Nup192p Is a Conserved Nucleoporin with a Preferential Location at the Inner Site of the Nuclear Membrane*

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Human Nup93, the homologue of yeast Nic96p, is associated with a 205-kDa protein whose intracellular location and function is unknown. We show here that the yeast open reading frame YJL039c, which is homologous to this human p205, encodes the so far largest yeast nucleoporin. Accordingly, green fluorescent protein (GFP)-tagged YJL039c was localized to the nuclear pores and therefore named Nup192p. Affinity purification of ProtA-Nic96p from glutaraldehyde-fixed spermatids reveals association with Nup192p. NUP192 is essential for cell growth. A temperature-sensitive mutant nup192-15 is neither impaired in nuclear import of a SV40 nuclear localization sequence-containing reporter protein nor in mRNA export, but association of Nup49-GFP with nuclear pores is inhibited at the non-permissive temperature. By immunoelectron microscopy, Nup192p-ProtA is seen at the inner site of the nuclear pores, at a distance of 60 ± 15 nm from the central plane of the pore. This suggests that Nup192p is an evolutionarily conserved structural component of the nuclear pore complex with a preferential location at the inner site of the nuclear membrane.

The nuclear pore complexes are huge structures embedded in the nuclear membrane that provide the major route for the passive diffusion of small molecules and active transport of large molecules between the nucleus and cytoplasm (1–4). In the electron microscope, the nuclear pore complex displays a modular organization, consisting of an octasymmetrical framework of eight spokes sandwiched between cytoplasmic and nuclear rings (5). The spokes embrace a central channel or “transporter” which gates nucleocytoplasmic transport in both directions. Attached to this core structure are peripheral elements, the cytoplasmic pore filaments, which extend from the cytoplasmic ring, and the nuclear basket attached to the nuclear ring and consisting of eight filaments that end in a distal ring (6–9). Electron microscopy also showed that the 8-fold symmetry and modular aspects of pore complex organization have been conserved during evolution although yeast NPCs1 are smaller as compared with Xenopus NPCs with respect to molecular mass and dimensions; in addition, some prominent structures present in vertebrate NPCs such as the luminal basket are absent in yeast (10, 11). The overall conservation in NPC morphology between yeast and vertebrates suggests that many components of the nuclear pore complex are conserved during evolution. Indeed, a significant number of NPC constituents are homologous between yeast and higher eukaryotes; however, often this homology is not easily noticed, and there are also cases in which no yeast homologue exists when a vertebrate Nup (e.g. Nup153) is known (12, 13). Conserved also is the machinery of soluble nucleocytoplasmic transport factors which interact with nucleoporins, in particular NPC constituents with FG/FXFG/GLFG repeat sequences (14). Accordingly, soluble transport factors such as importin/karyopherin α and β (Kap60p and Kap95p in yeast), the small GTPase Ran, and NTF2 have also been conserved in evolution (3).

One of the evolutionarily conserved subcomplexes of the nuclear pore is the Nsp1p complex in yeast and its higher eukaryotic counterpart, the p62 complex (12). In yeast, the core Nsp1p complex is built up by Nsp1p, Nsp49p, and Nup75p (15), to which Nic96p is further attached (16). In vertebrates, the p62 complex consists of p62, p58, p54, and p45 (17), in which p62 and Nsp1p, p54 and Nup57p, and p58 and Nup49p show a distinct sequence similarity (12). Furthermore, yeast Nic96p has homologues in human and Xenopus which were named Nup93. Interestingly, human Nup93 was localized to the nuclear basket by immunoelectron microscopy, and immunoprecipitation with anti-Nup93 antibodies revealed interaction with a pool of p62 and a novel protein of 205 kDa (18). Recently, Nsp1p and its interacting partner Nic96p were located by immunoelectron microscopy to distinct sites within NPCs, i.e. at both sites of the central gated channel and at the terminal ring of the nuclear basket (11).

To further study the interaction of Nic96p with other nuclear pore proteins in yeast, we sought to analyze the putative Saccharomyces cerevisiae homologue (ORF YJL039c) of human p205, which is in a complex with Nup93 (18). We show here that YJL039c encodes the so far largest yeast NPC protein, which is essential for cell growth. By immunoelectron microscopy, Nup192p-ProtA was found to be localized at the nuclear basket. Temperature-sensitive mutants of nup192 reveal that the NPC reporter protein Nup49p-GFP was no longer assembled into nuclear pores. This suggests that Nup192p is a structural protein of the nuclear pores, most likely constituting a major component of the nuclear basket filaments.

EXPERIMENTAL PROCEDURES

Yeast Methods and DNA Recombinant Work—The yeast strains used in this study are listed in Table I. Cells were grown in minimal SDC or rich yeast extract/peptone/dextrose medium. Genetic manipulations of yeast were performed as described (19). The following yeast plasmids were used: pUN100, ARS/CEN plasmid with the LEU2 marker; YCplac33, ARS/CEN plasmid with the TRP1 marker; YCplac22, ARS/CEN plasmid with the URA3 marker; YCP211, ARS/CEN plasmid with the ADE2 marker. Manipulation and analysis of DNA such as restriction analysis, end-filling, ligations, DNA sequencing, and polymerase chain reaction amplifications were performed according to Maniatis et
Gene and Plasmid Constructions—The NUP192 gene was excised from the cosmids clone cos28 (provided by Dr. Francis Galibert, Faculté de Médecine, Rennes, France) as a 7-kb StuI/SthI fragment and inserted into plasmid pUN100. To disrupt the NUP192 gene, pUN100-NUP192 was cut with MluI/SnaBI, and a 5.65-kb fragment corresponding to the NUP192 gene was released and replaced by the HIS3 gene. It was cut with SphI/EcoRI that released a 2.3-kb fragment, containing the HIS3 gene and flanked by 0.5-kb 5′- and 0.6-kb 3′-untranslated region of NUP192. This nup192::HIS3 fragment was used to transform the diploid BY420 strain for homologous recombination. A haploid nup192::HIS3 shuffle strain was constructed which was complemented by plasmid-borne YCplac33-URA3-NUP192. For tagging of Nup192p at its C-terminal end, a NolI site was created just before the stop codon of NUP192 by a PCR-based method. Three tandem HA epitope tags or the ProtA tag, available as its C-terminal end, a nup192::HIS3 MATα,ade2, his3, leu2, trpl, ura3, can1/can1, nup192::HIS3/NUP192 (BY420 derived)

NUP192 shuffle

nup192–15

MATa or a, ade2, his3, leu2, trpl, ura3, nup192::HIS3 (YCplac33-URA3-NUP192) (pUN100-LEU2-nup192–15 or YCplac22–TRP1-nup192–15)

NUP192-GFP ProtA-NIC96

MATa,ade2, his3, leu2, trpl, ura3, URA3-ProtA-NIC96 (pUN100-LEU2-NUP192-HA)

nup133

MATa or a, ade2, his3, leu2, trpl, ura3, nup133::HIS3

nup192 /nup133

MATa or a, ade2, his3, leu2, trpl, ura3, nup192::HIS3, nup133::HIS3 (pUN100-LEU2-NUP192-GFP, pAS211-ade2)

RESULTS AND DISCUSSION

We recently reported that mammalian Nup93, the vertebrate homologue of yeast Nic96p, is associated with p205 (18). Because a S. cerevisiae ORF (YJL039c) is homologous to human p205 (18), we tested whether this yeast YJL039c encodes a homologue of human p205. Meanwhile, an uncharacterized Schizosaccharomyces pombe ORF (GenBank accession number 4176539) appeared in the data libraries which is significantly homologous to S. cerevisiae ORF YJL039c. This allowed multiple sequence alignment between the various yeast and higher eukaryotic p205-related ORFs (Fig. 1A). Interestingly, when the S. cerevisiae and S. pombe ORFs were analyzed for transmembrane segments, several potential membrane-spanning sequences were predicted (Fig. 1B). Accordingly, these proteins contain hydrophobic stretches of amino acids scattered along the entire protein sequence (see also later).

The yeast YJL039c gene encoding a 192-kDa protein (p192) was tagged with GFP. Fluorescence microscopy of p192-GFP revealed a punctate nuclear envelope location with a staining pattern highly resembling the in vivo labeling of bona fide GFP-tagged nucleoporins (Fig. 1C). To demonstrate that p192 physically associates with nuclear pores in yeast, p192-GFP was expressed in nup133 cells which have clustered nuclear pores (24). p192-GFP co-clusters with nuclear pores in nup133 cells (Fig. 1C). Accordingly, p192 was named Nup192p. To find out about the in vivo role of NUP192, gene disruption of ORF YJL039c was performed. This showed that NUP192 is essential for yeast cell growth (Fig. 2A). The lethal phenotype of the nup192::HIS3 disruption mutant could be complemented by plasmid-borne NUP192 or NUP192-ProtA. Thus, NUP192 belongs to a group of nucleoporins that perform a unique and non-redundant function. In yeast, nucleoporins lacking FG-type repeat sequences are often not essential for cell growth (e.g. NUP188, NUP170, NUP157, NUP133, NUP120, NUP85, NUP84, POM152) and thus perform an overlapping function (25). The fact that NUP192 has not shown up so far in genetic screens for overlapping or redundant interactions at the nuclear pores (e.g. synthetic lethal screens) may be explained by the difficulty in obtaining by random mutagenesis a specific mutation within the essential NUP192 that causes synergistic impairment in combination with another mutated nucleoporin without completely inactivating the NUP192 function. Furthermore, because no apparent defect in nucleocytoplasmic transport could be found in nup192 thermosensitive mutants, it makes sense in retrospect that nup192 mutants were not among mutant collections that are impaired in nuclear protein import and mRNA export.

To study the in vivo role of NUP192, several thermosensitive mutants of NUP192 were generated by random mutagenesis (Fig. 2B). One of these thermosensitive mutants (nup192–15), which grows well at the permissive temperature (23 °C) and arrests at 37 °C (Fig. 2B, lane 6), was analyzed in more detail. The growth arrest was seen in nup192–15 thermosensitive mutants after an 8–10-h shift to the non-permissive temperature; accordingly, nuclear protein import of a classical NLS-containing GFP-reporter protein and Pus1p-GFP, as well as nuclear mRNA export, was analyzed in the nup192–15 thermosensitive mutant after a shift for several hours to the re-
Fig. 1. Nup192p is an evolutionary conserved yeast NPC protein. A, multiple sequence alignment of Nup192p (YJL039c) and its homologues in *S. pombe* (AL035260), human (D86978), and *Caenorhabditis elegans* (CEK12D12) using ClustalW1.7 and Boxshade Software. B, prediction of transmembrane segments within *S. cerevisiae* (*S. c.*) and *S. pombe* (*S. p.*) Nup192p by using TMpred Software. Positive values in the graphs depict the probability of transmembrane helices. C, subcellular location of Nup192p-GFP in nup192Δ cells and nup133Δ cells. Nuclear envelope and nuclear pore location is revealed by fluorescence microscopy.
strictive temperature; however, no inhibition of these nuclear protein import pathways (Fig. 3A) and mRNA export (Fig. 3B) was noticed. It is worth mentioning that the nic96–1 thermo-sensitive mutant did not reveal defects in nuclear protein import and mRNA export, thus resembling the nup192–15 mutant (16, 26). Interestingly, the nic96–1 mutant is impaired in NPC biogenesis (26). We therefore tested whether the assembly of the NPC-associated protein GFP-Nup49p is altered in nup192–15 cells upon shift to the restrictive temperature. The punctate nuclear envelope signal of GFP-Nup49p significantly decreased after 8–10 h at 37 °C; in a few cells, aggregation of GFP-Nup49p was also noticed (Fig. 3C). As a control, nup192–15 cells complemented by plasmid-borne wild-type NUP192 showed a normal nuclear envelope distribution and signal intensity of GFP-Nup49p when grown for 8–10 h at 37 °C (Fig. 3C). This suggests that assembly of GFP-Nup49p into the nuclear pores is impaired in the nup192–15 mutant. By using a similar fluorescence-based strategy, it was recently shown that certain NPC assembly mutants have a reduced GFP-Nup49p signal as compared with wild-type cells (27). We tested other GFP-tagged nucleoporins for the association with the nuclear envelope in nup192–15 versus NUP192 cells. In the case of Nic96p-GFP, Nup57p-GFP, and Nup82p-GFP, we also observed that the nuclear envelope location was significantly diminished at the restrictive temperature in nup192–15 as compared with NUP192 cells (data not shown).

To find out whether Nup192p is associated with Nic96p, ProtA-tagged Nup192p was affinity purified. Because the protein was predicted to contain eight strong transmembrane helices (see also Fig. 1B), we purified Nup192p-ProtA in the absence and presence of 1% Triton X-100. However, under both conditions, Nup192p-ProtA could be purified similarly well, yielding Coomassie-stainable amounts (Fig. 4A, lanes 9 and 10). This suggests that Nup192p is not an integral membrane protein, and the presence of hydrophobic sequences may reflect a different structural feature than facilitating membrane insertion. Interestingly, Nic96p contains several hydrophobic stretches of amino acids in its primary sequence that are not...
Nup192p Is the Largest Yeast Nucleoporin

**Fig. 2.** NUP192 is essential for yeast cell growth. A, tetrad analysis of a heterozygous diploid strain disrupted for the ORF YJL038c (nup192::HIS3/NUP192)*. This revealed a 2:2 segregation for viability for the pUN100-transformed strain, but a 4:0 or 3:1 segregation, when plasmid-borne NUP192-Prota was present (lanes 1–4, individual spores from complete tetrad). B, generation of temperature-sensitive nup192 mutants. The NUP192 gene was mutagenized, and derived thermosensitive mutants (nup192::HIS3 + pLEU2-nup192 ts) were characterized by growth on yeast extract/peptone/dextrose-plates at 23 °C (2 days) and 37 °C (3 days). Lane 1, wild-type (NUP192) strain; lanes 2 and 3, nup192–12 thermosensitive strain; lanes 4 and 5, nup192–13 thermosensitive strain; lanes 6 and 7, nup192–15 thermosensitive strain; lanes 2, 4, and 6, cells lacking pURA3-NUP192; and lanes 3, 5, and 7, cells carrying pURA3-NUP192 ts, thermosensitive.

**Fig. 3.** The thermosensitive nup192–15 mutant is impaired in assembly of GFP-Nup49p into nuclear pores. A, analysis of nuclear protein import in nup192–15 cells. Nuclear accumulation of the nuclear reporter proteins SV40 NLS-GFP-λaC and GFP-Pus1p in nup192–15 thermosensitive cells was measured after shifting the cells for 12 h to 37 °C by in situ hybridization using a fluorescently labeled oligo(dT) probe. Cells were also stained for DNA, and Nomarski pictures were taken. A control served the mex67–5 thermosensitive mutant which is strongly impaired in mRNA export at the non-permissive temperature. B, analysis of GFP-Nup49p assembly into nuclear pores in nup192–15 cells. It was grown at the indicated temperatures and for the indicated times before pictures were taken. The nup192–15 thermosensitive mutant was also transformed with the wild-type NUP192 gene inserted into a single copy ARS/CEN plasmid (pNUP192).

membrane-spanning helices, but mutations therein strongly impair the Nic96p function (16).

Affinity purified Nup192p-Prota does not reveal a major band which could correspond to Nic96p (Fig. 4A, Coomassie). On the other hand, Nup192p-Prota had the tendency to degrade during purification yielding many ProtA-containing breakdown bands, as revealed by Western blotting (Fig. 4A, anti-ProtA). This made it difficult to estimate by Western blotting whether Nic96p is present in the eluate in substoichiometric amounts. Therefore, we affinity purified ProtA-Nic96p and looked for co-purification of a HA-tagged version of Nup192p by Western blotting using anti-HA antibodies. In this case, a small pool of Nup192p-HA could be detected in the ProtA-Nic96 eluate (Fig. 4B). We fixed yeast spheroplasts with glutaraldehyde to stabilize protein complexes prior to affinity purification of ProtA-Nic96p. This allowed us to obtain a better co-enrichment of HA-tagged Nup192p in purified ProtA-Nic96p preparation derived from 0.1% glutaraldehyde-treated spheroplasts (Fig. 4B, lane 7). It appears that Nup192p was not chemically cross-linked to Nic96p because the putative cross-link product should migrate at −300 kDa on the SDS-polyacrylamide gel. The band observed on the SDS-polyacrylamide gel migrates at −190 kDa, which is the expected size of Nup192p-HA. Accord-
Nup192p is the largest yeast nucleoporin. Nup192p which is likely to be the functional homologue of an evolutionarily conserved human protein (p205) that was found in association with human Nup93. This yeast Nup, although essential, escaped genetic screens so far. However, we could find it in an unusual way by employing reverse genetics that were based on the information available from p205, the potential human homologue of Nup192p. This assumption turned out to be correct and allowed us to identify a yeast nucleoporin on the basis of its sequence homology to its putative mammalian counterpart. All the subsequent work with yeast Nup192p revealed that it is a NPC protein and involved in the assembly of the NPC marker protein GFP-Nup49p into nuclear pores. The location of Nup192p at the nuclear basket and its interaction with the Nic96p complex finally led us to suggest that Nup192p and Nic96p are essential components of the nuclear basket and are required for NPC assembly. Finally, we propose that human Nup93 and its interacting protein, p205 (hNup192), perform a similar role in the higher eukaryotic cells.

In conclusion, we have identified the so far largest yeast nucleoporin Nup192p which is likely to be the functional homologue of an evolutionarily conserved human protein (p205) that was found in association with human Nup93. This yeast Nup, although essential, escaped genetic screens so far. However, we could find it in an unusual way by employing reverse genetics that were based on the information available from p205, the potential human homologue of Nup192p. This assumption turned out to be correct and allowed us to identify a yeast nucleoporin on the basis of its sequence homology to its putative mammalian counterpart. All the subsequent work with yeast Nup192p revealed that it is a NPC protein and involved in the assembly of the NPC marker protein GFP-Nup49p into nuclear pores. The location of Nup192p at the nuclear basket and its interaction with the Nic96p complex finally led us to suggest that Nup192p and Nic96p are essential components of the nuclear basket and are required for NPC assembly. Finally, we propose that human Nup93 and its interacting protein, p205 (hNup192), perform a similar role in the higher eukaryotic cells.

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