Cloning and characterization of human phosphomannomutase, a mammalian homologue of yeast SEC53

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Phosphomannomutase (PMM) catalyzes the conversion of mannose-6-phosphate to mannose-1-phosphate, which is a substrate for the synthesis of GDP-mannose. This nucleotide sugar is then used in the synthesis of dolichol-phosphate-mannose, which is essential for N-linked glycosylation and thus the secretion of several glycoproteins as well as for the synthesis of glycosyl-phosphatidyl-inositol (GPI) anchored proteins. In the yeast Saccharomyces cerevisiae, SEC53, a gene required for viability, encodes PMM. Given the importance of PMM in glycoprotein synthesis, it is surprising that very little is known about the enzyme in higher eukaryotes. Recently, an autosomal recessive human disease, Carbohydrate-deficient glycoprotein syndrome type I (CDGS-I) has been correlated with severely reduced PMM activity. Here we report the isolation of a cDNA encoding human PMM, a protein of 29 kDa that is 55% identical and 66% similar to yeast Sec53p. Northern blot analysis shows a single 1.4 kb transcript that is ubiquitously expressed, although levels vary markedly among tissues. Expression of the human cDNA in a temperature-sensitive mutant sec53 yeast strain confers growth at the restrictive temperature, strongly suggesting that this gene encodes a functional PMM. Finally, when expressed in BHK cells, PMM is localized exclusively to the cytosol corresponding to its localization in yeast.

Key words: carbohydrate-deficient glycoprotein syndrome type I (CDGS-I)/dolichol-P-mannose/phosphomannomutase/SEC53/secretion

Introduction

The molecular machinery of the secretory pathway in the yeast Saccharomyces cerevisiae has been extensively characterized through the use of genetic tools (Rothblatt et al., 1994). In a seminal study Novick et al. (Novick et al., 1980) identified 23 complementation groups in a screen for mutants with temperature-sensitive defects in secretion. This and subsequent work has led to the isolation and characterization of numerous genes that are essential to secretion in yeast (Rothblatt et al., 1994). Many of the gene products have turned out to be either constituents of protein coats mediating vesicular trafficking, small GTPases regulating this traffic, components of the fusion machinery, or molecules involved in translocation of nascent polypeptide chains into the ER (Rothblatt et al., 1994). Initially sec53p appeared to belong to this last group (Ferro-Novick et al., 1984a,b). However, further characterization of the sec53 mutant revealed a defect in glycosylation of nascent polypeptide chains after translocation into the ER (Feldman et al., 1987). This finding was intriguing as sec53p had been found to be essentially confined to the cytosol (Bernstein et al., 1985). Consistent with this observation the defect in the sec53 mutant was shown to reside in the cytosol whereas the membranes remained fully functional at the restrictive temperature. When it was further demonstrated that the defect in the cytosol from the sec53 mutant was only observed after a significant lag upon transfer from the permissive to the restrictive temperature, it was inferred that sec53 encoded an enzyme in the synthesis of a precursor required for glycosylation (Feldman et al., 1987; Hibbs and Meyer, 1988). This enzyme was subsequently demonstrated to be PMM (Kepes and Schekman, 1988), an enzyme which converts mannose-6-phosphate to mannose-1-phosphate, a substrate for synthesis of GDP-mannose. The mannose moiety of GDP-mannose is transferred to the lipid carrier dolichol phosphate. Dolichol-P-mannose is then used in the assembly of the lipid-linked oligosaccharide precursor Glc3Man9GlcNAc2 which is donated by the lipid carrier to an asparagine in the synthesis of core-glycosylated nascent polypeptides in the ER (Kornfeld and Kornfeld, 1985). Dolichol-P-mannose is also the intermediate in the O-mannosylation reaction in yeast (Trueheart and Fink, 1989). Moreover, the mannosyl residues in the anchor of glycosyl-phosphatidyl-inositol (GPI) anchored proteins are also derived from dolichol-P-mannose (Benghezal et al., 1995; Conzelmann et al., 1990). Bearing this ubiquitous requirement for dolichol-P-mannose in glycosylation reactions in mind, it is hardly surprising that SEC53 is an essential gene in yeast.

In humans a recessive genetic disease referred to as carbohydrate-deficient glycoproteins syndrome type I (CDGS-I) results in underglycosylation of secretory proteins, lysosomal enzymes and membrane glycoproteins due to a defect in lipid-linked oligosaccharide biosynthesis (Powell et al., 1994; Krasnewich et al., 1995). Recently, it has been reported that cells from patients with this disease are severely deficient in PMM activity (Van Schaftingen and Jaeken, 1995). In this article, we report the cloning of a cDNA encoding a human homolog of sec53, hPMM, which is ubiquitous but differentially expressed. We show that the human cDNA can complement the defect of the conditional lethal sec53-6 mutant at the restrictive temperature 32°C, and that an epitope tagged derivative of hPMMp is localized exclusively to the cytosol when expressed in BHK cells, consistent with its localization in yeast.

Results

The nucleotide sequence and the predicted amino acid sequence of hPMM, human PMM, are shown in Figure 1A. Figure 1B shows a schematic presentation of the two clones, 63 and 4–1, used to generate the cDNA (see Materials and meth-
Fig. 1. (A) Nucleotide sequence of human PMM (GenBank U86070). The sequence is numbered relative to the start site of translation (position +1). The underlined sequence indicates the Kozak box-like sequence specifying ribosome binding. The deduced amino acid sequence is shown below in single letter code.

(B) (See on facing page.) Schematic representation of the two clones 63 and 4-1 used to assemble the full length cDNA for the human PMM. Clone 63 which contained the Kozak box-like sequence and the initiator ATG had an internal deletion of 388-nts. These were obtained from clone 4-1, which was lacking the first eight nucleotides of the coding sequence. Except for these differences two clones were identical. The sites indicated in the figure were used to assemble the full length cDNA and derivatives thereof as detailed in Materials and methods.
Cloning and characterization of human phosphomannomutase

ods). The 1242-nts sequence comprises a Kozak sequence, a 786-nts open reading frame, and a 447-nts 3′-utr. While this manuscript was in preparation, the sequence of two human cDNAs highly homologous to sec53 and almost identical to the sequence reported here were submitted directly to the database (GenBank D85231 and GenBank U62526). One of these submissions (GenBank U62526) also reports the localization of the gene to chromosome 22q13.

Alignment of the deduced peptide sequence from the human clone with PMM from the yeast *Saccharomyces cerevisiae*, Sec53p (Bernstein et al., 1985), and the yeast *Candida albicans*, PMM1 (Smith et al., 1992) using the PILEUP program of the Wisconsin Package (GCG, 1995), showed extensive homology among all three sequences (Figure 2). Thus, the predicted amino acid sequence of the human clone is 55.2% identical, and 66.3% similar to Sec53p, and 51.2% identical, and 67.5% similar to PMM1 from *Candida albicans*, whereas the yeast sequences are 77.4% identical and 88.9% similar. Interestingly, none of the eukaryotic PMMs show any significant similarity to bacterial PMM, or to other eukaryotic hexophosphatase mutases.

The extensive homology to Sec53p indicated that the human cDNA encoded a PMM. To test this possibility, RSY12 yeast expressing a conditional lethal sec53-6 allele were transfected with either the human cDNA inserted in the yeast expression vector pRF81 or vector only. RSY12 cells fail to grow and are essentially devoid of any PMM activity at 30°C and temperatures above (Ferro-Novick et al., 1984b; Kepes and Schekman, 1988). However, when hPMM was expressed in RSY12 the cells grew faster at the restrictive than at the permissive temperature, whereas no growth was observed at the restrictive

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![Fig. 2. Alignment of the predicted amino acid sequences of PMMs from human and yeast, Saccharomyces cerevisiae and Candida albicans. The analysis was performed using the PILEUP and PRETTY programs of the Wisconsin Package (GCG, 1995).](image-url)
temperature with pRF81 alone (Figure 3). Thus, hPMM complements the growth defect in RSY12 showing that the isolated cDNA encodes a functional homolog of yeast PMM.

A Northern blot analysis of the tissue distribution of human PMM mRNA revealed a ubiquitously expressed single transcript of 1.4 kb (Figure 4). The expression levels varied considerably with high levels detected in kidney, heart, and brain, intermediate levels in lung, pancreas, and liver, and low levels in skeletal muscle and placenta. Control experiments excluded that this pattern was due to differences in the amounts of total mRNA from these tissues (data not shown).

It has been reported that Sec53p in yeast is localized to the cytosol (Bernstein et al., 1985). To determine whether the human cDNA encoded a protein of the predicted size and to assess the subcellular distribution thereof in mammalian cells, an epitope-tagged derivative was generated in which 30-nts encoding the human c-myc epitope EQKLISEEDL were appended to the 3'-end of the coding sequence of hPMM. This construct in the mammalian expression vector pCB7 was transfected into BHK cells and hygromycin-resistant clones expressing myc-tagged hPMMp were identified by immunofluorescence. The diffuse staining pattern observed was consistent with a localization of hPMMp-myc in the cytoplasm (Data not shown). This was further examined by immunoprecipitation and Western-blotting of membrane- and cytosol-fractions of transfected BHK cells. As shown in Figure 5, the hPMMp-myc encoded a protein of ~29 kDa which was exclusively (>99%) found in the cytosol-fraction, consistent with the previously reported localization of PMM in S. cerevisiae (Bernstein et al., 1985).

Discussion

Carbohydrate-deficient glycoprotein syndromes are a family of genetic diseases in which secretory, membrane, and lysosomal proteins are underglycosylated. One form of this disease (type II) has been related to a deficiency in N-acetylgalactosaminyltransferase II (Charuk et al., 1995), an enzyme of the medial Golgi (Kornfeld and Kornfeld, 1985). In contrast, patients with CDGS-I exhibit a more pronounced defect in which the assembly of dolichol-linked oligosaccharide precursors is affected (Powell et al., 1994; Krasnewich et al., 1995). Recently, analysis of fibroblasts, lymphocytes, and liver biopsies from CDGS-I patients revealed that these cells exhibited dramatically reduced PMM activity (Van Schaftingen and Jaeken, 1995). Very recently, however, it has been questioned whether PMM deficiency is the only cause of CDGS-I as exogenous mannose was found to correct the defect in fibroblast from some patients with CDGS-I (Panneerselvam and Freeze, 1996).

In this report, we describe the cloning of a human cDNA with a predicted amino acid sequence that is 55.2% identical and 66.3% similar to sec53p, a PMM from S. cerevisiae. By Northern blot analysis a ubiquitously expressed single transcript of 1.4 kb was detected. However, expression levels varied considerably with kidney, heart, and brain containing high amounts, whereas lung, pancreas, and liver contained intermediate amounts, and low levels of transcript were detected in skeletal muscle and placenta. This finding is somewhat surprising as liver and in particular pancreas are highly secretory organs and may therefore imply the existence of a second isoform in these tissues. In this regard it is interesting that in a genetic study of 25 families the CDGS-I locus was mapped to a region contained within chromosome 16p13.3-p13.12 (Martinsson et al., 1994), while in a recent submission of a hPMM sequence to GenBank (U62526) the gene was reported to lie in chromosome 22q13. This apparent discrepancy could either...
reflect that CDGS-I may be caused by defects in genes with distinct functions as mentioned above or the existence of a second isoform of hPMM. Alternatively, other hexophosphate mutases may supplement PMM-activity in the tissues with low levels of hPMM expression. In yeast cells where both phosphoglucomutase genes PMG1 and PMG2 have been deleted, multi-copy complementation with SEC53 leads to a sharp increase in the level of phosphoglucomutase activity (Bole et al., 1994).

The cDNA isolated in this work restores growth at the restrictive temperature of yeast RSY12 cells harboring the conditional lethal sec53–6 allele. It thus clearly represents a functional human homolog of SEC53 and should thus prove a useful tool in the analysis of genetic defects associated with CDGS-I. If PMM-deficiency is indeed the cause of CDGS-I, the glycosylation defects in cells from these patients should be diminished following expression of hPMM. Moreover, the hPMM cDNA characterized here may eventually be utilized in treating patients with CDGS-I.

Materials and methods

Materials

Components of the two-hybrid genetic screen system, including a serum-starved WI-38 fibroblast library were generous gifts from Dr. Roger Brent and colleagues, Department of Molecular Biology, Massachusetts General Hospital, Harvard University. A detailed description of this system and its use is readily accessible (Golemis et al., 1994). The UNI-ZAP XR human fetal brain library was from Stratagene. The RSY12 yeast strain expressing the conditional lethal sec53–6 allele was kindly provided by Dr. Randy Schekman, Department of Molecular and Cell Biology and Howard Hughes Medical Institute, University of California at Berkeley. The genotype of RSY12 is sec53–6[3]-[112] ura3–52 MATa. The yeast expression vector pRF81 was generously provided by Dr. Russ Finley, Department of Molecular Biology, Massachusetts General Hospital. The mammalian expression vector pCB7 which confers resistance to hygromycin was obtained through Dr. Mike Roth, Dept. Biochemistry, University of Texas Southwestern Medical Center at Dallas. 9E10 monoclonal (IgG,) antibody against the epitope EQLKISEEDL from human c-myc was a gift from Kim Morrison, Harvard Medical School, Memorial Faculty.

The primers used for this work, obtained from the Molecular Biology Core at The Center for Inflammatory Bowel Disease, Massachusetts General Hospital, were as follows: 1: 5'–cggaattccagccatggcagtcaccgcccaggcagcccgcagacggagacgcct–3'; 2: 5'–tcgttcccaaagaagtggatggtgtcga–3'; 3: 5'–tgctgtccgatgctctttctgcaagaagttgctctctgagttgctctctgagttgct–3'; 4: 5'–tgctccgatgctctttctgcaagaagttgctctctgagttgctctctgagttgct–3'; 5: 5'–tcgttcccaaagaagtggatggtgtcga–3'. All other materials were obtained from Sigma or other commercial sources as listed below.

Cloning procedures

These were carried out using standard procedures (Ausubel et al., 1994). A two-hybrid screen of a serum-starved WI-38 fibroblast library with a bait consisting of amino acids 1–42 of the cytoplasmic tail of the human transferrin receptor fused to LexA yielded 12 leu, lacz+ clones, 5 of which contained an ~800 bp insert which using the BLAST algorithm (Altshul et al., 1990) showed homology to yeast sec53, PMM. Both strands of one of these clones, #63, were sequenced entirely by primer walking and found to be comprised of 845-bp with an open reading frame of 399-nucleotides. The deduced peptide sequence (133aa) from clone 63 showed extensive homology to sec53p but also strongly suggested an internal deletion of ~400 bp -100 nts downstream of the initiator ATG. Clone 63 was thus used to screen a UNI-ZAP XR human fetal brain cDNA library and eight clones were obtained, all of which were missing the 5′-end of the coding sequence, but comprised the remaining sequence of clone 63 and an additional 388-nucleotides between nts 87 and 88 in clone 63. The longest clone, 4–1, which stopped eight nucleotides short of the initiator ATG was used as template in a PCR reaction in which the upstream 56-mer, primer 1, comprising an EcoRI site, the Kozak-like box and the eight missing nucleotides, as deduced from the clones obtained in the two-hybrid screen, in addition to a recognition sequence. The sequence for the downstream 27-mer, primer 2, for this reaction was chosen anti-parallel to nts 640–666 in the cDNA. A complete cDNA, hPMM in the yeast expression vector pRF81, was assembled from an EcoRI-BsuRI fragment of the 5′-end of the PCR product and a BsuRI-Sall fragment of the 3′-end of clone 4–1, confirmed by dideoxy sequencing, and ligated into the EcoRI and XhoI sites of pRF81. hPMM was inserted into the library vector for the two-hybrid system pG4-5 using an EcoRI–Sphl fragment of the 5′-end of hPMM in pRF81 and a Sphl–Sall fragment of the 3′-end of clone 4–1. With full-length interaction with the bait was detected (leu+ lacZ–), suggesting that the original interaction was a result of an deletion which maintained an open reading frame in the clones, including clone 63, isolated from the serum-starved WI-38 library.

Expression of hPMM in BHK cells

A PCR product encoding the entire hPMMp with a C-terminal myc-epitope was amplified using the 56-mer, primer 3, identical to primer 1 except that the EcoRI site has been replaced by a BamHI site, and the 60-mer, primer 4, comprising in anti-parallel, an Xbal site, a stop codon, the myc-tag sequence, and the 18-terminal nts in the coding region of hPMM, as up- and down-stream primers, respectively. The product of the PCR reaction was digested with BamHI and XbaI, ligated into the BgIII and XbaI sites of pCB7 and sequenced. BHK cells, propagated in DMEM containing 10% FCS, in an atmosphere of 5% CO2, were transfected with the construct using lipofectASER (GibcoBRL) according to the manufacturer's instructions. Clones were selected by growing the cells in medium containing hygromycin (Boehringen Mannheim, Germany) for 12 days and recovered through the use of cloning rings. Ten clones were screened by immunofluorescence using the 9E10 antibody to the myc epitope and Texas red-conjugated goat anti-mouse IgG (Jackson Immunological, Bar Harbor, ME).

Two clones expressing intermediate levels of myc-tagged hPMMp along with a pool of the 10 clones were used to determine the subcellular distribution of myc-tagged hPMMp by immunoprecipitation and western blotting. These protocols have previously been described in detail (Hansen and Casanova, 1994). In brief, 100 mm confluent plates of either nontransfected BHK cells, or BHK cells transfected with hPMMp-myc were rinsed with PBS and scraped into 1 ml of ice-cold 3 mM imidazole, 300 mM sucrose, pH 7.4, homogenized by passage 40 times through a 30-gauge syringe, and the nuclei were pelleted by centrifugation. The postnuclear supernatant was centrifuged 1 h at 105,000 × g in a Beckman tabletop ultracentrifuge. The membranes (pellets) were re-suspended in 1 ml of SDS lysis buffer (0.5% SDS, 50 mM Tris pH 8.1, 1.0 mM NaCl, 5 mM EDTA, and 0.2% Na2S4O5) and the cytosol fraction (supernatant) was adjusted to that of the SDS lysis buffer, and both fractions boiled 3 min. Following addition of 0.5 ml Triton dilution buffer (2.5% Triton X-100, 100 mM Tris, pH 8.6, 100 mM NaCl, 5 mM EDTA, 0.02% Na2S4O5, the samples were subjected to immunoprecipitation using 9E10, a mouse monoclonal antibody to the myc-tag, and protein G-Sepharose. The precipitated proteins were resolved by SDS–PAGE on 10% gels, transferred to nitrocellulose, and the membranes probed with 9E10 followed by sheep anti-mouse/HRP. Immunoreactivity to 9E10 was detected by enhanced chemiluminescence, and the results quantified by densitometry using a Hewlett-Packard ScanJetic RX scanner and I%Lab Gel densitometry software (Signal Analytics Corp., Vienna, VA).

Acknowledgments

In addition to the people who kindly provided reagents for this work, we are grateful to Dr. Rosemarie Foster, Dr. Jeffrey Selstein, the Cancer Center, and Anthony Zervos, the Cutaneous Biology Center, Massachusetts General Hospital for ample helpful advice. This work was supported by a Senior Research Fellowship from the Danish Cancer Society (SHH) and by a grant from Fabricant Einar Willumsens Mindelegat, Dagmar Marshalls Fond (SHH) and the National Institutes of Health, NIH AI32291 and NIH DK33506 (J.E.C.).

Abbreviations

PMM, phosphomannomutase; CDGS-I, carbohydrate-deficient glycoprotein syndrome type I.

References


Received on January 7, 1997; revised on February 18, 1997; accepted on February 19, 1997.