Structural Basis of the Nic96 Subcomplex Organization in the Nuclear Pore Channel

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SUMMARY
Nic96 is a conserved nucleoporin that recruits the Nsp1-Nup49-Nup57 complex, a module with Phe-Gly (FG) repeats, to the central transport channel of the nuclear pore complex (NPC). Nic96 binds the Nsp1 complex via its N domain and assembles into the NPC framework via its central and C domain. Here, we report the crystal structure of a large structural nucleoporin, Nic96 without its N domain (Nic96ΔN). Nic96ΔN is composed of three domains and is a straight molecule that—although almost entirely helical—exhibits strong deviations from the predicted α-solenoid fold. The missing N domain projects midway from the Nic96 molecule, indicating how the Nsp1 complex might be located with respect to the rod-like Nic96. Notably, Nic96ΔN binds in vitro to FG repeats of the Nsp1 complex. These data suggest a model of how Nic96 could organize a transport module with coiled-coil domains and FG repeats in the central pore channel.

INTRODUCTION
The macromolecular machinery that enables bidirectional transport between the nucleus and the cytoplasm is a huge multiprotein assembly in the nuclear membrane, the nuclear pore complex (NPC). The overall architecture of the NPC is conserved and has been determined by various electron microscopic approaches (Reichelt et al., 1990; Beck et al., 2004; Rout and Blobel, 1993). Accordingly, the eight-fold symmetrical NPC consists of a central spoke complex, which is embraced by a cytoplasmic and a nuclear ring. Attached to the spoke-ring array are peripheral elements, the cytoplasmic pore filaments, and the nuclear basket (Beck et al., 2004). The spoke-ring complex forms the central channel through which nucleocytoplasmic transport occurs. The NPC, which has a calculated molecular mass of 40–60 MDa in yeast (Rout et al., 2000), is composed of a relatively small number (∼30) of different proteins (nucleoporins, Nups), which exist in multiple (8, 16, or 32) copies within the octagonal NPC scaffold. A significant number of these nucleoporins carry phenylalanine-glycine-rich repeat domains (FG repeats), which directly function in nucleocytoplasmic transport, whereas the nucleoporins that lack FG repeats are thought to be the main structural constituents of the NPC core (Stewart, 2007).

It has been calculated that the maximum mass flow through a single NPC is up to 80 MDa per second (Ribbeck and Gorlich, 2001). Transport substrates larger than 40 kDa are actively transported in a complex with transport receptors (karyopherins). The translocation through the NPC relies on the interaction of these transport receptors with the FG nucleoporins (Bayliss et al., 2000; Rexach and Blobel, 1995). Low-affinity interaction of the karyopherins with the FG domains allows the cargo-transporter complex to pass through the NPCs by facilitated diffusion, whereas the terminating step is energy dependent and requires hydrolysis of RanGTP (Görlich and Kutay, 1999). The exact mechanism of the translocation through the NPC is still unclear, but several models are currently discussed. These models differ mainly in how the FG repeats are arranged within the NPC scaffold. The selective phase model predicts the existence of an evenly distributed FG meshwork in the central channel formed by weak interactions of the FG repeats (Ribbeck and Gorlich, 2001). Cargo-transport complexes are capable of diffusing through this hydrophobic meshwork by dissolution of the FG self-interactions. Another model is based on the idea that the probability of entering an NPC transport channel will increase if the residence time of cargo-transport complexes at the transport entrance is extended (Rout et al., 2000). If a cargo has reached the entrance it can diffuse through a channel to the other side of the NPC. A further hypothesis, the “olive spaghetti” model, is a combination of these prior models, where the FG nucleoporins fill the inner nuclear pore wall, leaving a central channel of ∼10 nm (Macara, 2001). A recent study showed that a special class of FG nucleoporins, harboring Gly-Leu-Phe-Gly (GLFG) repeat motifs, can self-interact via this hydrophobic motif (Patel et al., 2007).

By combination of biochemical analyses and immuno-electron microscopy (EM), insight into the NPC composition, including the relative location of nucleoporins within the overall NPC framework, was obtained (Rout et al., 2000). Of the ∼30 yeast nucleoporins, about two-thirds are organized in biochemically stable NPC subcomplexes. One of the first NPC modules to be identified and studied was the Nup62 (formerly p62) complex in metazoan (Davies and Blobel, 1987; Dabauvalle et al., 1990) and its homologous counterpart, the Nsp1 complex in yeast (Grandi et al., 1993; Grandi et al., 1995). The Nsp1 complex is composed of four subunits, Nsp1, Nup49, Nup57, and Nic96, which are held together by coiled-coil interactions (Grandi et al., 1995). Within
this module, the Nsp1-Nup49-Nup57 core heterotrimer carries
FG domains that function in transient interaction with the shut-
tling transport receptors. Recently, the α-helical coiled-coil
domains of two subunits of the homologous Nup62 complex,
Nup58 and Nup45, have been crystallized and suggested to be
involved in adjusting the pore diameter (Melcak et al., 2007).

Yeast Nic96 belongs to a class of structural Nups without FG
repeats that occurs in at least 16 copies per NPC (Rout et al.,
2000). As expected for such an integral part of the NPC core
structure, it could be recently demonstrated for the vertebrate
homolog of Nic96, Nup93, to have the highest residence time
at the NPC of all Nups. Single Nup93 molecules stay integrated
in the NPC for about 70 hr, which is in strong contrast to highly
mobile Nups like Nup153, whose residence times range in the
order of seconds (Rabut et al., 2004).

Nic96 is essential in yeast, and mutations impair the formation
of NPCs and nuclear import (Zabel et al., 1996). Likewise, human
Nup93 binds to the Nup62 complex and is involved in NPC
biogenesis (Grandi et al., 1997). Nsp1 and its vertebrate ortholog
Nup62 are required for nucleocytosolic transport (Finlay et al.,
1991; Grandi et al., 1995). The interaction of Nic96 with Nsp1
could be narrowed down to the N-terminal domain, which
harbors coiled-coil motifs (Grandi et al., 1995). Except for the
interaction with the Nsp1 module, Nic96 was reported to bind
to the large structural nucleoporins Nup188 and Nup192 (Zabel
et al., 1996; Kosova et al., 1999; Nehrbsass et al., 1996). However,
these interactions found in pull-down experiments were weak,
yielding only substoichiometric amounts of Nic96 bound to
Nup188 or Nup192.

Structural knowledge of nucleoporins at the atomic level is
limited, with only a few X-ray structures of Nup domains reported
to date (Vetter et al., 1999a; Hodel et al., 2002; Berke et al., 2004;
Weirich et al., 2004; Melcak et al., 2007). Therefore, compu-
tional modeling has been the only means of predicting the
structure of the other NPC constituents. Notably, these analyses
predicted that most nucleoporins are constructed from a small
number of protein folds such as coiled-coil, α-helical solenoid,
β propeller, and natively unfolded FG repeat domains (Devos
et al., 2004, 2006).

To investigate the molecular architecture of Nic96, which is
a bridging molecule between the NPC core structure and the
heterotrimeric Nsp1 module that lines the transport channel,
we solved the crystal structure of Nic96 lacking its N-terminal
coiled-coil domain. Notably, the Nic96 structure has no signifi-
cant homolog in the PDB database and therefore could not be
predicted by previous computational modeling. In combination
with in vivo and in vitro data, the presented structural data clar-
ifies how Nic96 might be involved in NPC organization at the
boundary between the spoke-ring array and the FG meshwork
lining the central transport channel.

RESULTS

Recombinant Expression and Crystallization
of Yeast Nic96ΔN
The Nic96-containing Nsp1 complex consisting of Nic96, Nsp1,
Nup49, and Nup57 was purified from yeast and digested with
trypsin to identify stable proteolytic breakdown products to
be tested in crystallization trials. Whereas Nsp1, Nup49, and
Nup57 were largely degraded, a stable fragment of Nic96 truncated
from the N terminus was obtained (Nic96ΔN, Figure 1A and see Figure S1 available online). The N-terminal domain of
Nic96 (residues 1–189) was previously shown to contain coiled-
coil motifs, which mediate the interaction with the heterotrimeric
Nsp1–Nup49–Nup57 core complex (Grandi et al., 1995). The
observed proteolytically stable Nic96ΔN is similar to a previously
described Nic96 construct consisting of the central and C-termi-
nal domain that was shown to be involved in NPC targeting and/or
assembly (Grandi et al., 1995; Zabel et al., 1996). Subse-
quently, Nic96ΔN was expressed in E. coli and isolated to homo-
geneity using standard chromatography methods. Size exclusion
chromatography and dynamic light scattering indicated that
purified Nic96ΔN was monomeric (data not shown). Nic96ΔN
was crystallized in space group P1 at two different conditions,
one of which yielded crystals of higher quality (for further crystal-
lization details, see the Experimental Procedures).

Overall Structure of Nic96ΔN
The yeast Nic96ΔN (residues 190–839) crystals contain two
monomers in the P1 cell (Figure S2A). Although the contacts
are quite extensive, the biochemical data clearly suggest that
Nic96 is monomeric. In general, the structure reveals a highly
elongated protein, mainly composed of α helices (Figures 1B
and 1C). The atomic structure of the 650 residue-long Nic96ΔN
exhibits four distinct regions composed of (1) the N-terminal
end (residues 204–267, red), (2) a globular head domain (resi-
dues 268–477, orange), (3) a central domain, which is rod-like
(residues 478–731, blue), and (4) a C-terminal tail domain that
exhibits four distinct regions composed of (1) the N-terminal
end (residues 204–267, red), (2) a globular head domain (resi-
dues 268–477, orange), (3) a central domain, which is rod-like
(residues 478–731, blue), and (4) a C-terminal tail domain that
is also globular and comprises α helices 27–32 (residues 732–839,
grey) (Figure 1B). All residues that are defined and there-
fore included in the model are listed in Table 1. Notably, the
N-terminal of Nic96ΔN protrudes away from the middle of
the molecule and runs back into the head domain (Figure 1B
and Figure S2C). Thus, the first three N-terminal helices of
Nic96ΔN (residues 204–267) cofold with the central part of
Nic96 (residues 478–731) and both parts together form the
central domain with its 16 α helices. The N-terminal end also
contributes to the hydrophobic core of the molecule and is an
integral part of this domain. Exceptions from the otherwise ex-
clusively α-helical fold are residues 590–602 that form a β hairpin
insertion interrupting helix 20 and a second short β hairpin in
the head domain (residues 327–332). Moreover, Nic96ΔN contains
a number of loop regions, with many of them being disordered
in the crystal structure. The longest disordered loop spans
13 residues (517–529) between helices 16 and 17 (data not
shown).

To investigate whether the Nic96ΔN structure is related to
known protein folds, a DALI search for structural similarities
(http://www.ebi.ac.uk) was performed for the three subdomains
of Nic96ΔN. Accordingly, no meaningful structural relative was
found for the head domain, suggesting the structure represents
a new fold type. The central domain (without the N-terminal end)
forms a quite irregular α-α-superhelix with various breaks and
insertions (Kobe and Kajava, 2000). It bears limited structural
resemblance to tetratricopeptide repeat proteins, but the DALI
superimpositions are always limited to only a few pairs of helices.

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The irregularity of the Nic96 repeats becomes evident upon comparison with a "canonical" protein of the armadillo-repeat family of the $\alpha$-$\alpha$-superhelix fold, importin $b$. Accordingly, superposition of six helix-turn-helix motifs of the central domain of Nic96 (Figure 1E, blue) yielded an average rmsd of 2.4 Å. The first and last helices of the central domain have no partner helix to form an ARM- or HEAT-like helix-turn-helix motif, and helices 20a and 20b show even larger structural differences and are omitted for clarity. For comparison, 18 repeat motifs of importin $b$ (gray) differ by only 1.26 Å in the rmsd value upon superposition of the C$\alpha$ atoms (Figure 1E, gray). The tail domain shows limited structural similarity to RPR domains, which are found in proteins involved in the regulation of nuclear pre-mRNA; however, it might also be interpreted as very irregular extension of the central domain fold.

The irregularity of the Nic96 repeats becomes evident upon comparison with a "canonical" protein of the armadillo-repeat family of the $\alpha$-$\alpha$-superhelix fold, importin $\beta$. Accordingly, superposition of six helix-turn-helix motifs of the central domain of Nic96 (Figure 1E, blue) yielded an average rmsd of 2.4 Å. The first and last helices of the central domain have no partner helix to form an ARM- or HEAT-like helix-turn-helix motif, and helices 20a and 20b show even larger structural differences and are omitted for clarity. For comparison, 18 repeat motifs of importin $\beta$ (gray) differ by only 1.26 Å in the rmsd value upon superposition of the C$\alpha$ atoms (Figure 1E, gray). The tail domain shows limited structural similarity to RPR domains, which are found in proteins involved in the regulation of nuclear pre-mRNA; however, it might also be interpreted as very irregular extension of the central domain fold.

Taken together, the structural analyses revealed that the architecture of Nic96 deviates extensively from the predicted simple $\alpha$-solenoid fold. The head domain has no structural homolog in the PDB database, and the central domain is an irregular $\alpha$-$\alpha$-superhelix, in which the N-terminal end forms an additional layer of helices and might also qualify for a new fold.
Electron Microscopy of Nic96ΔN

The highly elongated Nic96ΔN molecule as seen in the crystal structure appears to be fairly rigid without obvious hinge regions that could point to dynamic conformational changes in the context of the structural framework of the NPC (Figures 1B and 1C). To determine whether Nic96ΔN in solution also exhibits a rigid structure, we performed EM of the negatively stained protein isolated from E. coli. Characteristic class averages of Nic96ΔN after iterative alignment and classification of ∼4000 particles revealed that the Nic96 molecules have the same straight and lengthened appearance as found in the crystal structure (Figure 1D). Thus, the EM analysis supported that Nic96ΔN is not structurally flexible. However, a hinge region accessible to trypsin cleavage (Figure S1) might exist between the rigid rod and the missing N-terminal domain that projects midway from the Nic96 molecule (Figure 1B and Figure S2C) and recruits the Nsp1-Nup49-Nup57 heterotrimer.

Surface Properties of Nic96ΔN

A Nic96ΔN monomer was calculated to have an overall accessible surface area of about 29,400 Å² (AREAIMOL, ccp4i, version 5.0.2), and two neighboring monomers in the crystals share 9000 Å² of their surface in head-to-tail contacts mediated by a number of hydrogen bonds (Figure S2B and data not shown). This head-to-tail packing becomes possible due to a peculiar feature of the Nic96ΔN molecule: the protein displays a strong polar surface charge distribution, which generates a significant dipole moment across the entire molecule. This dipole moment is due to two oppositely charged poles, the strongly positively charged N-terminal head domain and the negatively charged C-terminal tail domain separated by the central region that is of mixed surface charge (Figure 1C). The direction of the dipole vector is aligned with the longitudinal axis of the molecule with a calculated vector size of 2576 Debyes (D) and 537 e*A⁻, respectively (calculated for all 650 residues after nondefined residues were placed into the model). Notably, the dipole moment of Nic96ΔN is unusually high when compared to other proteins, which on average have a 4-fold lower dipole moment (638.5 D for 11,981 proteins analyzed).

Refined nic96 Mutants Designed on the Basis of the Nic96ΔN Crystal Structure

Based on the crystal structure of Nic96ΔN, we sought to construct refined nic96 mutants lacking structurally distinct regions (e.g., head, tail, coiled-coil) to better define the contribution of each domain for the overall Nic96 function. Thus, precise domain deletion constructs were generated in full-length Nic96, which lack (1) the C-terminal tail domain (Nic96Δtail, residues 610–839), (2) the head domain (Nic96Δhead, residues 204–610), or (3) the N-terminal coiled-coil domain (Nic96ΔN, residues 1–189) (Figure 2A). Subsequently, these mutants were labeled with GFP and expressed in a nic96 gene disruption mutant (Figure 2B). Consistent with previous findings, the C-terminal domain of Nic96 is of minor importance for the essential Nic96 function (Zabel et al., 1996), whereas the deletion of the head domain caused a significant growth defect at all tested temperatures (Figure 2B). Also in accordance with earlier observations (Grandi et al., 1995), the deletion of the coiled-coil N domain from Nic96 caused a severe growth retardation that was even stronger than deletion of the head domain (Figure 2B).

Our X-ray data allowed reinterpretation of two previously described temperature-sensitive mutants, nic96-1 (L260P, P332L) and nic96-2 (W334R) (Zabel et al., 1996), that map in the globular head domain and the central domain (L260), respectively. The W334R mutation could abolish a hydrophobic contact to residues in helices 6 and 7 (W334) and hence affect the overall folding of the hydrophobic core. The L260P mutation interrupts an α helix and thus could also have an effect on the overall hydrophobic core of the central domain. Moreover, this latter mutation illustrates the importance of the correct cofolding of the N-terminal end and the central part of Nic96ΔN. The P332L mutation might affect folding of an extended loop region with a β hairpin insertion at the surface of the head domain (Figure S2D). Interestingly, unlike most of the surface loops in the crystal structure, this extended loop is well defined in the electron density map of Nic96ΔN.

Finally, a Nic6 deletion mutant lacking a hydrophobic stretch (nic96Δ322–342; Grandi et al., 1995) from the head domain can now be better interpreted in the structural sense. Interestingly, the nic96Δ322–342 strain was not viable (Grandi et al., 1995), whereas deletion of the entire head domain (nic96Δhead, residues 204–610) allowed cell growth, albeit very slowly (Figure 2B). Altogether these data suggest that the nic96Δ322–342 mutant protein was misfolded and hence behaved like a strain lacking Nic96.

Head and Coiled-Coil Domains of Nic96 Contribute to Efficient NPC Targeting

To find out how the various domains in Nic96 contribute to NPC targeting, we analyzed the respective GFP-labeled constructs by fluorescence microscopy. Cells expressing GFP-tagged Nic96Δ head exhibited a punctate nuclear rim staining with faint staining of the cytoplasm, which was similar to wild-type Nic96 (Figure 2C). In contrast, cells expressing GFP-Nic96Δhead exhibited a reduced nuclear envelope staining and increased cytoplasmic staining (including cytoplasmic spots), suggesting that assembly into the NPC was affected. Consistent with these findings, the Nic96Δhead and Nic96Δtail constructs, when tagged with ProtA to facilitate their affinity purification, were still associated with the Nsp1-Nup49-Nup57 heterotrimer (Figure 2D). This suggests that the coiled-coil N domain—which is present in those constructs—is still able to recruit the Nsp1 complex independent of the other domains. Moreover, cells expressing GFP-Nic96ΔN exhibited a strongly diminished NPC location with an increased cytoplasmic signal. In addition, GFP-Nic96ΔN was often seen concentrated in a single spot close to the nuclear envelope that also contained another NPC marker, Nup120, labeled with the red fluorescent reporter mCherry (Figure S3). Altogether these data demonstrate that different Nic96 domains (head, coiled-coil) contribute to optimal nuclear envelope localization.

Surface Point Mutations in the Head and Tail Domain of Nic96ΔN

To analyze whether the overall net charge of the Nic96 head and tail region is of functional importance (e.g., for electrostatic interaction with neighboring nucleoporins), we mutated charged surface residues in these domains. For the positively charged head,
we generated a triple mutant, in which the surface lysine residues K312, K318, and K321 were changed to glutamates. In a similar way, the negative charge of the tail domain was changed by the double mutant D669R and D671R. However, all these mutations did not affect cell growth when compared to wild-type cells (data not shown). In contrast, combining the Nic96 triple head mutant (K312E, K318E, K321E) with the nup188 gene disruption caused a synthetic lethal phenotype (Figure 2E). Altogether these genetic data imply that the analyzed surface charges in the head and tail domain of Nic96 do not play an essential function but become crucial when another structural nucleoporin, which is linked to Nic96, is defective. Thus, the oppositely charged head and tail domains of Nic96 are of functional significance and may facilitate protein-protein interactions with neighboring structural nucleoporins. However, further mutagenesis is needed to understand in detail the importance of the observed asymmetry of charge distribution within the Nic96 rod.

Figure 2. Nic96 Mutants Generated on the Basis of the Crystal Structure
(A) Schematic drawing of the analyzed Nic96 deletion constructs tagged with GFP at the N terminus. The numbers above the graph indicate the position at which the deletions have been made.
(B) Growth of the Nic96 domain deletion constructs on YPD plates for 2 or 6 days.
(C) Subcellular localization of the indicated GFP-labeled full-length Nic96 and domain deletion constructs analyzed in the fluorescence microscope (left panel) or viewed by Nomarski microscopy (right panel). Scale bar, 5 μm.
(D) Affinity purifications of ProtA-tagged Nic96 full-length and domain deletion constructs (Nic96 Δtail, Nic96 Δhead) from nic96Δ cells. The indicated eluates were analyzed by SDS-PAGE and Coomassie staining. The position of Nic96 bait proteins is indicated by a red star, and copurifying Nsp1, Nup57, and Nup49 are shown by arrows.
(E) Growth of a nic96 charge mutant (nic96 K312E K318E K321E) mapping in the head domain on 5-FOA-containing plates. This nic96 head domain mutant was expressed in either NUP188 or nup188Δ cells. No growth on 5-FOA indicates synthetic lethality. To reveal equivalent loading of cells, it was also spotted on SDC-Leu plates.

Nic96ΔN Binds to the GLFG Repeat Domains of Nup49, Nup57, and Nup145N

A recent study reported binding of GLFG domains of nucleoporins to a subset of non-FG nucleoporins including Nic96 by using a fluorescence-based visual assay (Patel et al., 2007). In view of our finding that the central domain of Nic96—although only partially—superimposes to a region in importin β, which is also known to bind to FG repeats of nucleoporins (Bayliss et al., 2000), we sought to further explore whether Nic96ΔN can directly bind the FG repeats of different nucleoporins. To this end, we performed an in vitro binding assay using recombinant Nic96ΔN immobilized on GSH beads via the GST tag to which different purified FG repeat fragments were added in the presence of an E. coli lysate to compete for unspecific binding (Figure 3A). This analysis demonstrated that Nic96ΔN binds to the GLFG repeats of Nup49 and Nup57, which are subunits of the Nsp1 complex. Moreover, Nic96ΔN also interacted with the GLFG repeats of another nucleoporin, Nup145N. However, Nic96ΔN only marginally bound to the FXFG repeats of Nsp1 (Figure 3A).

Moreover, reciprocal binding experiments were performed using immobilized GLFG repeats isolated under native conditions from E. coli and recombinant Nic96ΔN (Figure 3B). Previously, we showed that GST-GLFGNup100 repeats exhibit specific binding to transport receptors like Mex67-Mtr2 or Arx1, but not to other tested proteins (Strässer et al., 2000; Bradatsch et al., 2007). When GST-GLFGNup100 was incubated with Nic96ΔN premixed with an E. coli lysate, significant binding of Nic96ΔN to the GST-GLFG repeats, but not to GST-FXFGNsp1 repeats or GST alone, was observed under these “fishing” conditions (Figure 3B). This binding of Nic96ΔN to GST-GLFGNup100 repeats showed a saturation behavior (Figure S4).
Last, we tested synthetic GLFG peptides derived from Nup57 either with one or two GLFG motifs and labeled with a His tag in pull-down experiments. This analysis showed that a single GLFG peptide can interact with Nic96ΔN, but the binding was strongly increased when the peptide with two GLFG motifs was used (Figure 3C). Only residual background binding of Nic96ΔN to empty Ni-NTA beads was observed. Recombinant Ran, which served as a negative control, showed no specific binding to the GLFG peptide-coated beads (Figure 3C). Altogether, these data reinforced the possibility that Nic96ΔN harbors a binding site or sites for GLFG but not FXFG peptides, which may have implications for a role of Nic96ΔN in the organization of FG domains within the active transport channel (see the Discussion).
DISCUSSION

Previously, due to the lack of experimentally determined structures, bioinformatic methods were used to predict the fold of the nucleoporins that constitute the NPC (Devos et al., 2004, 2006). This method predicted only a small number of distinct fold types for the proteins of the NPC (Devos et al., 2006). Nic96 was predicted to be a member of the α-solenoid Nups (Devos et al., 2006), which consist of only α helices arranged in a superhelical manner (as, e.g., found in karyopherins that are composed of HEAT or ARM repeats; Vetter et al., 1999b). In contrast to the prediction, the crystal structure of yeast Nic96ΔN has revealed an irregular helical fold that was not foreseen. Nic96 is a straight molecule composed of three regions with different folds, where at least the head domain has no related structure in the PDB database. Thus, the hypothesis that the NPC assembly is formed from a limited number of evolutionarily conserved structural folds in a “lego-like” fashion (Devos et al., 2006) should be treated with caution. Indeed, our structural findings reinforce the need to solve the crystal structures of the other nucleoporins.

To investigate whether Nup93, the mammalian homolog of Nic96, exhibits a similar structural appearance and dipole moment, we calculated a homology model of Nup93ΔN based on the structure of Nic96ΔN. The resulting model (Figure S5) has good internal scoring (Threader, version 3.5, http://bioinf.cs.ucl.ac.uk/threader/, z score = 6.49 [significant is >4]) at 23.6% sequence identity. The internal Threader database comprising >6000 unique folds from the PDB revealed no other significantly similar structure. Accordingly, Nup93 is very likely to exhibit the same overall structure as Nic96ΔN, implying that the overall fold is conserved during evolution. Unlike Nic96, Nup93 is predicted to have a much smaller dipole moment (data not shown), pointing to a yeast-specific peculiarity.

The X-ray structure of Nic96ΔN reveals a potential arrangement of the full-length Nic96 and the Nsp1-Nup49-Nup57 heterotrimer. As the N-terminal end of Nic96ΔN and likely the missing N-terminal coiled-coil domain project from the center of the elongated Nic96 molecule, the Nsp1-Nup49-Nup57 heterotrimer—upon binding to the coiled-coil domain—could be oriented perpendicularly to the longitudinal axis of the Nic96 molecule (Figure 4A). Thus, the entire Nic96-Nsp1-Nup49-Nup57 complex could exhibit a T-shaped architecture. However, there appears to be a hinge region between the coiled-coil N-terminal domain and adjacent Nic96ΔN molecule. Thus, it is possible that this hinge region allows for topological flexibility of the coiled-coil domain and its attached Nsp1-Nup49-Nup57 complex with respect to the rod-like Nic96 molecule.

The observation that Nic96ΔN can bind to FG repeats of nucleoporins suggests that this structural nucleoporin might also be involved in organizing the natively unfolded FG repeats in the central transport channel. It was recently reported that the Nup84 complex, which is composed solely of nucleoporins lacking FG repeats, can interact with several different FG Nups (Allen et al., 2002). These interactions may help to maintain a “seal” between the filamentous FG repeat meshwork and the structural core of the NPC scaffold (Allen et al., 2002). In current models it is assumed that the density of FG domain filaments within the central transport channel of the NPC influences the exclusion size of the permeability barrier.

Altogether the findings can be interpreted with the following model for the organization and topology of the Nsp1 complex within the NPC scaffold (Figure 4B). (1) Eight Nic96 molecules are circularly arranged (but a perpendicular orientation could also be possible) at the boundary between the structural core of NPC and the central pore channel that contains the FG repeat meshwork. (2) The Nic96 moiety, which corresponds to Nic96ΔN, assembles via its head and tail domains into the NPC scaffold by interaction with other structural Nups (e.g., Nup188, Nup192, Nup84 complex). (3) Via its N domain that harbors coiled-coil motifs, Nic96 recruits the Nsp1-Nup49-Nup57 module, whose
natively unfolded FG repeats project into the pore channel that forms the permeability barrier for nucleocytoplasmic transport. (4) Nic96 can directly bind to GLFG repeat motifs of Nup57 and Nup49 and other nucleoporins, ensuring that the pore channel is also sealed around the NPC structural core. (5) The hinge region between the Nic96 rod domain and the coiledcoil N domain could allow for topological flexibility. Thus, the Nsp1-Nup49-Nup57 complex bound to the coiled-coil N domain of Nic96 might adopt other orientations than depicted. Recently, it was proposed that eight Nup58/45 tetrameric assemblies (Nup58/45 is the mammalian ortholog of yeast Nup49) are circularly arranged to form a ring in the pore channel and sliding of two Nup58/45 dimers against each other would result in an overall extension of the Nup58/45 tetramer and hence induce a dilation of the central pore complex (Melck et al., 2007). To date, it remains to be shown whether the Nsp1-Nup49-Nup57 coiled-coil complex exhibits a circular arrangement as proposed for the analogous p62 complex.

In conclusion, the crystal structure of Nic96 gives insight into the possible organization of FG domains at the border between the central transport channel and the structural channel wall.

EXPERIMENTAL PROCEDURES

Construction of Mutant Alleles and Genetic Analysis

Plasmids used in this work are listed in Table S1 and were generated by standard recombinant DNA methods. TAP integration and deletion strains were constructed in DS1-2b (MAT α trp1-169 his3-120 ura3-52 leu2-α1). Nic96 constructs were transformed in nic96Δ and nic96Δ/nup188Δ shuffle strains, and the shuffle plasmid was lost on 5-FOA plates. Growth of the single and double mutants was performed on either 5-FOA or YPD plates (Bassler et al., 2001). Double mutants were predicted to contain two molecules per asymmetric unit corresponding to a Matthews coefficient of 3.9 and a solvent content of 67.9%, respectively.

Electron Microscopy and Image Processing

For negative staining, 7 μl of sample was placed on a freshly glow-discharged, carbon-coated grid and stained with uranyl acetate (2% w/v) and dried. Micrographs were recorded under low-dose conditions on a 2 k × 2 k Tietz-CCD camera (TVIPS F224) at 200 kV with a nominal magnification of 50,000×. For image processing, a total of 4000 particle images were selected, boxed, and normalized in the gray value distribution. Particle images were aligned with respect to each other by alignment by classification (Dubé et al., 1993) omitting the first step of particle centering. Alignment was followed by multistatistical analysis (MSA) as implemented in IMAGIC V (van Heel et al., 1996).

Expression and Purification of Nic96 from E. coli

Nic96ΔN (coding for residues 189–650 of the full-length protein) was PCR cloned into the BamHI/Xhol sites of pET24d. Heterologous expression of the Nic96 construct as GST fusion protein was performed at 22 °C overnight in E. coli BL21 CodonPlus cells (Stratagene) grown in 2 × YT enriched LB medium after induction with 0.5 mM isopropyl-β-D-thiogalactopyranoside at a cell density of OD600 = 0.6. The harvested cells were disrupted using a microfluidizer (Microfluidics, lysis buffer: 300 mM NaCl, 50 mM Tris [pH 8.0], 5% glycerol, 5 mM β-mercaptoethanol, 1 mM EDTA, and protease inhibitors), and the cleared crude extract was loaded onto a GST column. After intensive washing (wash buffer: 300 mM NaCl, 50 mM Tris [pH 8.0], 5 mM β-mercaptoethanol, and 1 mM EDTA), the GST tag was cleaved off by incubation with TEV protease at 4 °C on the GSH column overnight. Nic96ΔN was isolated to homogeneity using a Superdex S200 size exclusion column (elution buffer: 200 mM NaCl, 10 mM Tris [pH 8.0], and 5 mM β-mercaptoethanol). Peak fractions were concentrated to 10–25 mg/ml and flash frozen in liquid nitrogen for subsequent crystallization studies.

Selenomethionated protein was generated using the feedback inhibition method (Doublie, 1997) with an expression and purification procedure as described for native Nic96. Complete incorporation of selenomethionine was verified by MALDI-TOF analysis.

Crystallization and Structure Determination of Nic96ΔN

High-throughput crystal screening was performed (Hampton Research screens, Mosquito robot) and the following refined crystallization condition yielded native Nic96ΔN crystals of space group P1 (hanging drop, 20 °C): 3%–10% PEG 3350, 0.1 M Bis-Tris (pH 6.5), 0.05 M lithium sulfate, 3% 1.6 hexadimethonium, and 0.01 mM DTE at a protein concentration of 20–25 mg/ml. A second crystallization condition led to native Nic96ΔN crystals of the same space group and dimensions but of a lower quality: 2%–10% PEG 8000, 0.1 M sodium cacodylate, 0.1 M sodium acetate, and 3% 1.6 hexadimethonium. Selenomethionated Nic96ΔN was crystallized at concentrations of 6–12 mg/ml using the first native condition.

For X-ray diffraction experiments, all crystals were frozen in mother liquor containing 15%–20% glycerol after stepwise equilibration. All X-ray data were collected at beamline PX-II of the Swiss Light Source (SLS, Villigen, CH) using a MAR Research CCD detector at wavelengths of 0.9792 Å (native dataset) and 0.9794 Å (SeMet SAD dataset). All data were processed using XDS (Kabsch, 1993) and DENZO (Otwinowski and Minor, 1997). Based on space group, unit cell parameters, and self-rotation function, the crystals were predicted to contain two molecules per asymmetric unit corresponding to a Matthews coefficient of 3.9 and a solvent content of 67.9%, respectively.

Initial phases were obtained from a SAD SeMet dataset with 22 of 24 SeMet sites (two monomers/A) identified by Solve (Terwilliger and Berendzen, 1999). Subsequent improvement of these phases was achieved using Arp/Warp (Morris et al., 2003). The resulting refined model was used for phasing of the native dataset at 2.6 Å resolution. To minimize Rfree, simulated annealing was performed using CNS (Brunger et al., 1998) as well as restrained and TLS refinement in REFMAC (Murshudov et al., 1997; Winn et al., 2001; Painter and Merritt, 2006). Both monomers of the asymmetric unit were built with Coot (Emsley and Cowtan, 2004) separately without NCS restraints due to differences mainly in the loop regions (overall rmsd, 1.5 Å). Ramachandran statistics of the refined native dataset are as follows: 74.8% (core region), 16.4% (additional allowed), 4.9% (generously allowed), and 3.8% (disallowed regions).

Due to the better resolution and no major structural differences between the final native and SeMet models, all structure analysis was performed with the 2.6 Å native dataset. For data and model statistics, see Table 1.

Binding of GLFG Repeats to Nic96ΔN

HISα-TEV-Nup57-GLFG (1–233), HISα-TEV-Nup49-GLFG (1–246), HISα-TEV-Nsp1-1FXFG (1–601), and HISα-TEV-Nup145-GLFG (1–219) were purified under denaturing conditions, reconstituted on Ni-NTA beads (Qiagen), and eluted with 150 mM imidazole, 150 mM NaCl, 50 mM K(OAc), 2 mM Mg(OAc)2, and 0.1% NP-40 (pH 7.5). Immobilized GST-Nic96ΔN was incubated with an excess of GLFG repeats in the presence of an E. coli lysate (~1 mg repeats were mixed with ~4 mg lysate) for 1 hr at 16 °C, and after several washing steps the bound fractions were eluted with SDS sample buffer. Binding of Nic96ΔN to GST-GLFGhap1100 (1–640), GST-FOXhap1100 (274–564), and GST was performed as recently described (Strässer et al., 2000).

Synthetic peptides (Biosyntan GmbH, Berlin, Germany) were derived from Nup57 containing either one GLFG motif (Nup57-1, -2, -3) or two GLFG motifs (Nup57-4) and an N-terminal 6× His tag. Ten microliters of peptide-coated and
washed beads (150 mM NaCl and 20 mM Tris [pH 7.5]) were incubated with 100 μg of purified Nic96ΔN and purified recombinant Ran as negative controls, respectively. After incubation at 4°C for several hours, the beads were washed several times in 1 ml steps (150 mM NaCl, 20 mM Tris [pH 7.5], and 30 mM imidazole) to elute proteins that are unspecifically bound to the Ni-NTA beads, which were controlled by a setup using empty Ni-NTA beads. The bound fractions as well as the peptides were eluted by SDS sample buffer and analyzed by SDS-PAGE, Coomassie staining, or silver staining in the case of the lower molecular weight area of the gel (GLFG peptides), respectively. Due to the small size of 26–33 amino acids, the peptides were only weakly stained.

**Supplemental Data**

Supplemental Data include five figures, one table, Supplemental Experimental Procedures, and Supplemental References and can be found with this article online at [http://www.molecule.org/cgi/content/full/29/1/48/DC1/](http://www.molecule.org/cgi/content/full/29/1/48/DC1/).

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Accession Numbers
The atomic coordinates of the Nic96 structure have been deposited with the Protein Data Bank (PDB) under the accession code 2RFO.