represent the mean ± standard deviation from three experiments.

(D) Rescue of LLO precursor levels with exogenous Dol-P. GlcNAc transferase activity in fibroblasts was measured. Microsomal fractions from fibroblasts were incubated with radioactive GlcNAc, and then Dol-PP-GlcNAc₃ formation was analyzed by TLC. Extracts from patients’ fibroblasts produced a reduced amount of Dol-PP-GlcNAc₃. However, the addition of exogenous Dol-P rescued this defect.

See also Figure S2.
**Srd5a3 Mutation Is Lethal in Mouse and Results in an Activation of the Unfolded Protein Response Pathway**

We found that mice homozygous for a LacZ gene trap (Gt) insertion in intron 3 of Srd5a3 were recovered at embryonic stages, up to embryonic day 12.5 (E12.5) but not beyond (Figures 4A and 4B). At E10.5, Srd5a3Gt(betaGeo)703Lex/Gt(betaGeo)703Lex embryos (abbreviated Srd5a3Gt/Gt) were smaller and failed to undergo axial rotation observed at E8.5 in WT littermates. Analysis with β-gal colorimetric staining in asymptomatic heterozygous carriers showed strong expression in the yolk sac, eyes, heart, and neural tube (Figures S4A–S4D). In keeping with this, homozygous mutants frequently presented dilated hearts (Figure 4A) and open neural tubes, which is consistent with the broad phenotypes observed in patients.

To identify the misregulated pathways underlying these developmental defects, we carried out expression microarray analysis in Srd5a3Gt/Gt embryos versus WT littermates before morphological differences appeared (Figure 4C). Whole-transcriptome analysis revealed that among the 50 most upregulated transcripts, 20% are involved in the regulation of the unfolded protein response (UPR) or are activated in this pathway (Tables S1 and S2). An activation of the UPR pathway in E8.5 Srd5a3Gt/Gt embryos was confirmed by real-time RT-PCR and with an E9.5 mouse embryonic fibroblast cell line treated with tunicamycin used as a positive control (activates the UPR pathway by blocking N-glycosylation; Figure 4D). A marker of this pathway, BiP, is upregulated at the transcript and protein levels in Srd5a3Gt/Gt embryos, with a particularly high expression in neuroepithelial cells (Figure 4E). Srd5a3 expression was not detected in mutant embryos; however, in cells inhibited for N-glycosylation by tunicamycin treatment, its expression increased significantly (Figure 4D). We also confirmed UPR activation by determining enrichment of gene ontology (GO) categories by all the genes significantly misregulated in Srd5a3Gt/Gt embryos compared with littermate controls. UPR was the biological process most significantly enriched for the genes upregulated (Table S3), whereas genes involved in general cellular metabolic processes and specific embryonic developmental program like regionalization were the most significantly downregulated (Table S3). These observations suggest that Srd5a3 is required for ER protein folding, a primary role of N-glycan during development.

**DFG10 and SRD5A3 Are Necessary for Conversion of Polyprenol to Dolichol in Yeast, Mouse, and Human**

During the de novo synthesis of dolichol in eukaryotes, the farnesyl pyrophosphate (FPP), a product of the mevalonate pathway, is elongated by its successive condensation with isopentenyl pyrophosphate (IPP), catalyzed by a cis-isopentenyltransferase named dehydrodolichyl diphosphate synthase (DHDD) (Figure 5A). According to the current model, when the chain reaches target length, the pyrophosphate and phosphate groups are removed, although the phosphatases are not yet identified (Kato et al., 1980; Wolf et al., 1991). The alpha-isoprene unit of polyprenol is subsequently reduced by an NADPH-dependent
Figure 4. Characterization of Homozygous Srd5a3<sup>GT/GT</sup> Gene Trap Mouse Embryos

(A) Phenotype at E10.5 shows failure to rotate, ventral body wall defect (arrow), and dilated heart (arrowhead). The scale bar represents 1 mm.

(B) Graphic representation of the genotype obtained from the progeny of heterozygous mating, with lethality appearing between E11.5 and E13.5.

(C) Genes overexpressed in Srd5a3<sup>GT/GT</sup> at E8.5 detected with 44k mouse genome oligo microarray (1 is the log 2 of a 2-fold expression increase; error bars represent the mean ± standard deviation from four independent experiments). Among ten of the most upregulated genes, five (in blue) are involved in the unfolded protein response pathway (UPR). Morphology of heterozygous and homozygous Srd5a3 mutant embryos at E8.5, before embryo axial rotation. The scale bar represents 500 μm.

(D) Real-time RT-PCR confirming activation of the UPR pathway (Errors bars are means ± standard deviations, asterisks indicate p < 0.05, n = 3).
SRD5A3 Promotes the Reduction of Polyprenol to Dolichol

We next tested whether SRD5A3 was capable of reducing polyprenol to dolichol. We assessed polyisoprenoid levels in yeast transformed with vectors expressing the human and the yeast enzymes (Figures 6A–6E), cultured in minimal media and harvested during the log phase. The important accumulation of polyprenol detected in the dfg10-100 strain (Figure 6B) is efficiently and specifically corrected in yeast transformed with the SRD5A3 gene (Figure 6D) compared to other normal exogenous dolichol biosynthesis. One postulated an initial dephosphorylation of dolichol (Heller et al., 1992; Rossignol et al., 1983). A second phosphorylation of dolichol as the major pathway for the production of dolichols involved has not been identified. Finally, a dolichol kinase (Ddk), mutated in CDG-I, transfers phosphate from CTP to dolichol (Allen et al., 1978; Kranz et al., 2007). The unidentified polyprenol reductase enzyme made SRD5A3 a likely candidate for this function.

To explore a disruption of the final step of dolichol biosynthesis in SRD5A3 or DFG10 mutated cells and to unequivocally identify the last step of dolichol synthesis, we used liquid chromatography–mass spectrometry (LC-MS) (Garrett et al., 2007) to analyze polyprenols in WT and dfg10-100 yeast strains, E11.5 WT and Srd5a3G2G2 mouse embryos, and fibroblasts and leukocytes from controls and patients. Polyprenol was not detected in any samples of WT origin as reported (Swiezewska and Danikiewicz, 2005) but was easily detected in the yeast and mouse mutants, in the same molar range as the dolichol naturally present in control samples (Figures 5B and 5C), suggesting a block in the polyprenol reduction step. In reference to an internal standard, by correcting polyprenol isotopic contribution, we detected a 70% decrease in dolichol in the dfg10-100 mutant compared to the WT yeast from the same background (Figure 5B, right end). Given these striking results in both mouse and yeast, we were surprised to find no clear change in prenol profiles in patient fibroblasts or leukocytes (data not shown). Because one possible explanation might be that the normal exogenous fetal calf serum used in tissue culture supplied dolichol to overcome this metabolic block, we instead analyzed directly patient fresh plasma. We found an increased level of polyprenoids in patients’ samples versus controls and in other CDG-I patients with a significant increased of polyprenols-18,19,20/dolichols-18,19,20 ratios (Figure 5D), indicating a defect in polyprenols metabolism in all organisms tested.

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**DISCUSSION**

**Biological Activity of SRD5A3**

SRD5A3 sequence predicts a steroid 5α-reductase domain, and some enzymes with this domain are able to reduce a variety of steroid hormones with a delta4,5,3-oxo structure (Russell and Wilson, 1994). Mutation of the *SRD5A2* gene in human causes male pseudohermaphroditism as a result of an enzymatic block of testosterone to dihydrotestosterone conversion (Andersson et al., 1991), and mutation of the *Srd5a1* gene in mice affects reproduction and parturition, suggesting involvement in androgen metabolism (Mahendroo and Russell, 1999). Interestingly, a previous study suggested that cell extract containing overexpressed SRD5A3 was able to reduce testosterone to dihydrotestosterone (Uemura et al., 2008), albeit at a slow rate.

However, both our biochemical and clinical investigations in the patients with SRD5A3 mutations indicate that the nature of the substrate of the SRD5A3 enzyme is not related to the steroids. Our patients displayed no abnormal sexual abnormalities that would have suggested a primary defect of steroid metabolism. Moreover, karyotype analysis excluded the possibility of sex reversal in all (data not shown). These observations lead us to hypothesize that the in vivo substrate of SRD5A3 could be a different lipid. Polyprenols share a common origin with cholesterol because they are also built from isoprene units.

Another enzyme with a predicted steroid 5α-reductase domain, Tsc13/GPSN2, has been shown to be an enoyl reductase involved in the elongation of very long chain fatty acids (Kohlwein et al., 2001). This study also illustrates that the predicted steroid 5α-reductase domain is involved in the reduction of a nonsteroid lipid and suggests that the full spectrum of lipid reduction mediated by steroid 5α-reductase-like enzymes needs further evaluation.

**Current Models for Dolichol Biosynthesis**

Several mechanisms have been proposed for the last steps of dolichol biosynthesis. Some postulated an initial dephosphorylation of polyprenol diphosphate followed by reduction to Dol-P, then dephosphorylation of Dol-P to produce dolichol (Chojnacki and Dallner, 1988). However, several studies demonstrated the phosphorylation of dolichol as the major pathway for the production of Dol-P (Heller et al., 1992; Rossignol et al., 1983). A second proposal suggested that the final condensation reaction of Pol-PP uses isopentenol instead of isopentenyl-PP. In this reaction, Pol-PP is directly transformed to a one isoprene unit longer...
Figure 5. Analysis of Polyprenols and Dolichols, in Yeast, Mouse, and Human with LC-MS

(A) De novo biosynthesis and recycling of dolichol in eukaryotic cells with the yeast (in blue) and human (in red) enzymes involved. Isopentenylpyrophosphate (IPP) is the building block for all polyprenoids. IPP molecules are added sequentially in trans-configuration on dimethylallyl pyrophosphate (DMAPP) via the farnesyl pyrophosphate synthase (ERG20/FDPS) to form geranyl pyrophosphate (GPP) and then farnesyl pyrophosphate (FPP). More IPP units are then added in cis-configuration on FPP by the cis-prenyl transferases (RER2, SRT1/DHDDS), producing long polyprenoids that are embedded in the ER membrane. Once the final length is reached, both phosphate residues are released by unidentified phosphatases. The alpha-isoprene unit of the polyprenol is subsequently reduced by an NADPH-dependent microsomal reductase. For this step, the corresponding aldehydes have also been suggested as intermediates (Sagami et al., 1996). Finally the dolichol-specific kinase (SEC59/DK) transfers a phosphate from CTP to dolichol. Dol-P is used to build the lipid linked oligosaccharide (LLO). Once the oligosaccharide structure is transferred to specific asparagine residues, Dol-P is released on the luminal leaflet of the ER and is dephosphorylated by a pyrophosphatase (CWH8).
dolichol, thus circumventing the dephosphorylation steps (Ekström et al., 1987). The third proposal is the most widely accepted (Figure 5A) based on the finding of high concentrations of polyrenol during the initial phase of dolichol biosynthesis (Ekström et al., 1984) and the detection of a basal polyrenol reductase activity, in vitro (Sagami et al., 1993). However, the reductase postulated in this reaction had not been identified, and thus these models could not be directly evaluated. Our results suggest that SRD5A3 is the polyrenol reductase, which is consistent with the last model, confirming that the reduction of polyrenol is the major pathway for dolichol biosynthesis.

Residual Dolichol in SRD5A3 Mutants

Dolichol was still detected in human, mouse, and yeast SRD5A3/DFG10 mutants, suggesting the existence of another de novo biosynthetic pathway for dolichol production. The presence of dolichol in these mutants is not explained by dietary contribution, which was reported to be negligible in rat (Keller et al., 1982), and the nature of the mutations in human, mouse, and yeast suggests that these organisms have null mutations for this gene. These observations indicate the existence of an alternative pathway for de novo synthesis of dolichol in eukaryotic cells. Disruption of LLO biosynthesis due to mutation in Dpgat1 in mouse results in embryonic lethality at E5 (Marek et al., 1999), a more severe phenotype than that observed in Srd5a3 mutant mouse embryos, consistent with an alternative pathway. One candidate is the TSC13 gene, the only other gene in S. cerevisiae encoding a steroid 5α-reductase domain (Pfam database). We tested whether the tac13 mutant had abnormal CPY glycosylation and whether the dfg10/tsc13 double mutant showed further increase of the polyrenol/dolichol ratio, but found no effect of either (Figure S3), suggesting that the alternative pathway for dolichol synthesis is independent of these genes. Interestingly, among the pathways activated in embryonic mouse mutants was the mevalonate pathway, including the isoprenoid biosynthetic enzymes (Tables S1 and S2). This could suggest a positive feedback mechanism, which might help organisms overcome a partial block of these pathways.

Phenotypic Spectrum Resulting from Disruption of Dolichol Metabolism

Tissues affected in patients with SRD5A3 mutations, such as nervous system, ocular structures, skin, or coagulation factors, reflect sensitivity for alteration in N-glycosylation. Such congenital defects and the detection of a restricted expression pattern of Srd5a3 in mouse embryo suggest a spatial-temporal requirement during development. N-glycan number and branching regulate surface glycoprotein levels, affecting cell proliferation and differentiation (Lau et al., 2007). N-glycosylation may help regulate specific developmental pathways yet to be discovered. A comparable multisystem disorder has been recently mapped to the same locus, suggesting that these patients have the same genetic defect (Kahrizi et al., 2009).

Although we find defects in the N-glycosylation pathway, dolichol is also required for the synthesis of O-mannose-linked glycans, C-mannosylation, and glycoporphospholipid anchor synthesis, and some of the pathology may derive from these defects, not explored here. Furthermore, little is known about the glycosylation-independent functions of dolichol, considered as a general membrane component in mammalian cells (Rip et al., 1985).

The pathogenesis and phenotypic specificity of CDGs deserves further investigations. However, our results point to an unsuspected role for a steroid reductase-like enzyme in the pathogenesis of one type of CDG, presumably mediated by a requirement for dolichol synthesis.

EXPERIMENTAL PROCEDURES

Genome Mapping

All patients were enrolled according to approved human subjects protocol at respective institutions. DNA was extracted from peripheral blood leukocytes by salt extraction, genotyped with the Illumina Linkage IVb mapping panel (Murray et al., 2004), and analyzed with easyLINKAGE-Plus software (Hoffmann and Lindner, 2005). Parameters were set to autosomal recessive with full penetrance and disease allele frequency of 0.001. Genomic regions with LOD scores over 2 were considered as candidate intervals. Linkage simulations were performed with Allegro 1.2c under the same parameters, with 5000 markers at average 0.64 cM intervals, codominant allele frequencies, and parametric calculations (Hoffmann and Lindner, 2005).

Mutation and CDG Screening

We performed direct bidirectional sequencing of the five coding exons and splice junction sites of SRD5A3 via BigDye Terminator cycle sequencing (Applied Biosystems). We screened 31 patients with CDG-Ix and seven patients from a cohort with CDG-ix and either strong clinical overlap such as severe congenital eye malformation and/or indications for a dolichol-phosphate biosynthesis defect. Clinical description of patients 08-0486, 08-0487, and 07-0419 was previously reported, corresponding respectively to patients 3, 5, and 7 (Morava et al., 2008) and 25, 26, and 27 (Morava et al., 2009). CDG was diagnosed by affinity chromatography and mass-spectrometry analysis of transferrin (O’Brien et al., 2007) or with transferrin isoelectric focusing (de Jong et al., 1994).
Figure 6. **In Vivo and In Vitro Polyprenol Reduction Promoting Activity of SRD5A3**

(A–E) LC-MS analysis of lipid extracts from yeast cultured in minimal media.  
(A) Only dolichol is detected in WT yeast strains transformed with pYX212 empty vector.  
(B) In dfg10-100 strain transformed with pYX212 empty vector, accumulation of polyprenol relative to dolichol is evident. An additional compound (arrows) was tentatively identified as polyprenal, previously suggested as an intermediate in yeast during in vitro dolichol biosynthesis (Sagami et al., 1996).  
(C) In the dfg10-100 strain transformed with the WT DFG10 gene, no polyprenol accumulation was detected.

15 mM MgCl2, 0.8 mM DTT, 26% glycerol, and 150 µg protein in a final volume of 60 µl. After 15 min at 24 °C, the reaction was stopped with chloroform/methanol (3/2, by volume) and processed by phase partitioning (Sharma et al., 1982). Radioactive glycolipids were separated on silica gel 60 plates (Merck) developed in chloroform/methanol/water (65/25/4, by volume). Radioactivity was detected and quantified by phosphorimager (Molecular Dynamics).

**Assay II**

For determination of the transfer to exogenous Dol-P, the reaction contained 3.6 µg Dol-P, 2.4 mM diheptanoyl-phosphatidylcholine, 36 mM Tris-HCl (pH 7.5), 0.1 µg UDP-[14C]GlcNAc (specific activity 282 µCi/ mmol), 11 mM MgCl2, 0.7 mM DTT, 25% glycerol, and 150 µg protein in a final volume of 60 µl. Incubation and processing of the reaction was as in assay I.

**Construction of DsRed-SRD5A3 and GFP-SRD5A3 Expression Plasmids**

The ORF of SRD5A3 was amplified from a human fetal brain complementary DNA (cDNA) library and cloned in phGFP II-N (Stratagene) and pDsRed2-C1 (Clontech) vectors. Site-directed mutagenesis was performed with QuickChange II site-directed mutagenesis kit (Stratagene) according to the manufacturer’s instructions.

**Analysis of Srd5a3**

Frozen embryos (129/SvEvBr × C57Bl6/J mix) carrying a gene trap insertion in one allele of Srd5a3 were obtained from Lexicon and transferred in pseudopregnant female (Renard and Babinet, 1984). Genotyping was performed by PCR with yolk sac-extracted DNA. Whole-transcriptome analysis was performed with TMHMM, a program for predicting membrane-spanning segments based on hidden Markov model (http://www.cbs.dtu.dk/services/TMHMM/). Pfram database was used to identify proteins with steroid 5α-reductase domain (PF02544).

**Bioinformatics**

Phylogenetic tree representation was done with phylogeny (Dereeper et al., 2008), (http://www.phylogeny.fr/version2_cgi/index.cgi). Topology prediction was performed with TMHMM, a program for predicting membrane-spanning segments based on hidden Markov model (http://www.cbs.dtu.dk/services/TMHMM/). Pfram database was used to identify proteins with steroid 5α-reductase domain (PF02544).

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**SUPPLEMENTAL INFORMATION**

Supplemental Information includes Extended Experimental Procedures, six figures, and three tables and can be found with this article online at doi:10.1016/j.cell.2010.06.001.

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