Integral membrane proteins associated with the nuclear pore complex (NPC) are likely to play an important role in the biogenesis of this structure. Here we have examined the functional roles of domains of the yeast pore membrane protein Pom152p in establishing its topology and its interactions with other NPC proteins. The topology of Pom152p was evaluated by alkaline extraction, protease protection, and endoglycosidase H sensitivity assays. The results of these experiments suggest that Pom152p contains a single transmembrane segment with its N terminus (amino acid residues 1–175) extending into the nuclear pore and its C terminus (amino acid residues 196–1337) positioned in the lumen of the nuclear envelope. The functional role of these different domains was investigated in mutants that are dependent on Pom152p for viability. The requirement for Pom152p in strains containing mutations allelic to the NPC protein genes NIC96 and NUP59 could be alleviated by Pom152p’s N terminus, independent of its integration into the membrane. However, complementation of a mutation in NUP170 required both the N terminus and the transmembrane segment. Furthermore, mutations in NUP188 were rescued only by full-length Pom152p, suggesting that the luminal structures play an important role in the function of pore-side NPC structures.

Bidirectional transport between the nucleus and the cytoplasm is mediated by nuclear pore complexes (NPCs) (1, 2). These massive structures extend across the nuclear envelope (NE) and are bound to the pore membrane domain (see Refs. 1–3). Integral membrane proteins associated with the NPC are speculated to play an important role in the molecular organization of the NPC and its biogenesis. In metazoan cells, two pore membrane proteins have been identified and characterized: gp210 and Pom121p (4–7). Although the N terminus in each protein is viable, suggesting that Pom152p might be a member of a group of functionally overlapping proteins. This phenotype was utilized in genetic screens to identify a number of genetically interacting nucleoporins including Nup170p (13), Nup188p (14, 15), Nup59p (16), and the previously identified nucleoporin Nic96p (13, 17). Like Pom152p, each of these proteins is an abundant constituent of the yeast NPC. Their abundance has led us to hypothesize that they are components of the repetitive substructures that form the 8-fold symmetrical core of the NPC (15). However, the functional basis for the observed genetic interactions between Pom152p and these nucleoporins remains unclear.

In a continuing effort to understand the function of Pom152p, we have conducted a series of experiments aimed at defining its topology in the pore membrane and examining functional interactions between its different domains and several of the abundant nucleoporins. Our data demonstrate that Pom152p is a type II integral membrane protein with its N-terminal domain positioned on the pore side of the membrane and its C terminus located within the lumen of the NE. With this information, we tested a series of deletion mutations to identify regions of Pom152p that are capable of rescuing the viability of strains containing mutations allelic to NUP170, NIC96, NUP188, and NUP59. Although the N terminus in each
case is necessary to rescue the mutants examined, differing requirements exist for the transmembrane segment and the luminal domain.

EXPERIMENTAL PROCEDURES

Yeast Strains and Media—The yeast strains used in this study are listed in Table I. All strains were grown in either YPD (1% yeast extract, 2% bactopeptone, and 2% glucose) or synthetic minimal media supplemented with the appropriate amino acids, bases, and 2% glucose. 5-FOA (Toronto Research Chemicals)-containing plates were made as described previously (18). All strains were grown at 30 °C. Procedures for yeast manipulation were conducted as described previously (18).

Plasmids—The plasmids used in this study are as follows: (a) pRS315, CEN/LEU2 (19); (b) pPOM152, pRS315 containing the POM152 gene (13); and (c) pPOM152-HA, pRS315 containing HA-tagged POM152 (termed pPM1-HA in Ref. 12). Several deletion variants were constructed from pPOM152 or pPOM152-HA by restriction enzyme digestions or by using PCR techniques and are listed in Fig. 1. In the case of the deletion constructs p170-301-HA and p170-1337-HA, the POM152 promoter and the first two codons of POM152 are followed in frame by the codon for amino acid residue 170.

Preparation of Crude Nuclei, Nuclei, and NEs—Yeast nuclei and NEs were isolated using the procedure of Kilmartin and Fogg (20) with more recent modifications (21). For the isolation of crude nuclei, we used the following mini-scale procedure. Cells were grown in the appropriate media to mid-log phase. All of the following steps were performed at 30 °C. Procedures for yeast manipulation were conducted as described previously (18).

Trypsin Digestion—Yeast NE (or nuclei) containing Pom152p-HA or the p170-1337-HA deletion variant were diluted in BTM buffer and sedimented through a sucrose cushion (1M sucrose in BTM buffer) at 100,000 × g for 15 min. The pellet was then resuspended in BTM buffer and divided into equal aliquots. These aliquots were incubated with different concentrations of trypsin (from 0 to 500 μg/ml) in the presence or absence of 1% of Triton X-100 for 20 min at 0 °C. The reaction was stopped by the addition of trichloroacetic acid (to 10%) or solution P (to 1%) and soybean trypsin inhibitor (Boehringer Mannheim). To examine the membrane association of protease protected fragments of Pom152p, the membranes were sedimented through a sucrose cushion (1M sucrose in BTM buffer) at 100,000 × g for 15 min after trypsin digestion and the addition of trypsin inhibitors. The pellet was then washed with BTM buffer, and alkaline was extracted as described above.

Endoglycosidase H Treatment—Crude yeast nuclei (10 μg of total protein) were washed several times with BTM buffer and then resuspended in endoglycosidase H (Endo H) buffer (50 mM sodium citrate, pH 5.5, 0.1% SDS, and 1 mM dithiothreitol). Samples were incubated at 75 °C for 20 min to denature proteins and solubilize membranes. 5 milliunits of Endo H (Boehringer Mannheim) or a mock solution as a control and phenylmethylsulfonyl fluoride to a final concentration of 1 mM were added. Samples were then incubated overnight at 37 °C. Proteins in the reaction mixtures were then recovered by trichloroacetic acid precipitation and solubilized in SDS sample buffer.

Western Blot Analysis—Proteins were separated by SDS-polyacryl-
amid gel electrophoresis and then electrophoretically transferred to nitrocellulose membranes (Hybond-C, Amersham). Antibody incubations using the monoclonal antibodies mAb 12CA5 and mAb118C3 and subsequent washes were performed in phosphate-buffered saline containing 0.1% Tween-20. Binding was detected using enhanced chemiluminescence with horseradish peroxidase-labeled donkey anti-rabbit IgG (Amersham). For the Western blot analysis of total cell lysates, cells from liquid cultures were collected by centrifugation. Pellets were resuspended in 2M NaOH containing 8% β-mercaptoethanol and incubated on ice for 10 min. Proteins were precipitated with trichloroacetic acid, washed with acetone, resuspended in SDS sample buffer, sonicated, and separated by SDS-polyacrylamide gel electrophoresis.

**RESULTS**

Expression of POM152 Deletions and Mapping of the mAb118C3 Binding Site—A series of POM152 deletion mutations were constructed to examine the functions of different domains of Pom152p. Schematic representations of these deletions are shown in Fig. 1. The majority of these constructs contain two tandem repeats of an epitope derived from the HA antigen inserted after amino acid residue 295 (12) that are recognized by the monoclonal antibody mAb 12CA5. Plasmids containing POM152-HA and the different deletion variants were introduced into a pom152-null strain (PMY17). Synthesis of the encoded proteins was evaluated by Western blotting of total cell extracts using mAb 12CA5 (Fig. 2A). With the exception of the p1-1219 construction, which lacks the HA tag, each of the deletion variants was detected with mAb 12CA5 and was visible migrating at an apparent molecular mass near that predicted by their sequence.

Using these deletion variants, we have mapped by Western blotting the region of Pom152p that is recognized by mAb118C3 (Fig. 2B), a previously identified monoclonal antibody that binds specifically to Pom152p (21). We observed that C-terminal truncations as short as 118 amino acid residues (including p1-1219 and p1-1025-HA) abolished mAb118C3 binding (Fig. 2B). Conversely, all deletion constructions that contained the last 301 amino acid residues of Pom152p (including p170-1337-HA, p176-263-HA, and p295-1036) bound to mAb118C3, suggesting that this antibody binds to an epitope within this region. Moreover, taking into account that variant p1-1219 is functionally expressed in yeast (see the complementation data below), it is likely that the epitope for mAb118C3 is located within this region. Moreover, taking into account that variant p1-1219 is functionally expressed in yeast (see the complementation data below), it is likely that the epitope for mAb118C3 is located within this region.

**Topology of Pom152p in the Pore Membrane**—On the basis of its primary structure, we previously proposed that Pom152p is a type II integral membrane protein containing a single transmembrane segment (TMS) with a pore-side N-terminal domain and a luminal C-terminal segment (12). However, this and other plausible models have not been experimentally tested.

As a first step in defining its topology, we have attempted to identify the region(s) of Pom152p that acts as a transmembrane segment(s). Hydrophathy analysis of Pom152p (Fig. 3A) revealed two hydrophobic regions of sufficient length to serve as TMSs extending from amino acid residues 111–131 and 176–195. To test the ability of each of these regions to act as a membrane anchor, alkaline extraction experiments were performed on crude nuclear fractions isolated from yeast expressing deletion constructions of POM152 encoding one or both of the potential TMSs. Consistent with previous results (12), full-length Pom152p was resistant to extraction and was quantitatively present in the membrane pellet, whereas peripheral nucleoporins, including Nup116p, were extracted (Fig. 3B). Similarly, a truncated form of Pom152p containing the N terminus and both of the potential TMSs, p1-301-HA, was resistant to alkaline extraction and remained associated with the membrane. However, the ability of Pom152p to integrate into the membrane was dependent on the 176–195 segment alone. A deletion construct lacking the 111–131 segment and containing the 176–195 segment (p170-1337-HA) was resistant to alkaline extraction. Conversely, a deletion mutant lacking the 176–195 segment and containing the 111–131 segment was quantitatively extracted from the membrane (p176-263-HA; Fig. 3B). These results suggest that Pom152p contains a single TMS. We therefore concluded that the molecule is divided into three segments: (a) an N-terminal segment (consisting of amino acid residues 1–175), (b) a TMS (amino acid residues 176–195), and (c) a long, C-terminal segment (amino acid residues 196–1337).

The presence of a single membrane anchoring domain suggested four possible orientations for Pom152p in the membrane (Fig. 4). Models I and II represent a situation in which the hydrophobic segment crosses the membrane, whereas in models III and IV the anchoring domain loops into the membrane. To assess the topology of Pom152p, we performed limited pro-
tease digestion experiments of NEs containing Pom152p-HA. We predicted that the cisternal domain of Pom152p should be largely protected by the nuclear membrane, whereas a cytoplasmic, pore-exposed segment would be accessible to the protease and would be at least partially degraded.

As shown in Fig. 5, the digestion of intact NEs with trypsin produced a fragment of Pom152p-HA with an apparent molecular mass 7 kDa less than that of the wild-type protein (termed Pom152p-7). The Pom152p-7 fragment was largely resistant to proteolysis at various concentrations of trypsin, and it was recognized by both mAb 12CA5 and mAb118C3. Because these antibodies bind epitopes that lie near opposite ends of the C-terminal segment, it is likely that Pom152p-7 contains the majority, if not all, of this segment. Under similar conditions, the nucleoporins Nup53p and Nup188p were rapidly degraded (data not shown). The Pom152p-7 fragment, however, was quickly degraded in the presence of Triton X-100. In this case, several distinct fragments were detected with mAb 12CA5 and mAb118C3, suggesting that the C terminus was now accessible to the protease. To a far lesser degree, these smaller fragments were also present in intact NEs treated with high concentrations of trypsin (50 and 500 μg/ml). This likely reflects the accessibility of the protease to the perinuclear cisterna in unsealed NEs.

To further evaluate whether the Pom152p-7 fragment is the result of a cleavage at the N terminus or the C terminus, we performed similar trypsin digestion experiments on nuclear fractions isolated from cells expressing Pom152p-HA or a deletion variant lacking the N terminus (p170-1337-HA). As shown in Fig. 6A, the molecular mass of p170-1337-HA was unaffected by trypsin treatment, whereas full-length Pom152p was cleaved to the Pom152p-7 form. These results demonstrate that the cleavage that generates Pom152p-7 occurs at the N terminus and that the TMS and the C terminus are protected. Interestingly, the protease-resistant Pom152p-7 truncation migrated more slowly than the p170-1337-HA deletion, suggesting that the Pom152–7 fragment contains a portion of the N terminus.

On the basis of these results, we would predict that Pom152p-7 is anchored to the membrane. To test this, alkaline extractions were performed on NEs after treatment with a high concentration of trypsin (100 μg/ml). As shown in Fig. 6B, Pom152p-7 was present within the pellet fraction, suggesting that it was anchored to the membrane. Similarly, smaller fragments recognized by mAb 12CA5 were also bound to the membrane, which is consistent with the location of the HA epitope.

Fig. 3. A, hydropathy plot of Pom152p. Hydrophobic values of individual amino acid residues were averaged within a 23-amino acid residue window as described by Kyte and Doolittle (26) using the Strider program. Arrows indicate the position of two hydrophobic peaks centered within two potential transmembrane segments extending from amino acid residues 111–131 (1) and 176–195 (2). B, membrane integration of Pom152p deletion constructs. Crude nuclei were isolated from PMY17 cells expressing various POM152 deletion constructs. Nuclei were then extracted with 20 mM NaOH. After centrifugation, polypeptides in aliquots of the supernatant (S) and the membrane pellet (P) fractions were analyzed by Western blotting using the anti-HA antibody mAb 12CA5 (α-HA). The efficiency of the extraction was evaluated by probing the p1-301-HA-containing samples with an anti-GLFG polyclonal antibody (α-GLFG) (the position of Nup116p is indicated). Molecular mass markers are indicated in kilodaltons.
near the TMS.

The results of the experiments described above are consistent with a model for the topology of Pom152p in which the N terminus is located on the pore side of the membrane and the C terminus is positioned in the lumen of the NE (Fig. 4, II). However, we could not exclude the possibility that the C-terminal segment is positioned at the pore side of the membrane and is highly protected from proteolysis by other NPC proteins (Fig. 4, IV). Two observations argue against this model: (a) Pom152p binds concanavalin A, suggesting that it is glycosylated (12); and (b) concanavalin A binding to Pom152p can be abolished by treatment with endoglycosidase H (data not shown). The specificity of this enzyme for oligosaccharide modifications made in the lumen of the endoplasmic reticulum/NE suggests that in fact a domain of Pom152p lies within the NE lumen. To clearly establish that the C-terminal segment is glycosylated, Endo H treatment was performed on Pom152p-

Fig. 4. Hypothetical models for the topology of Pom152p in the pore membrane. Four potential topological models for Pom152p within the pore membrane are shown. N and C indicate the position of the N terminus and the C terminus of the protein. O and □ indicate the approximate positions in Pom152p-HA of epitopes recognized by the monoclonal antibodies mAb118C3 and mAb 12CA5, respectively. A thick black line denotes the transmembrane segment.

Fig. 5. Accessibility of Pom152p to exogenous protease. NEs were isolated from PMY17 cells containing Pom152p-HA and treated with different concentrations of trypsin in the absence (-) or presence (+) of 1% Triton X-100. After incubation for 15 min at 0 °C, all digests were terminated by the addition of trichloroacetic acid. The trichloroacetic acid precipitates were analyzed by Western blotting using the monoclonal antibody mAb118C3 or mAb 12CA5. Molecular mass markers are indicated in kilodaltons.

Fig. 6. A, resistance of p170-1337-HA to trypsin digestion. Crude nuclei were isolated from PMY17 cells containing Pom152p-HA and p170-1337-HA (lacking the N terminus). Samples were treated with (+) or without (-) trypsin (50 μg/ml). Immediately after addition (0) or after a 15-min incubation at 0 °C (+), all digests were terminated by the addition of trichloroacetic acid. The trichloroacetic acid precipitates were analyzed by Western blotting using the monoclonal antibody mAb 12CA5 (α-HA). B, membrane association of the Pom152p-7 trypsin fragment. NEs containing Pom152p-HA were treated with (+) or without (-) trypsin (100 μg/ml). Trypsin-digested membranes were then subjected to alkaline extraction. After centrifugation, aliquots of the supernatant (S) and membrane pellet (P) fractions were analyzed by Western blotting using mAb 12CA5 (α-HA). Molecular mass markers are indicated in kilodaltons.
HA- and p170-1337-HA-containing nuclear fractions. The presence of N-linked oligosaccharides was assessed by a change in the molecular mass of the proteins after Endo H digestion. As shown in Fig. 7, Endo H treatment reduced the molecular masses of both Pom152p-HA and p170-1337-HA by ~7 kDa. This reduction in mass corresponds to the elimination of three to four polysaccharide chains from the N-glycosylation sites. This is in agreement with the number of potential sites (four) in the C-terminal region. These results confirm that the C-terminal segment is N-glycosylated and is localized in the lumen. Finally, in similar experiments performed with a Pom152p mutant lacking the TMS (pA176-263-HA; Fig. 7), Endo H treatment had no effect on the protein’s mass, suggesting that the TMS is necessary for translocation of the C-terminal segment into the lumen of the endoplasmic reticulum/NE (Fig. 7). Together, these data demonstrate that Pom152p is a type II integral membrane protein.

**Domain-specific Complementation of Pom152p Synthetic Lethal Mutants**—We have previously identified a battery of mutants allelic to the nucleoporin genes NUP59, NUP170, NUP188, and NIC96, which are dependent on the presence of Pom152p for their viability (13, 14, 16). This may reflect the ability of Pom152p to perform a function similar to these nucleoporins and/or stabilize structures effected by various nucleoporin mutations. To determine what regions of Pom152p are required to complement these various mutants, different deletion constructs were tested for their ability to replace the wild-type Pom152p. Because each of the mutant strains is dependent on a URA3-containing plasmid, pCH1122-POM152 (URA3), they fail to grow on 5-FOA-containing plates (13, 14). Functional complementation of these mutants by various truncated forms of Pom152p was scored by their ability to rescue growth on 5-FOA-containing media. However, in the absence of the TMS, the majority of the C-terminal domain was required, because deletions in this segment (p1025 and p424) abolished complementation of psl7 and psl40. For the complementation of the psl21 (nup170-21) mutant, the N terminus and the TMS were both necessary and sufficient. Surprisingly, strains containing mutations allelic to NUP188 (psl4 and psl44) were rescued only by full-length POM152. Although capable of complementing other psl mutants, all deletions of the C terminus that we tested failed to complement psl4 and psl44. These results suggest an important role for the luminallly disposed C-terminal domain.

**DISCUSSION**

The data presented in this study provide experimental evidence for the topology of Pom152p. In addition, we have used this information to identify functional domains of Pom152p that are necessary for its genetic interactions with other NPC proteins. Using Pom152p and various deletion constructs in combination with alkaline extraction, protease protection, and endoglycosidase H assays, we have demonstrated that Pom152p is a type II integral membrane protein with its N-terminal 175 amino acid residues positioned within the nuclear pore and its C-terminal 1141 amino acid residues located within the lumen of the NE. Pom152p is anchored to the membrane by a single TMS extending from amino acid residues 176–195. This segment is both necessary and sufficient for its attachment to the membrane. Adjacent to the TMS, two positively charged amino acid residues are positioned on its N-terminal side, and two negatively charged amino acid residues flank the C-terminal side. This charge distribution flanking the TMS is similar to that observed in other type II membrane proteins and has been used as a tool for predicting membrane protein topology (22–24).

As is the case for other type II membrane proteins, Pom152p does not contain a cleavable N-terminal signal sequence for integration into the endoplasmic reticulum membrane (12). Thus, the TMS of Pom152p is likely to function both as a signal sequence and as a stop-transfer sequence to integrate the protein into the membrane. Our data showing that all of the constructs containing the TMS are integrated into the membrane support this idea. Moreover, if a small region containing the TMS is deleted, the remainder of the protein (pA176-263-HA) is no longer resistant to alkaline extraction, and the C-terminal segment is not glycosylated (Figs. 3 and 7).

The N-terminal 175 amino acid residues of Pom152p are located on the pore side of the nuclear envelope membrane. This region is thus positioned to directly interact with nucleoporins that compose the core domain of the NPC. These are
likely to include a subset of the abundant nucleoporins that have been localized to the NPC core including Nup188p, Nup170p, Nup157p, and Nic96p (13, 14). Consistent with this observation, in mutants that are allelic to the NUP59, NUP170, NUP188, and NIC96 genes and require Pom152p for viability, the N terminus of Pom152p is uniformly required for complementation. Interestingly, we observed that the N-terminal truncation of Pom152p that is resistant to trypsin (Pom152p-7) migrates on SDS-polyacrylamide gels with an apparent mass larger than a deletion mutant lacking the N terminus (p170-1337-HA). This result can not be explained by the positioning of trypsin cleavage sites (data not shown). Instead, it is likely due to the partial protection of the N terminus, perhaps by its association with other NPC proteins or the periphery of the pore membrane.

Whereas the N terminus was necessary for the complementation of all of the POM152 synthetic lethal mutants that we examined, the requirements for the TMS and the lumenally disposed C-terminal segment were dependent on the particular nucleoporin mutants examined. For example, the complementation of the nic96-7 and nup59-40 mutant alleles required the N terminus in combination with either the TMS or the complete C-terminal segment. The necessity for either of these two segments suggests that they can act indirectly to sequester two or more N-terminal segments in a conformation that is capable of binding nucleoporins. For wild-type Pom152p, the spatial orientation of N termini may be established by lateral interactions between adjacent TMSs (either homotypic or heterotypic) and the C-terminal segments of neighboring Pom152p molecules. By comparison, the complementation of the nup170-21 mutant strictly required both the N terminus and the TMS, suggesting that integration into the membrane is essential for rescuing this mutant. Such varying requirements for the context in which the N terminus is presented may reflect the involvement of Pom152p in different stages of NPC biogenesis.

It is likely that the large luminal domain of Pom152p (≈85% of the molecule) contributes a significant amount of mass to NPC structures located in the lumen of the NE. In this regard, it is similar to the mammalian pore membrane protein gp210 (5, 8), which has 95% of its mass positioned on the luminal side of the membrane. This region of gp210 likely contributes to luminal structures such as the lumenal spokes or radial arms, and it has been proposed that this region plays a role in pore formation and the maintenance of NPC structure (5, 8, 10). Moreover, antibodies to the luminal domain of gp210 partially inhibit nuclear import of classical nuclear localization signal-containing substrates (25). Surprisingly, the complementation of mutants allelic to NUP188 requires full-length Pom152p including the lumenally disposed C-terminal segment. Even a deletion as short as 118 amino acid residues from the C terminus abolishes complementation. These results further underscore the importance of the luminal region of Pom152p in the function of its N terminus. Moreover, they represent a functional link between structures located within the lumen of the NE and those positioned on the pore side of the membrane.

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