POM152 Is an Integral Protein of the Pore Membrane Domain of the Yeast Nuclear Envelope

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Abstract. We have identified a concanavalin A-reactive glycoprotein of 150 kD that coenriches with isolated yeast nuclear pore complexes. Molecular cloning and sequencing of this protein revealed a single canonical transmembrane segment. Epitope tagging and localization by both immunofluorescence and immunoelectron microscopy confirmed that it is a pore membrane protein. The protein was termed POM152 (for pore membrane protein of 152 kD) on the basis of its location and cDNA-deduced molecular mass. POM152 is likely to be a type II membrane protein with its NH₂-terminal region (175 residues) and its COOH-terminal region (1,142 residues) positioned on the pore side and cisternal side of the pore membrane, respectively. The proposed cisternally exposed domain contains eight repetitive motifs of ~24 residues. Surprisingly, POM152 deletion mutants were viable and their growth rate was indistinguishable from that of wild-type cells at temperatures between 17 and 37°C. However, overproduction of POM152 inhibited cell growth. When expressed in mouse 3T3 cells, POM152 was found to be localized to the pore membrane, suggesting a conserved sorting pathway between yeast and mammals.

Nuclear pore complexes (NPCs)¹ are macromolecular assemblies that serve to regulate nucleocytoplasmic communication (for review see Forbes, 1992). They reside in circular openings (nuclear pores) across the nuclear envelope (NE) (for review see Franke, 1974; Gerace and Burke, 1988). The nuclear pore membranes are morphologically and biochemically distinct domains of the NE that border the nuclear pores. So far, two integral membrane proteins have been identified in higher eukaryotes that are specifically located in the pore membrane domain, namely gp210 (Gerace et al., 1982; Wozniak et al., 1989) and POM121 (Hallberg et al., 1993). Both membrane proteins have a single transmembrane segment. However, whereas most of the mass of gp210 is located on the cisternal side of the pore membrane (Wozniak et al., 1989; Greber et al., 1990), the bulk of POM121 faces the pore side of the pore membrane (Hallberg et al., 1993). Gp210 could contribute either to the "luminal" spokes or the radial arms that have been identified in ultrastructural analysis (Hinshaw et al., 1992; Akey and Radermacher, 1993; for review see Akey, 1992). Conversely, the pore-exposed bulk of POM121 is most likely an integral part of the pore side components of the NPC as it shares a repetitive pentapeptide motif (Hallberg et al., 1993) that has also been identified in some NPC proteins (Davis and Fink, 1990; Nehrbass et al., 1990; Starr et al., 1990; Sukegawa and Blobel, 1993).

One of the most likely functions of these pore membrane proteins is the anchoring of the NPC in the nuclear pore (Gerace et al., 1982; Wozniak et al., 1989; Hallberg et al., 1993). Such proteins may also play a role in regulating nucleocytoplasmic traffic through the NPC. Greber and Gerace (1992) have shown that a monoclonal antibody against the cisternal domain of gp210 can reduce the rate of protein import into the nucleus. Furthermore, integral pore membrane proteins may be involved in the circumscribed fusion of the inner and outer nuclear membrane to form new nuclear pores (Maul, 1977; Wozniak et al., 1989). Such fusion processes could also be involved in the elimination of nuclear pores by restoring the double membrane.

Integral proteins of the pore membrane domain have not previously been identified in yeast. However the recent isolation of NPCs from yeast has allowed the identification of a predominant, constituent concanavalin A (ConA)–binding glycoprotein. We have determined that this protein is an integral protein of the pore membrane domain. The protein was termed POM152 on the basis of its cDNA-deduced primary structure and calculated molecular mass of 151,670 daltons. Unexpectedly, deletion mutants of the POM152 gene are viable. When expressed in mouse 3T3 cells the yeast protein specifically localized to the mammalian pore membrane.

¹Abbreviations used in this paper: Con A, concanavalin A; HA, hemagglutinin; NE, nuclear envelope; NPC, nuclear pore complex; POM, pore membrane protein; SDS-HA, SDS-hydroxylapatite; SM-URA, synthetic medium lacking uracil.
Materials and Methods

Strains and Media

The yeast strains used in this study are listed in Table I. They were grown as previously described (Sherman et al., 1986) in either YPD (1% yeast extract, 2% bactopeptone, and 2% glucose) or synthetic minimal media (SM) supplemented with the appropriate amino acids and either 2% glucose or 2% galactose. Standard procedures for yeast genetic manipulations were as described in Sherman et al. (1986). Transformations of yeast using lithium acetate were performed as described in Ito et al. (1983).

Fractionation of Yeast NPC Proteins

Approximately 5 mg of enriched yeast nuclear pore complexes, isolated from Saccharomyces uvarum as described by Rout andBlobel (1993), were solubilized in 2% SDS, 100 mM sodium phosphate buffer, pH 6.8, 100 mM DTT, and 0.5 mM PMSF. Polypeptides were fractionated by SDS-hydroxyapatite chromatography as previously described (Courvalin et al., 1990) except that the linear elution gradient was 0.2-0.75 M sodium phosphate, pH 6.8, containing 0.1% SDS and 1.0 mM DTT. For SDS-PAGE analysis, aliquots from fractions were diluted twofold in SDS-sample buffer and loaded directly onto the gel.

Further separation of polypeptides from SDS-HA fractions containing a Con A-binding protein of an estimated mass of 150 kD was achieved by reverse-phase HPLC. Fractions from the SDS-HA eluate containing p50 were pooled and directly loaded onto an Aquapore butyl (C-4) column (100 × 10 mm, Brownlee Labs, Applied Biosystems Inc., Foster City, CA) equilibrated with 60% formic acid. After a 5-min linear increase to 66% acetonitrile in 60% formic acid, the column was eluted with a 1-h linear gradient of 66-33% acetonitrile in 60% formic acid. In preparation for electrophoresis, aliquots of the eluted fractions were dried in a Speed Vac Concentrator (Savant Instruments Inc., Hicksville, NY). Dried pellets were solubilized in SDS-sample buffer, heated at 65°C for 20 min, and then analyzed by SDS-PAGE.

For cleavage and sequencing of p50, HPLC fractions containing this protein were pooled, prepared for SDS-PAGE as above, and separated on a 6% polyacrylamide gel. Polypeptides were then electrophotoreographically transferred to polyvinylidene difluoride membrane and visualized with 0.1% Ponceau red in 1% acetic acid. p50 was excised and cleaved with endoproteinase Lys-C as described (Fernandez et al., 1992) and several internal peptides were subjected to NH2-terminal sequence analysis.

Sodium Carbonate Extraction of Yeast NEs

Yeast NEs were isolated from S. uvarum using the procedure of Kilmartin and Fogg (1982). All manipulations were done at 4°C. 0.25 mg of NE proteins were suspended in 0.5 ml of 10 mM bisTris, pH 6.5, 0.1 mM MgCl2, 1 mM DTT, and 0.1 mM PMSF. An equal volume of 0.2 M sodium carbonate, pH 11.5, was added to the suspended NEs and the sample was incubated for 15 min. Extracted proteins were separated from the NE membrane by centrifugation at 436000 g for 30 min in a TLA 100.2 rotor (Beckman Instruments Inc., Palo Alto, CA). The supernatant was collected and proteins were precipitated with 10% TCA. This precipitate, the membrane pellet, and the starting NE fraction were solubilized in SDS-sample buffer in preparation for SDS-PAGE. The gels were either stained with Coomassie blue or the polypeptides electrophoretically transferred to nitrocellulose, probed with 14C-labeled Con A (Sigma Chemical Co., St. Louis, MO), and visualized by fluorography as previously described (Wozniak et al., 1989).

Isolation and Sequencing of the Gene Encoding p50

The sequence of a peptide fragment of p50 corresponding to amino acid residues 332-353 was used to determine the exact cDNA sequence of p50 in this region using the PCR procedures (Lee et al., 1988). Synthesis, isolation, subcloning, and sequencing of the PCR products were performed as previously described (Radu et al., 1993) with the following modifications.

The two partially degenerate oligonucleotides were synthesized corresponding to the sense sequence of amino acid residues 332-337 and the antisense sequence of amino acids 349-353. The template for PCR was Saccharomyces cerevisiae genomic DNA (0.5 µg per reaction) and the annealing temperature was adjusted to 50°C.

On the basis of the sequence of the PCR product a 41-mer oligonucleotide complementary to the sense strand was synthesized. This oligonucleotide was end labeled with γ[^32]P] ATP (New England Nuclear, Boston, MA) using T4 polynucleotide kinase (New England Biolabs, Beverly, MA) and used to screen a S. cerevisiae genomic DNA library in lambda DASH (>450,000 pfus) (Stratagene Cloning Systems, La Jolla, CA). Phage lifts were performed as described (Benton and Davis, 1977). Prehybridization, hybridization, and washing of filters were conducted as described (Radu et al., 1993). Five overlapping clones were isolated that represent a 7.9-kb fragment of genomic DNA containing the gene encoding p50 (shown schematically in Fig. 6 A). Inserts from these clones were excised with Sall and subcloned into pBluescript II SK (−) (Stratagene Cloning Systems). Double-stranded sequencing of plasmid DNA (Mierendoff and Pfeffer, 1987) was performed with synthetic oligonucleotide primers using Sequenase (United States Biochemical Corp., Cleveland, OH). For determining the sequence across a single internal Sall site, lambda DNA (10 µg) was sequenced directly using the same procedure. A 5,482-bp fragment, which was bidirectionally sequenced, containing the p50 (now termed POMI52) open reading frame is shown in Fig. 4.

For expression purposes, a cDNA bordered by BamHI sites and containing the complete POMI52 gene was assembled in pBluescript II SK (−) from three restriction fragments isolated from separate lambda clones: a 1.3-kb BamHI/BglII fragment from the 5’ end of the gene, a 2.8-kb BglII/SalI internal fragment, and a 2.6-kb Sall/BamHI 3’ fragment (see schematic, Fig. 6 A). The resulting plasmid is termed pBPM1.

Table I. Yeast Strain Genotype

| Strain  | Genotype                  | Derivation                        |
|---------|---------------------------|-----------------------------------|
| W303    | Mata/Mata ade2-1/ade2-1   | Integrative transformation of     |
|         | ade2-1 ura3-1/ura3-1      | W303 with BamHI fragment of      |
|         | his3-11,15/his3-11,15     | pPOM1-HIS                         |
|         | trpl-1/trpl-1             |                                    |
|         | leu2-3,112/leu2-3,112     |                                    |
|         | can1-100/can1-100         |                                    |
| PMY1    | Mata/Mata ade2-1/ade2-1   | Segregant of sporulated PMY1      |
|         | ade2-1 ura3-1/ura3-1      | Transformation of PMY17 with       |
|         | his3-11,15/his3-11,15     | pPOM1-HA                           |
|         | trpl-1/trpl-1             |                                    |
|         | leu2-3,112/leu2-3,112     |                                    |
|         | can1-100/can1-100         |                                    |
|         | pPOM1/HA(LEU2)            |                                    |
| PMY17   | Mata ade2-1 ura3-1        | Transformation of W303 with       |
|         | his3-11,15/trpl-1         | pPMGal                              |
|         | leu2-3,112/can1-100       |                                    |
|         | can1-100/can1-100         |                                    |
| PMGal   | Mata/Mata ade2-1/ade2-1   |                                    |
|         | ade2-1 ura3-1/ura3-1      |                                    |
|         | his3-11,15/his3-11,15     |                                    |
|         | trpl-1/trpl-1             |                                    |
|         | leu2-3,112/leu2-3,112     |                                    |
|         | can1-100/can1-100         |                                    |
|         | pPMGal(URA3)              |                                    |
Epitope Tagging of POM152

For immunolocalization studies of POM152, an epitope tag encoding two tandemly repeated 12-amino acid residue peptides, 10 from the influenza virus hemagglutinin (HA) molecule (Wilson et al., 1984; Field et al., 1988) plus two flanking glycines added as spacers, was inserted into POM152 after amino acid residue 293 (see Fig. 4). This was accomplished by synthesizing two complementary oligonucleotides, yHA-1 and yHA-2, with the following sequence:

\[
\begin{align*}
\text{yHA-1} & : G Y P Y D V P D Y A S G \\
\text{yHA-2} & : Y Y C T I C T G T A C C T A A G G C C G \text{anti-sense strand}
\end{align*}
\]

An excess of this oligonucleotide pair was ligated into a unique BglII site on a transformation vector (pJJ217; Jones and Parkash, 1990) flanked by genomic DNA sufficient for recombination at the target site. Transformation was accomplished by DNA sequencing. The tagged POM152 gene and the wild-type allele were identified by restriction digestion analysis. Disruption of the POM152 Gene

Deletion and disruption of the POM152 gene was performed by integrative transformation using the procedure of Rothstein (1991). The construct used for transformation (pPM1-HIS) was assembled by pbEsscript II SK(-) and consisted of a BamHI/XhoI DNA fragment containing the HIS3 selectable marker isolated from the vector pJ217 (Jones and Parkash, 1990) flanked by genomic DNA sufficient for recombination at the POM152 locus. Flanking the HIS3 gene on the 5' side was a 523-bp amplification product extending from a BamHI site 5' of the POM152 gene to an Mbol site at nucleotide 915. The 3' fragment was derived from a 2.6-kb Sal/BamHI restriction fragment containing the 3' end of the POM152 gene and sequences downstream to a BamHI site (see Fig. 6A for map of this construct).

A BamHI/BamHI fragment of pPM1-His was transformed into the S. cerevisiae diploid strain W303 and His+ transformants were isolated. Heterozygous diploids carrying the integrated pom152-1::HIS3 disrupted gene and the wild-type allele were identified by Southern blotting. Cells (pMY1) were sporulated and tetrad analysis was performed. The expected 2:2 segregation of the His+ marker was observed. The absence of the wild-type POM152 gene in the His+ haploids was confirmed by Southern analysis of genomic DNA (5 μg) isolated from each of four segregants. Probing was conducted using the 5' PCR product described above (labeled with 33P by random priming) under conditions described for library screening.

Indirect Immunofluorescence and Immunoelectron Microscopy

The yeast haploid strain PMY17-HA containing the tagged POM152 gene (pom152-2::HA) was used for both immunofluorescence and immunoelectron microscopy. Cells from early log phase cultures were prepared for indirect immunofluorescence using the procedure of Kilman and Adams (1984) with the modifications of Wente et al. (1992). Probing for the epitope-tagged POM152 with an mAb specific for the HA peptide (12CA5; Delaware Antibody Co., Richmond, CA) and detection with FITC-labeled goat anti-mouse IgG (Cappel Laboratories, Organon Teknika Corp., Durham, NC) was performed as previously described (Wente et al., 1992). Photographs were taken through a 100× objective on Kodak T-Max 400 film (Eastman Kodak Co., Rochester, NY) and processed at 1600 ASA.

For immunoelectron microscopy, spheroplasts (Byers and Goetsch, 1991) from early log phase cultures of PMY17-HA cells were washed and fixed essentially as described in Wente et al. (1992). Spheroplasts were then pelleted, dehydrated in graded alcohol, and embedded in Lowicryl according to the manufacturer's instructions (Electron Microscopy Sciences, Fort Washington, PA). Ultrathin sections were collected on Formvar carbon-coated nickel grids. The sections were probed with mAb 12CA5 (see above) diluted 1:2 in PBS for 2-3 h at room temperature. After washing with PBS, the sections were incubated with goat anti-mouse IgG bound to 10 nm gold (Sigma Chemical Co., St. Louis, MO) as described (Wente et al., 1992). Spheroplasts were then s~ onto appropriate plates containing SM-URA galactose. Washed cells were subsequently incubated in SM-URA containing 2% galactose (SM-URA galactose). Cells were then stained with uranyl acetate and blot and carbon coated nickel grids. The immunolabeling was the same as described for the Lowicryl sections except the antibody was diluted 1:5 in PBS containing 0.5% BSA. The grids were stained and processed according to Griffiths et al. (1983).

The A gold staining of isolated NPCs, samples of the enriched NPC fraction were prepared for negative stain electron microscopy as described (Rout and Blobel, 1993). However, the final uranyl acetate staining step was replaced with a wash of the grids in Con A blot buffer, Control incubations also contained 0.5 M methyl cellosolve and 0.05% triton X-100. After extensive washing in Con A blot buffer, the grids were fixed in 2.5% glutaraldehyde in the same buffer (30 min, room temperature) and negatively stained with 4% uranyl acetate. All images were recorded on Kodak electron microscope film.

Expression of pom152::HA in Mouse NIH 3T3 Cells

A full-length cDNA containing the coding region of pom152-2::HA was assembled from two PCR products, a 5' product, 2.4 kb, a 3' segment of 2.165 bp. The 5' product was synthesized using a sense primer consisting of a 5' XbaI site followed by nucleotides 842-859 of the POM152 gene (including the initiation codon) and an anti-sense primer corresponding to nucleotides 3,241-3,257. The 3' segment was synthesized using a sense primer encoding nucleotides 2,719-2,742 and an anti-sense primer encoding a 5' BamHI site followed by nucleotides 4,865-4,882 (including the termination codon). The temperature for both reactions was the pPM-HA plasmid pom152-2::HA. Both amplification products overlap across a region of the POM152 gene that contains a unique EcoO109I site (at nucleotide 3,088). The two products were cleaved with Eco0109I and either XbaI or BamHI and together assembled in the multiple cloning site of the mammalian expression vector pSVL (Pharmacia LKB Biotechnology, Piscataway, NJ). The pom152-2::HA/pSVL plasmid was introduced into 3T3 cells by direct microinjection (Capechi, 1980). Expression was detected 2-3 days after injection by indirect immunofluorescence using the 12CA5 antibody as described above.

GALI-directed Overexpression of POM152

The PCR-derived pom152-2::HA cDNA was subcloned into the yeast expression vector pRS426 (Christianson et al., 1992) of the GAL1 promoter to produce the plasmid pPMMGal. This plasmid and pRS426 were independently transformed into the diploid strain W303 and viable Lira+ transformants were selected. Single colony transformants were propagated on synthetic medium lacking uracil and containing 2% glucose (SM-URA glucose). To examine the effect of POM152 overexpression on cell growth, cells were transferred to SM-URA containing 2% galactose (SM-URA galactose) or SM-URA glucose media and incubated for 12 h at 30°C. Cells were then streaked onto appropriate plates containing SM-URA galactose or SM-URA glucose, incubated for 48 h at 30°C, and photographed. For immunofluorescence studies, cells were grown to early log phase in SM-URA glucose. Cells were then washed 3× with water and 2× with SM-URA galactose. Washed cells were subsequently incubated in SM-URA galactose for 10 h at 30°C. Indirect immunofluorescence was conducted using the 12CA5 antibody as described above.

Results

Isolated Yeast Nuclear Pore Complexes Contain a 150-kD Con A-binding Protein

Recently, an enrichment procedure has been developed for the isolation of NPCs from the yeast Saccharomyces (Rout and Blobel, 1993). Analysis of the fractions from the enrichment procedure by SDS-PAGE and immunoblotting confirmed the coenrichment of known NPC proteins with the isolated NPCs (Rout and Blobel, 1993). When parallel blots were performed with 13C-Con A (Fig. 1), a single band of ~150 kD (pl50) (Fig. 1, compare lanes 7, 10, 16, and 21) was similarly found to coenrich with the NPCs, coincident with a

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Figure 1. Coenrichment of a Con A-binding protein of ~150 kD with yeast NPCs. Five separate sucrose gradients that sequentially led to enriched NPCs (see Rout and Blobel, 1993) were analyzed by SDS-PAGE and 14C-Con A blotting to identify glycoproteins that cofractionate with NPCs. Gradients and gradient fractions were prepared exactly as described in Rout and Blobel (1993). Coomassie blue staining of similar fractions can be seen in this reference. Fractions from each gradient are grouped as indicated by the lines beneath the gel with the first gradient on the left and the last on the right. The numbers below the lines represent the equivalents (in arbitrary n units) loaded per lane relative to n units of starting cells. Spheroplasts and subsequent fractions containing NPCs starting with crude nuclei (lane 3) and continuing to highly enriched NPCs (lane 21) are indicated above the gel. A single Con A-binding protein of 150 kD (arrowhead, lane 21) is visible coenriching with NPCs. Molecular mass markers in kilodaltons are shown to the left of the gel.

Figure 2. p150 is an integral membrane protein of yeast nuclear envelopes. Yeast NEs (lane NE) were extracted with 0.1 M sodium carbonate, pH 11.5, and then sedimented by centrifugation to yield a supernatant fraction (lane s) containing peripheral proteins and a membrane pellet containing integral proteins (lane p). Proteins of these two fractions, as well as of enriched NPCs (NPC), and of a fraction from the C-4 HPLC eluate containing p150 (Fr.68) (see Fig. 3 B) were separated by SDS-PAGE and either stained with Coomassie blue (panel A) or transferred to nitrocellulose and probed with 14C-Con A (B). Coomassie blue staining revealed a protein of similar mobility as p150 (A, lane Fr.68) in the NPCs, the NE, and the extracted membrane pellet fractions (A, lanes NPC, NE, and p). Consistent with this, 14C-Con A revealed that p150 was present in the NE fraction (B, lane NE) and that it remained associated with the extracted membrane fraction (B, lane p). No Con-A binding protein of similar mobility is visible in the supernatant (B, lane s). Molecular mass markers in kilodaltons are shown to the right.

To determine whether p150 is in fact an integral membrane protein, we extracted isolated yeast NEs with 0.1 M sodium carbonate, pH 11.5, and by subsequent centrifugation separated peripheral from integral membrane proteins in a supernatant (s) and pellet fraction (p), respectively (Fig. 2 A). Indeed, a Coomassie staining band of ~150 kD was present in the NE and resisted high pH extraction (Fig. 2 A, compare lanes p and NE). This membrane-integrated protein comigrated with the p150 protein present in isolated NPCs and in a highly enriched fraction of p150 (Fig. 2 A, lanes NPC and Fr.68: for the origin of Fr.68 see Fig. 3 B). Probing of the SDS-PAGE-separated proteins with 14C-Con A showed that the Con A-reactive p150 in the isolated NPCs and the NPC subfraction (Fig. 2 B, lanes NPC and Fr.68) comigrated with a Con A-reactive protein in the NE (Fig. 2 B, lane NE) that resisted extraction by alkali (Fig. 2 B, lane p). No Con
A-binding proteins of similar mass were visible in the high pH extract (Fig. 2B, lane s). These results suggested that the Con A-reactive p150 of the NPC fraction is an integral protein of the pore membrane.

To purify p150, enriched NPCs were solubilized in SDS and the proteins separated by SDS-HA chromatography (Fig. 3A). In column fractions analyzed by SDS-PAGE, p150 was visible in fractions 34–38 both by Coomassie staining (Fig. 3A, arrowhead) and by 12C-Con A blotting (data not shown). Further separation of polypeptides in the p150-containing fractions was accomplished by reverse phase HPLC (Fig. 3B). p150 was purified from HPLC fractions by preparative SDS-PAGE, transferred to PVDF membrane, and subjected to endoproteolytic cleavage. Amino acid sequence from several peptides was obtained (see Fig. 4). The NH₂-terminus was found to be blocked. The purity of the sequenced p150 was indicated by the fact that all the peptide sequences obtained were found in the deduced amino acid sequence from the cloned gene (see below).

Isolation of the Gene Encoding p150

The internal peptide sequence obtained from p150 allowed us to use PCR to synthesize a corresponding segment of the gene encoding p150. From the sequence of the PCR product a specific anti-sense oligonucleotide was designed and used to screen a yeast genomic library. Five overlapping genomic clones were isolated. Shown in Fig. 4 is a 5,482-bp contiguous sequence obtained by bidirectional sequencing of the isolated clones. An ATG at position 743, which is flanked by conserved bases found at translational start sites (Hamilton et al., 1987), initiates an open reading frame 4,011 bp in length. Flanking this region on the 5' and 3' sides are consensus sequences for transcription initiation (TATA) and termination (Struhl, 1987; Zaret and Sherman, 1982), respectively. Southern analysis of yeast chromosomes placed this gene on chromosome 13 (data not shown).

Analysis of the Deduced Amino Acid Sequence of p150

The open reading frame of the gene encoding p150 is 1,337 amino acid residues long with a calculated molecular mass of 151,670 Daltons, in agreement with the mass of p150 estimated by SDS-PAGE. 13 internal peptide sequences obtained from p150 are represented in the deduced amino acid sequence of this gene, further establishing its identity (Fig. 4). On the basis of its apparent localization to the pore membrane domain and its DNA-deduced molecular mass (~152 kD) we propose to term p150 as POM152.

The integral membrane character of POM152 suggested that it contains one or more transmembrane segments. Analysis of the hydrophobicity of the deduced amino acid sequence of POM152, conducted using the method of Kyte and Doolittle (1982) (data not shown), identified a 19-amino acid segment (residues 175–195) (see Fig. 4) lacking charged residues and of sufficient hydrophobicity to function as a transmembrane segment (Kyte and Doolittle, 1982). Consistent with this assignment, this segment is bordered by charged amino acid residues.

The deduced amino acid sequence of POM152 contains five consensus sites (NXS/T) for N-linked oligosaccharide addition, two on the NH₂-terminal side of the transmembrane segment and three on the COOH-terminal side. The binding of Con A to POM152 suggests that at least one of these sites is glycosylated. This is further supported by the observation that endoglycosidase H digestion of POM152 abolished Con A binding and reduced its apparent molecular mass by ~3 kD (data not shown). Fortuitously, peptide sequence was obtained from a fragment of POM152 containing a putative glycosylation site located at residue 280 (see Fig. 4). The sequence of this fragment proceeds normally from residue 270 to 302 but is blank at the position of the predicted asparagine (residue 280) (data not shown). This suggests that in fact residue 280 is N-glycosylated (Evans et al., 1988).

The deduced amino acid sequence of POM152 appears unique by comparison with currently available protein data bases. No significant similarity exists between POM152 and the two identified mammalian pore membrane proteins,
Figure 4. Nucleotide and deduced amino acid sequence of the POM152 locus. Nucleotides are numbered on the right. Amino acid residues, represented by their one-letter code, are numbered above their symbol at intervals of 10 beginning with the initiator Met and extending through residue 1,337 of the open reading frame. Sequences obtained by peptide sequencing of POM152 are underlined. The potential transmembrane segment is boxed. The asparagine (residue 280) believed to be modified by oligosaccharide addition is indicated by a star. The point of insertion of the hemagglutinin epitope tag following amino acid residue 293 is indicated by an arrowhead. The predicted topology of POM152 in the pore membrane is shown on the right.
gp210 and POM121, with the exception of a short region adjacent to the NH₂-terminal side of the POM152 transmembrane segment that shows 43% similarity to amino acids cloned nucleoporins (Davis and Fink, 1990; Nehrbass et al., including gp210 (Wozniak et al., 1989) and the molecularly

Figure 5. Alignments of POM152 with POM121 and of the POM152 repeats. (A) A 19-amino acid residue segment of POM152 is compared with a similar region of POM121 by the method of Pearson and Lipman (1988). Identical or similar amino acids are boxed. The position in the protein of the last residue in each segment is shown on the right. A single gap is used to improve the alignment. (B) Alignment (by the Pearson/Lipman method) of eight repeated segments of ∼24 residues within POM152. Boxed are identical or similar residues present in five or more of the repeats. Gaps are inserted to improve the alignment. A consensus sequence is shown below which is derived from identical residues in five or more repeats (shown in bold). The position of the last residue in each repeat is shown at the right.

POM152

EFLYTLPSFNTAPRLTFPK 176

POM121

FFSNTPFTNPFPASSAKP 924

C-G-V-L-G-P-F-Y

A-C-G-O-S-D-N-V-E-F-I-D-O-P-L-P-M-K-L-A-N 413

OCVQQGQLVNGFLSGFCAPPYIYNT 650

LCLCDHSHSPVVALGDDPTTLTV 756

KHSGS-VTEIPKLDGCFFPTVKF 859

EVCDMTLEEQIVFLASLPPFILST 966

FLP-P-INLKF-LQGPPFS1TF 1077

EYCEDDY-TAYQLOVAPFMKY 1178

GREDDQAEVFSFHTPPFLTV 1276

Finally, using dot matrix analyses we have identified eight repetitive segments in POM152, each ∼24 amino acids in length. All eight segments lie on the COOH-terminal side of the transmembrane segment. The sequence of these repeating units and their alignments are shown in Fig. 5. With the exception of the distance between the first and second repeats, each of these repeating motifs begins at ∼100-amino acid intervals. The regions between these repeats show no apparent similarity to one another.

POM152 Deletion Mutants Are Viable

Southern blotting of yeast genomic DNA cleaved with various restriction enzymes showed that the POM152 gene is present in a single copy per haploid genome (data not shown). To define the phenotype associated with the disruption of this gene, we deleted it in the diploid strain W303 by gene replacement. This was accomplished by integrative transformation using a DNA fragment in which a 3.6-kb MboI/SalI fragment from within the POM152 reading frame was replaced with a DNA fragment encoding the HIS3 gene (Fig. 6 A). Stable His⁺ transformants were selected and analyzed by Southern blotting to identify those with the correct replacement. One transformant was sporulated and tetrads were dissected (Fig. 6 B). In all cases the segregants were viable and grew equally well. For all segregants, His⁺:His⁻ segregated 2:2. Southern blotting of one set of segregants confirmed the disruption (Fig. 6 C). A comparison of both haploid cells carrying the disruption and wild-type cells revealed no obvious morphological differences when examined by light and electron microscopy (data not shown). Both mutant and wild-type cells also grew equally well at temperatures ranging from 17 to 37°C (data not shown).

Immunolocalization of Epitope-tagged POM152

To establish the cellular distribution of POM152, the localization of epitope-tagged POM152 (POM152-HA) was examined by indirect immunofluorescence microscopy. The tag, which codes for two tandemly repeated, 10-amino acid epitopes derived from the hemagglutinin antigen (HA), was inserted at a unique BglII site (see Fig. 4). Haploid cells with a chromosomal disruption of POM152 (PMY17) were transformed with a single copy plasmid (pPM1-HA) carrying the epitope-tagged POM152 gene (pom152-2::HA) and its endogenous promoter to produce the strain PMY17-HA. Using an mAb against the tag (anti-HA), POM152-HA was visible as patches along the surface of the nucleus (Fig. 7 A). This pattern is characteristic for NPC proteins (Davis and Fink, 1990; Wente et al., 1992; Wimmer et al., 1992).

Definitive evidence for the location of POM152 at the NPC was obtained by immunoelectron microscopy. Spheroplasts from PMY17-HA cells were fixed and either embedded in Lowicryl and sectioned or directly frozen and cryosectioned. Sections were probed with the mAb against the epitope tag and binding was detected with 10-nm colloidal gold particles bound to goat anti-mouse IgG. The gold particles are localized along the NE at the NPC (Fig. 6 B). The positions of over 200 gold particles were quantified on these sections as described in Wente et al. (1992). This revealed a high degree of label specificity for the NE and the NPC (Table II).

In an attempt to sublocalize the native POM152, the enriched NPC fraction was probed with colloidal gold-labeled Con A and visualized by negative stain electron microscopy (Fig. 8 D). As POM152 is the predominant Con A-binding protein in the NPC fraction, the gold distribution should reflect the localization of this protein. We observe that ∼50% of the gold particles (n = 661) were found attached to the NPC rims (Fig. 8 D), representing a 50-fold higher specific activity (gold particles/rim 2) over background. Both the specific labeling and the background were dramatically reduced by the presence of the competitive inhibitor methyl α-D-mannopyranoside (data not shown). Thus, despite the polyspecific nature of the probe, it is likely that the binding of Con A to the NPC rim reflects the position of POM152, consistent with it being a pore membrane protein.

Overexpression of POM152 Inhibits Cell Growth

We have investigated the effects of POM152 overproduction on the growth of diploid W303 cells. pom152::HA was placed under the control of the inducible GAL1 promoter in
Figure 6. Deletion and disruption of the POM152 gene. The POM152 gene was disrupted by deleting an MboI/SalI fragment within the open reading frame (represented by the thick black line) and replacing it with the gene for the HIS3 selectable marker (A). Integrative transformation of the diploid yeast strain W303 was performed with a BamHI fragment from this construct. A heterozygous diploid strain was then sporulated and the tetrads were dissected. B shows the haploid segregants of six tetrads. In each case all four spores were viable. To confirm the success of the disruption Southern blots were performed using BamHI cut genomic DNA from four haploid segregants (C, lanes 2-5) and the parent diploid strain (C, lane 1). The transformed diploid strain contains both the wild-type gene (6.6 kb) and the disrupted gene (4.4 kb). Both His+ (i.e., disrupted) haploids (C, lanes 4 and 5) lack the wild-type POM152 gene.

Figure 7. Localization of epitope-tagged POM152 by indirect immunofluorescence microscopy. Spheroplasts from the haploid strain PMY17-HA (containing pom152-2::HA) expressing the HA-tagged POM152 gene product were fixed, permeabilized, and probed with a mAb (12CA5) directed against the HA epitope. Binding to POM152-HA was visualized with FITC-labeled goat anti-mouse IgG (Anti-HA). DNA-binding DAPI stain was used to define the nucleus in the same cells (DAPI). POM152-HA is visible along the nuclear surface in a punctate pattern characteristic of NPC localization. Bar, 5 μm.

pom152-2::HA
Figure 8. Localization of epitope-tagged POM152 to the NPC by immunoelectron microscopy. Spheroplasts from the haploid strain PMY17-HA expressing the HA-tagged POM152 gene product were fixed and either embedded in Lowicryl (A) or prepared for cryosectioning (B and C). Sections were probed with mAb 12CA5 and binding was detected with goat anti-mouse antibodies bound to 10-nm gold particles. Gold particles are visible along the nuclear envelope in association with the NPCs. Presented in D is an electron micrograph of a negatively stained enriched NPC fraction which had been probed with Con A linked to 20-nm colloidal gold particles. The particles are visible around the rim of the isolated NPCs. Bars, 0.2 μm.
Table II. Distribution of Gold Particles on Immuno-stained Spheroplasts Expressing POM152::HA

| Location     | Total number | Density |
|--------------|--------------|---------|
| Nucleoplasm  | 16           | 0.10    |
| NPCs         | 114          | 1.46    |
| Cytoplasm    | 77           | 0.08    |

The number and location of 10-nm gold particles were determined for 100 spheroplast thin sections. The density of the gold particles was calculated as described (Wente et al., 1992).

the high-copy, 2-μm plasmid pRS426. The resulting plasmid (termed pPMGai) and the pRS426 vector alone were independently transformed into W303 cells and the growth of individual transformants examined on medium containing either glucose or galactose (Fig. 9 A). In cells containing the pPMGai plasmid, galactose-induced overexpression of the pom152::HA gene product markedly inhibited cell growth (Fig. 9 A; compare the growth of three strains containing the pPMGai plasmid on SM-URA galactose to their growth on SM-URA glucose). When one of the overexpressing strains (PMGai) (Fig. 9 B) was examined by indirect immunofluorescence using the anti-HA antibody, POM152-HA was visible along the periphery of the nucleus and in patches adjacent to the plasma membrane suggesting that the protein is largely present in the NE and the endoplasmic reticulum network (Fig. 9 B). No signal was observed with the anti-HA antibody in cells containing the pRS426 plasmid (Fig. 9 C).

Expressed POM152 Localizes to Mammalian Pore Membranes

We have examined the subcellular distribution of the pom152::HA gene product in mouse 3T3 cells to determine whether it would accurately localize to the nuclear pore membrane. To do this the complete open reading frame of pom152::HA was inserted into a transient eukaryotic expression vector (pSVL). Plasmid DNA was introduced into 3T3 cells by direct microinjection into the nuclei of subconfluent, unsynchronized cultures. The localization of the expressed pom152::HA gene product was evaluated by immunofluorescence using the mAb against the tag (Fig. 10, Anti-HA) 21 h after injection. Indeed, POM152-HA was visible in a characteristic punctate pore membrane pattern (Wozniak and Blobel, 1992) both when viewing the nuclear surface (Fig. 10 A) and the nuclear rim (Fig. 10 B). No staining was visible in cells not expressing the pom152::HA gene product (data not shown). These data suggest that yeast POM152 can be accurately sorted and retained within the pore membrane domain of mammalian cells. At high levels of pom152::HA expression an additional endoplasmic reticulum-like staining pattern was also observed (data not shown). This is similar to what has been previously observed with elevated expression levels of the mammalian pore membrane protein gp210 (Wozniak and Blobel, 1992, unpublished data) and is consistent with the movement of POM152 to the pore mem-

Figure 9. Overexpression of POM152. Diploid yeast W303 cells were transformed with the plasmid pRS426 or pPMGai (pRS426 containing pom152::HA under the control of the Gal1 promoter). Cells derived from individual transformants were streaked onto SM-URA galactose and SM-URA glucose plates and tested for their ability to grow (A). As shown in A, cells containing pRS426 (v) grew equally well on both galactose- and glucose-containing medium. However, the growth of strains containing the pPMGai plasmid (1, 2, and 3) was markedly inhibited on galactose plates with no colony formation visible. The overexpression of the pom152::HA gene product was confirmed by indirect immunofluorescence. Cells from a strain carrying the pPMGai plasmid (v) or a control strain with the pRS426 plasmid (C) were grown for 10 h in SM-URA galactose, processed for immunofluorescence, and probed with the anti-HA antibody (12CA5). Binding was visualized with FITC-labeled anti-mouse IgG (Anti-HA). DAPI stain was used to define the nucleus in the same cells (DAPI). In the PMGai strain, POM152-HA was visible in the nuclear envelope and adjacent to the plasma membrane in an endoplasmic reticulum-type pattern (B, Anti-HA). No staining was visible in the pRS426-containing strain (C, Anti-HA). Bar, 5 μm.
Figure 10. POM152-HA is targeted to the pore membrane domain of mouse 3T3 cells. 3T3 cells expressing the pom152::HA gene product were fixed, permeabilized, and probed with mAb 12CA5. Binding to POM152-HA was visualized with Texas red-labeled goat anti-mouse IgG (Anti-HA). POM152-HA is visible at densely packed points along the nuclear surface when the focal plane is tangential to the nucleus (Nuclear surface) or as a punctate ring when the focal plane passes through the center of the nucleus (Nuclear rim). This pattern is characteristic of nuclear pore proteins. Bar, 10 μm.

bran membrane domain following its integration into the endoplasmic reticulum membrane.

Discussion

To date, the pore membrane domain of the yeast NE remains largely undefined. We report here the identification and the characterization of an integral membrane protein of this domain and term it POM152. POM152 coenriched with yeast NPCs indicating that it interacts with the NPC proteins (termed nucleoporins or NUPs), directly or indirectly. That this protein was originally observed as a coenriching constituent of the highly enriched NPC fraction (Rout and Blobel, 1993) further strengthens the likelihood that many of the other 80-90 coenriching proteins in this fraction are pore membrane (POMs) and nuclear pore complex (NUPs) components.

Although we have not experimentally determined the topology of POM152, our data suggest that it contains a single transmembrane segment. The COOH-terminal region (residues 196-1,337) of POM152 is likely to be entirely positioned on the cisternal side of the pore membrane; this region contains three consensus sites for N-linked glycosylation, and at least one of these sites appears to be glycosylated (see results), explaining its reactivity with Con A. Although this cisternal side-exposed region of POM152 shows no sequence similarity to other proteins in the data base, it shows eight repetitive segments of ~24 residues (see Fig. 6 B). Of note is a regularly spaced cysteine in five of the eight repeats. Moreover, all but one of these repeats begins at intervals ~100 residues apart. These repetitive segments might be involved in homophilic or heterophilic interactions on the cisternal side of the pore membrane. Homophilic or heterophilic interactions via repetitive (and topologically equivalent) domains are characteristic of many cell surface adhesion molecules (Edelman and Crossin, 1991). In the case of POM152, side interactions may help stabilize the sharply bent structure (by 180°) of the pore membrane on its cisternal side. Similarly, the cisternally interacting domains of POM152 or its homolog(s) (see below) may contribute, at least in part, either to the lumenal spoke domains or radial arms (see introduction).

The NH2-terminal portion (residues 1-175) of POM152 that precedes its transmembrane segment is likely to be exposed on the pore side of the pore membrane. A small segment of the region (residues 158-176) shows a 43% similarity (see Fig. 6 A) with a topologically equivalent (i.e., pore side exposed) region of the rat POM121 (residues 905-924). The functional significance of this homology, if any, remains to be established.

POM152 is not synthesized with a cleavable signal sequence for integration into the endoplasmic reticulum. As in the case of other type II membrane proteins, the transmembrane segment of POM152 is likely to function both as a signal sequence and as a stop transfer sequence to integrate the protein into the bilayer of the endoplasmic reticulum. It remains to be determined which domain of POM152 serves as the sorting determinant to localize it to the pore membrane domain. For gp210, the dominant sorting determinant has recently been localized to its transmembrane segment (Wozniak and Blobel, 1992). Our observation here that POM152, when expressed in mammalian cells, is correctly sorted to the pore membrane domain of these cells suggests that this pathway is conserved between yeast and mammals. Moreover, the conserved sorting suggests that POM152 might substitute for a mammalian structural and/or functional homolog.

Surprisingly, a yeast strain in which the POM152 gene was deleted was viable and its growth indistinguishable from that of wild-type cells at a wide range of temperatures. It is possible, however, that other growth conditions would reveal a phenotype. This remains to be investigated. Alternatively, a functionally homologous protein(s) may be present within the nuclear pore which can compensate for the loss of
POM152. Overexpression of the POM152 gene severely inhibited cell growth. However, it remains to be determined whether this phenotype is related to alterations in the function of the nuclear pore.

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