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In Vitro Reconstitution of a Heterotrimeric Nucleoporin Complex Consisting of Recombinant Nsp1p, Nup49p, and Nup57p

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The yeast nucleoporins Nsp1p, Nup49p, and Nup57p form a complex at the nuclear pores which is involved in nucleocytoplasmic transport. To investigate the molecular basis underlying complex formation, recombinant full-length Nup49p and Nup57p and the carboxyl-terminal domain of Nsp1p, which lacks the FXFG repeat domain, were expressed in *Escherichia coli*. When the three purified proteins were mixed together, they spontaneously associated to form a 150-kDa complex of 1:1:1 stoichiometry. In this trimeric complex, Nup57p fulfills the role of an organizing center, to which Nup49p and Nsp1p individually bind. For this interaction to occur, only two heptad repeat regions of the Nsp1p carboxyl-terminal domain are required, each region being about 50 amino acids in length. Finally, the reconstituted complex has the capability to bind to full-length Nic96p but not to mutant forms which also do not interact in vivo. When added to permeabilized yeast cells, the complex associates with the nuclear envelope and the nuclear pores. We conclude that Nsp1p, Nup49p, and Nup57p can reconstitute a complex in vitro which is competent for further assembly with other components of nuclear pores.

INTRODUCTION

The exchange of macromolecules between the cytoplasm and the nucleus is controlled by nuclear pore complexes (NPCs), supramolecular protein assemblies embedded in the double membrane surrounding the nucleus. This 125 MDa complex (Reichelt *et al.*, 1990) has been studied extensively with different electron microscopic techniques in a variety of organisms and a consensus model has been proposed (reviewed in Pante and Aebi, 1994; Akey, 1995; Davis, 1995; Goldberg and Allen, 1995). In this model, the nuclear pore consists of a spoke-ring assembly in which eight radial symmetrically arranged spokes are sandwiched inbetween a cytoplasmic and a nucleoplasmic ring. The spokes span the double membrane of the nuclear envelope, thus forming a gated channel with a central plug (or transporter) in its center. Filaments are attached from the rings that reach either into the cytoplasm or the nucleoplasm. The nucleoplasmic filaments are connected by a terminal ring, thereby yielding a basket-like arrangement. The overall structure of the NPCs appears to be conserved during evolution from yeast to higher eukaryotes.

Karyophilic proteins synthesized in the cytoplasm are actively imported into the nucleus through the NPCs (reviewed by Melchior and Gerace, 1995; Simos and Hurt, 1995). They therefore contain nuclear localization sequences (NLSs), which are often characterized by short stretches of basic residues (reviewed in Dingwall and Laskey, 1991). These NLSs are recognized by a heterodimeric NLS receptor which mediates binding to the NPCs (Görlich *et al.*, 1994, 1995; Imamoto *et al.*, 1995; Moroianu *et al.*, 1995; Radu *et al.*,

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1995b; Weis *et al.*, 1995). Other proteins including the small GTPase Ran, Ran-binding proteins that regulate the GTPase cycle of Ran, and a phosphoprotein of 15 kDa (named p10 or NTF2) are then involved in the translocation of the karyophile into the nucleoplasm, where it is released from the NLS receptor (reviewed in Goldfarb, 1994; Moore and Blobel, 1994; Tartakoff and Schneiter, 1995).

RNA export from the nucleus into the cytoplasm is also a signal-mediated process, but different RNA species seem to have different export signals. These signals are on the proteins bound to the RNA and on the RNA itself (reviewed in Izauralde and Mattaj, 1995; Gerace, 1995; Zapp, 1995). So far, export receptors which mediate binding of RNPs to the NPCs are not known. However, Ran and its regulatory proteins are implicated in RNA export, because snRNA and mRNA accumulate inside the nucleus if the Ran cycle is impaired (Kadowaki *et al.*, 1993; Bischoff *et al.*, 1995; Schlenstedt *et al.*, 1995).

Recently, nuclear export signals (NES) have been identified in several proteins nuclear (reviewed in Gerace, 1995). In the inhibitory protein of protein kinase C (PKI), a 10-amino acid stretch rich in hydrophobic residues was identified that mediates rapid export of proteins from the nucleus (Wen *et al.*, 1995). A similar NES in human immunodeficiency virus-1 Rev protein was identified by Fischer *et al.* (1995). In addition, it was found that the pathway for Rev export is also used by several RNPs. This export could be attributed to NES-containing proteins bound to these RNAs (Fischer *et al.*, 1995). Interestingly, the human Rab and the yeast Rip1p were found to interact with the NESs in a two-hybrid screen. Both proteins contained FG repeats, which are also found in several nuclear pore proteins. The human Rab protein, however, localized to the nucleoplasm (Bogerd *et al.*, 1995), whereas the yeast Rip1p was found at the nuclear envelope, possibly at the NPCs (Stutz *et al.*, 1995).

Understanding the mechanisms governing nucleocytoplasmic transport through the NPCs requires knowledge not only of the soluble factors, but also of the NPC proteins involved. In recent years, due to biochemical and genetic approaches, a large number of NPC proteins has been identified (reviewed by Rout and Wente, 1994; Davis, 1995; Doye and Hurt, 1995), and this number is growing rapidly. Among these is the yeast NPC protein Nsp1p (Nehrbass *et al.*, 1990), which is structurally related to the higher eukaryotic nucleoporin p62 (Starr *et al.*, 1990; Carmo-Fonseca *et al.*, 1991; Cordes *et al.*, 1991). Purification of p62 and Nsp1p revealed that both proteins form complexes with other nucleoporins. Whereas p62 copurifies with p58, p54, and p45 (Finlay *et al.*, 1991; Kita *et al.*, 1993, Buss and Stewart, 1995, Guan *et al.*, 1995; Hu *et al.*, 1996), Nsp1p is organized in a stable complex consisting of nucleoporins Nup49p and Nup57p (Grandi *et al.*, 1993). Another yeast nucleoporin, Nic96p, is more loosely associated to this heterotrimeric core complex (Grandi *et al.*, 1995b). The p62 complex and the yeast Nsp1p complex are involved in nucleocytoplasmic transport, because antibody depletion of p62 (Dabauvalle *et al.*, 1988; Finlay *et al.*, 1991) and mutants in the yeast proteins of the Nsp1p complex affect protein import into the nucleus (Nehrbass *et al.*, 1993; Wimmer *et al.*, 1992; Grandi *et al.*, 1993; Grandi *et al.*, 1995b).

To gain further insight into the biogenesis and structural organization of the yeast Nsp1p complex, we expressed Nsp1p, Nup57p, and Nup49p in bacteria and examined reconstitution of these recombinant nucleoporins into distinct supramolecular complexes in vitro.

MATERIALS AND METHODS

Expression and Purification of Recombinant Nucleoporins

The DNA encoding full-length Nup49p and Nup57p without the start methionine and the carboxyl-terminal domain of Nsp1p (corresponding to amino acids of 2–11 plus an additional alanine followed by residues 606–823) were cloned as *Xho*I/*Mlu*I restriction fragments (the restriction sites were generated using polymerase chain reaction (PCR) methodology) into the expression vector pET8c, previously cut with *Xho*I/*Mlu*I, which allowed in-frame fusion with the amino-terminally located six histidines (as described in Studier *et al.*, 1990; Grandi *et al.* 1995b).

In the case of the two Nsp1p carboxyl-terminal domains (used for the experiments reported in Figure 6A) which consisted of heptad repeat regions 1 and 2 (hep1+2) and heptad repeat regions 2 and 3 (hep2+3), DNA corresponding to amino acid residues 665–784 and residues 732–823, respectively, was amplified by PCR as described above and inserted into pET8c.

All constructs were efficiently expressed in *Escherchia coli* BL21 (2–10 mg protein/l culture) and mostly found in inclusion bodies. The inclusion bodies were dissolved by homogenization in protein buffer (PB; 150 mM NaCl, 50 mM KP_i, pH 8, 1 mM $MgCl_2$) containing 8 M urea, and after centrifugation the supernatant was brought to 4 M urea by mixing with an equal volume of urea-free PB.

This supernatant was applied to a Ni^{2+}-agarose column (QIAGEN, Hilden, Germany). The column was washed with PB containing 25 mM imidazole, and the bound proteins were eluted stepwise with PB containing 4 M urea plus 50 or 100 mM imidazole.

Reconstitution and Analysis of the Nucleoporin Complex

The purified nucleoporins, which were eluted from the Ni^{2+}-agarose column at a concentration between 5 mg/ml and 0.2 mg/ml, were mixed in approximately equimolar amounts (as judged from the intensity of Coomassie blue-stained bands separated by SDS-PAGE), and the urea was slowly removed by two consecutive dialysis steps, first against PB containing 1.5 M urea followed by PB free of urea. The dialyzed solution, which had a protein concentration of about 0.5 mg/ml, was centrifuged for 10 min in an Eppendorf centrifuge and the supernatant applied onto a Superdex G200 FPLC column (Pharmacia, Heidelberg, Germany). Forty fractions, each 0.4 ml, were collected with a flow rate of 0.4 ml/min and analyzed by SDS-PAGE followed by Coomassie blue or silver staining. The protein concentration in the peak fractions containing the reconstituted complex was typically 0.1 mg/ml.

Glutaraldehyde Cross-linking

One microgram of reconstituted complex or the single proteins was cross-linked by the addition of glutaraldehyde in final concentrations of 0.01%, 0.05%, and 0.1%. Cross-linking was carried out at room temperature for 30 s. The reaction was stopped by the addition of SDS-PAGE loading buffer and boiling at 95°C for 3 min.

Biotinylation of Proteins

The dialyzed recombinant proteins were reacted twice for 15 min at room temperature with a 1:10 volume of a 1 mg/ml biotin NHS (Sigma, Deisenhofen, Germany) in DMSO. The unreacted biotin-NHS was quenched by the addition of Tris-HCl (pH 9.4) to a final concentration of 100 mM and incubation for another 15 min.

Blot Overlay

One milligram of purified Nsp1p, Nup49p, and Nup57p was mixed with 1 mg of bovine serum albumin (BSA), and the proteins were separated by SDS-PAGE (10%) before being transferred onto nitrocellulose. The nitrocellulose membrane was blocked with 5% low-fat milk in phosphate-buffered saline and incubated with either 20 mg of purified and biotinylated Nsp1p and Nup49p or 20 mg of purified and unlabeled Nup57p for 2 h at room temperature. Control strips were incubated for the same time period without recombinant proteins. After incubation of the individual blots with either a rabbit polyclonal serum directed against the Nup57p followed by a goat anti-rabbit horseradish peroxidase (HRP) conjugate (Bio-Rad, Munich, Germany) or avidin-HRP (Sigma) in the case of biotinylated Nsp1p and Nup49p, blots were developed to yield a color reaction or by enhanced chemiluminescence, respectively.

In Vitro Transcription/Translation of Nic96p

The genes encoding wild-type Nic96p, Nic96pΔ28-63, and Nic96pΔ28-147 (Grandi *et al.*, 1995b) were amplified by PCR. The 5′ and 3′ primers contained *Xho*I and *Not*I restriction sites, respectively, which allowed insertion of the PCR products into the pGEM 11 vector (Promega, Heidelberg, Germany), previously cut with *Xho*I/*Not*I. This cloning of *NIC96* genes allowed in vitro transcription from the T7 RNA polymerase promoter. In vitro translation of *NIC96* transcripts in the presence of [^{35}S]methionine was carried out as described in the manual supplied by Promega. [^{35}S]methionine was purchased from Amersham-Buchler (Braunschweig, Germany).

Association of Nic96p Proteins with Recombinant Nsp1p, Nup49p, Nup57p or Reconstituted Complex

Five milliliters ml of reassociated complex or individual nucleoporins were incubated for 30 min at 30°C with 15 ml of in vitro transcribed and translated (Promega) Nic96p constructs. As a negative control, only buffer instead of bacterial expressed proteins was added. After dilution with 200 ml of immunoprecipitation buffer (IP; 2% Triton X-100, 40 mM NaCl, 5 mM $MgCl_2$, 20 mM Tris-HCl pH 8), 7 ml of Ni^{2+} agarose (QIAGEN) equilibrated in IP was added. After a 30-min incubation at room temperature and after five washes with IP, the pellets were resuspended in Laemmli's sample buffer. Proteins were separated by SDS-PAGE, gels were treated with intensifier (Amersham-Buchler), dried, and fluorography was performed overnight with Kodak X-OMAT film.

Electron Microscopy

For electron microscopy, 20-μl samples in 200 mM ammonium acetate containing 30% glycerol were sprayed onto freshly cleaved mica. After transfer into a BA 5 HM freeze-etch apparatus (Balzers AG, FL-9496 Balzers), samples were dried, rotary shadowed with platinum at a nominal angle of 4 degrees, rotating the sample at approximately 100 rpm, and backed with a thin carbon film at a 90-degree angle. The replicas were floated onto water, transferred to a copper grid, and examined at 100 kV in a Hitachi 7000 transmission electron microscope at 50,000× nominal magnification. The effective magnification was calibrated using negatively stained catalase crystals. Micrographs were recorded on Kodak SO-163 film.

Analytical Ultracentrifugation

Sedimentation velocity (SV) and sedimentation equilibrium (SE) centrifugation runs were performed in a Beckman XLA analytical ultracentrifuge equipped with an absorption optics system (Beckman Instruments, Palo Alto, CA). SV runs were carried out at 56,000 rpm and 20°C using a 12-mm double-sector (DS) Epon cell. Sedimenting material was assayed by its absorbance A at 230 nm and at 277 nm. Sedimentation coefficients were corrected to H_2O by a standard procedure. SE runs were carried out in a 12-mm DS charcoal-filled Epon cell. The cells were filled only up to 3 mm or less filling height at speeds adapted to the molecular weights measured.

Average molecular masses were calculated using a floating baseline computer program that adjusts the baseline absorbance such as to obtain the best linear fit of lnA versus r^2 (A, absorbance; r, radial distance in the cell; Van Holde, 1971). A partial specific volume V of 0.73 cm^2/g was used for all of the centrifugation runs.

Labeling of Reconstituted Nucleoporin Complex with Fluorescein Isothiocyanate

Two microliters of a 6 mg/ml FLUOS (Boehringer Mannheim, Mannheim, Germany) in DMSO solution were added to 100 μl of freshly reassembled nucleoporin complex and incubated at 30°C for 1 h. Unreacted FLUOS was quenched by the addition of 2 ml of 2 M Tris-HCl (pH 9.4) and further incubated at 30°C for 15 min. This mixture was kept on ice until it was used for incubation with semiopen yeast cells.

Fluorescence Microscopy with Semiopen Yeast Cells

Semiopen yeast cells were prepared according to Schlenstedt *et al.* (1993). Briefly, cells from a wild-type yeast strain were grown to 0.5 OD_{600}. After incubation with 50 mM PIPES (pH 9.4)/1 mM DTT, cells were spheroplasted with oxalyticase (Enzogenetics in YP (yeast extract, bactopeptone), 50 mM KP_i (pH 7.5), and 0.6 M sorbitol. After spheroplasting, cells were allowed to recover for 30 min in YP/0.7 M sorbitol buffer before incubation with permeabilization buffer (20 mM PIPES, pH 6.8, 150 mM potassium acetate, 2 mM magnesium acetate, 0.4 M sorbitol). Finally, cells were resuspended in permeabilization buffer containing 0.5 mM EGTA and frozen over liquid nitrogen in 100-μl aliquots. Cells were stored at −80°C.

To test for nuclear envelope targeting of the reconstituted nucleoporin complex, cells were thawed on ice and diluted with an equal volume of permeabilization buffer. Two microliters of mAb414 (Babco, Berkeley, CA) was added and cells were rotated for 40 min at room temperature. Cells were washed with permeabilization buffer and then 2 microliters of goat anti-mouse Texas Red conjugate and 2 microliters of fluorescein isothiocyanate-labeled reconstituted nucleoporin complex were added and rotated for another 40 min at room temperature. Cells were washed five times with permeabilization buffer before being viewed as wet mounts with a Leica confocal laser microscope.

RESULTS

Expression and Purification of Recombinant Nsp1p, Nup49p, and Nup57p

In yeast, Nsp1p physically associates with several other nuclear pore proteins including Nup49p,

Nup57p, Nic96p, and Nup82p (Grandi *et al.*, 1993, 1995a). Among these multiple interactions, a stable heterotrimeric core complex is formed among Nsp1p, Nup57p, and Nup49p (Grandi *et al.*, 1995b). To this core complex Nic96p is also bound, but this interaction is weaker and requires the structural integrity of the Nsp1p/Nup49p/Nup57p core complex (Grandi *et al.*, 1995b). To examine the requirements for the assembly of Nsp1p, Nup49p, and Nup57p into a core complex, we expressed the three nucleoporins in *E. coli* BL21 cells (Studier *et al.*, 1990). DNA encoding full-length *NUP57* and *NUP49* was cloned into pET8c, allowing bacterial expression of the proteins with six amino-terminal histidines. For *NSP1*, only the carboxyl-terminal domain was expressed this way (referred to here as Nsp1p), which in vivo carries all of the information for the interaction with Nup49p and Nup57p (Grandi *et al.*, 1993). 0.2–5 mg of the recombinant nucleoporins were affinity purified from inclusion bodies in the presence of 4 M urea on Ni^{2+}-NTA agarose. The identity and purity of recombinant Nsp1p, Nup49p, and Nup57p were verified by SDS-PAGE and Coomassie blue staining (Figure 1) or Western blotting using specific antibodies directed against Nsp1p and Nup57p. Since no specific antibodies for Nup49p are available, anti-GLFG antibodies were used for the detection of Nup49p (our unpublished observations).

In Vitro Assembly of Complexes from Recombinant Nsp1p, Nup49p, and Nup57p

To analyze whether purified recombinant Nsp1p, Nup49p, and Nup57p can form a nucleoporin complex in vitro, the purified proteins were mixed together in approximately stoichiometric amounts in the presence of 4 M urea. Assembly was then initiated by dialysis against a reconstitution buffer, thereby slowly removing the urea from the nucleoporin mixture (see MATERIALS AND METHODS). During dialysis, a portion of Nup49p precipitated, whereas Nsp1p and Nup57p remained mostly soluble. The reconstituted complex was then analyzed by FPLC on a Superdex G 200 gel filtration column and compared with the individual recombinant nucleoporins that had been treated in the same way. The various column fractions were analyzed by SDS-PAGE and Coomassie blue or silver staining.

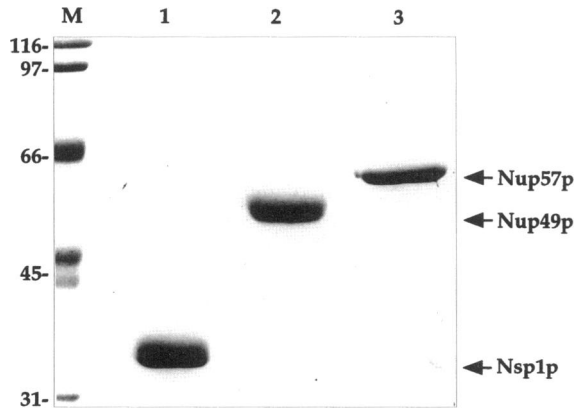

Figure 1. Expression of recombinant nucleoporins in *E. coli*. Full-length Nup57p and Nup49p and the Nsp1p carboxyl-terminal domain were overexpressed as amino-terminally tagged $(His)_6$-fusion proteins in *E. coli* and purified by affinity chromatography on Ni^{2+} NTA-agarose columns. The purified nucleoporins were analyzed by SDS-PAGE and Coomassie blue staining. Lane 1, Nsp1p (the carboxyl-terminal domain); lane 2, Nup49p; lane 3: Nup57p. Left, molecular weights of marker proteins (M) in kDa.

Figure 2 documents the elution profiles of the individual proteins and the reconstituted nucleoporin mixture from the Superdex G 200 gel filtration column: accordingly, recombinant Nsp1p peaked in fraction 38, Nup49p in fraction 36, and Nup57p in fraction 33. In contrast, the reconstituted nucleoporin mixture coeluted primarily in fractions 28 and 29 (Figure 2). A change in the elution profile is most evident for the small Nsp1p carboxyl-terminal domain, which shifts from fraction 38 where it peaks as an individual protein (Figure 2, top panel), to fraction 28 where it peaks in the reconstituted Nsp1p/Nup49p/Nup57p mixture (Figure 2, bottom panel). To show that complex assembly does not require corenaturation of the recombinant proteins in the same solution, we combined proteins that had been renatured individually. We found that a similar complex formed, as judged by the elution profile when individually dialyzed Nsp1p, Nup49p, and Nup57p were mixed for 1 h and then analyzed on the same gel filtration column (Figure 3).

Based on calibration of the Superdex G 200 column with molecular weight marker proteins, Nsp1p (predicted molecular weight 30 kDa) eluted at 47 kDa, Nup49p at approximately 67 kDa, Nup57p at 110 kDa, and the trimeric complex at approximately 230–260 kDa. These calibration data show that the individual recombinant nucleoporins Nsp1p, Nup49p, and Nup57p predominantly exist as either monomers or homodimers after dialysis and do not form higher order aggregates. However, if mixed in stoichiometric amounts the proteins assemble into a trimeric complex (see below).

To evaluate more precisely the molecular mass of the core complex, analytical ultracentrifugation of individual proteins and the reconstituted complex (i.e., pooled fractions 28–30; Figure 2, bottom panel) was performed. Unlike gel filtration, this method allows a precise determination of the molecular mass, independent of the shape of the sample. Sedimentation velocity runs were used to determine the S values, whereas the molecular masses of the individual proteins and the reconstituted complex were determined by sedimentation equilibrium runs. For Nsp1p, Nup49p, and

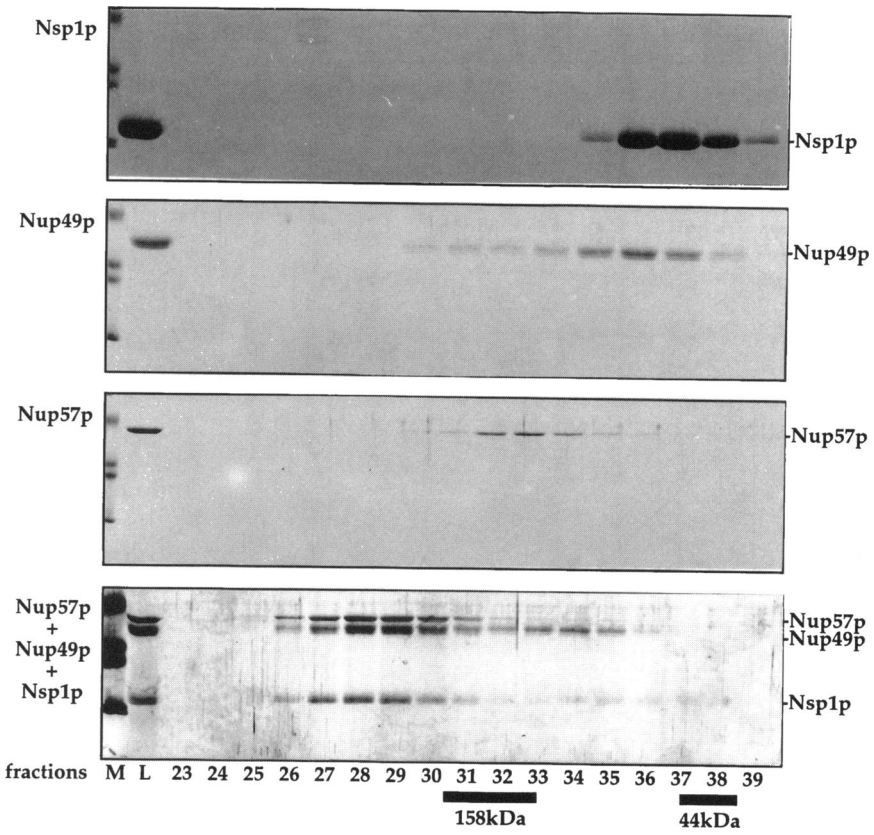

Figure 2. Recombinant Nup57p, Nup49p, and Nsp1p can assemble in vitro to form a heterotrimeric complex. The purified nucleoporins were renatured by dialysis either as single proteins or as a mixture of all three proteins. The dialyzed samples were loaded onto a Superdex G200 gel filtration column, and the collected fractions were analyzed by SDS-PAGE. The gels of single proteins were Coomassie blue stained, whereas the gel showing the reconstituted complex (Nup57p + Nup49p + Nsp1p) was silver stained. L, proteins loaded on gel filtration column; M, protein marker. The positions of the proteins are indicated on the right. Peaks of gel filtration calibration proteins are indicated as filled boxes below the fraction numbers.

Nup57p, S values of 1.3, 1.35, and 2.2 and molecular masses of 22, 36, and 55 kDa, respectively, were found (Figure 4). We found that the significantly smaller molecular mass of Nsp1p (22 kDa instead of the expected 30 kDa) was due to polyelectrolytic effects, since further sedimentation equilibrium runs with serial dilutions of Nsp1p allowed a concentration-dependent extrapolation of its molecular mass to 30 kDa (Figure 4). Such polyelectrolytic effects are a well-documented (for review, see Alexandrowicz and Daniel, 1963) phenomenon, leading to smaller molecular mass determinations by analytical ultracentrifugation for some proteins at higher concentrations.

An S value of 3.9 and molecular masses ranging between 121 and 152 kDa were obtained for the reconstituted Nsp1p/Nup49p/Nup57p complex (Table 1). These mass values are consistent with a heterotrimeric complex composed of one copy each of Nsp1p,

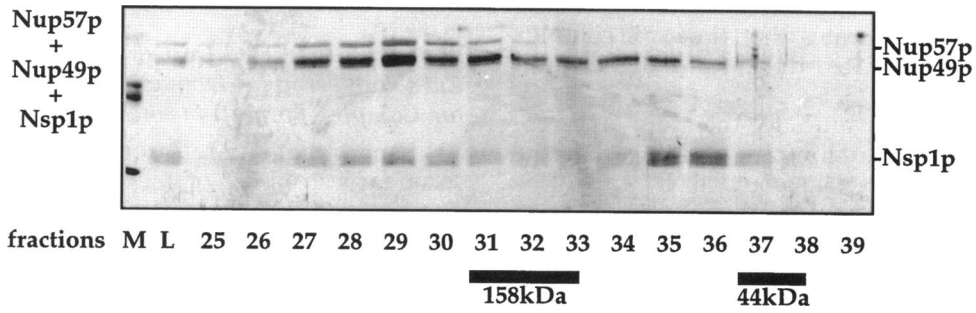

Figure 3. The heterotrimeric complex can be formed from the individually renatured Nup57p, Nup49p, and Nsp1p. Recombinant Nup57p, Nup49p, and Nsp1p were dialyzed individually, mixed for 1 h at room temperature, and applied to a Superdex 200 gel filtration column. Fractions were collected as described in the legend to Figure 2 and analyzed by SDS-PAGE and silver staining. L, proteins loaded on gel filtration column; M, protein marker;. The positions of the proteins are indicated on the right. Peaks of gel filtration calibration proteins are indicated as filled boxes below the fraction numbers.

	sedimentation velocity	sedimentation equilibrium
Nsp1p	1.3 S	22 kDa 30 kDa*
Nup49p	1.35 S	36 kDa
Nup57p	2.2 S	55 kDa
complex	3.9 S	120-152 kDa

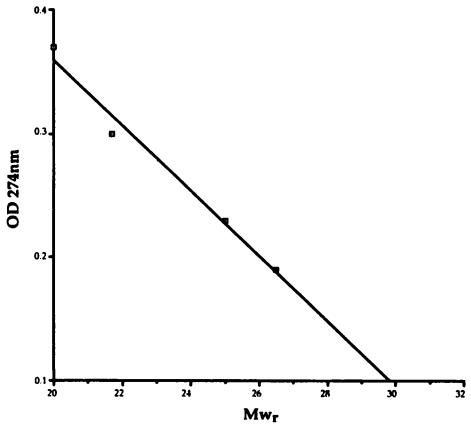

Figure 4. Biophysical properties of the recombinant proteins. Recombinant Nsp1p, Nup49p, and Nup57p as well as the reconstituted complex (pooled fractions 28–30 from Superdex G200 column) were subjected to SV and SE runs in an analytical ultracentrifuge equipped with absorption optics. This way, S values were obtained from the SV runs. The SE runs from molecular masses were determined for all samples. *, For Nsp1p, several SE runs were performed with serial dilutions of Nsp1p (as indicated by the OD values). This revealed that the molecular mass determination of Nsp1p is dependent on the protein concentration and allowed extrapolation of the molecular mass of the recombinant protein to (the expected) 30 kDa (see graph). The protein concentration was determined by measuring the OD values at 274 nm.

Nup49p, and Nup57p. However, about 50% of the signals were derived from smaller molecular weight components, which indicates that the core complex may have partly dissociated during prolonged ultracentrifugation.

The relatively small S values for the individual proteins as well as for the complex indicate that the samples are asymmetric. This could well explain the differences between the molecular masses estimated by gel filtration and the analytical ultracentrifugation, because asymmetric proteins would appear larger by gel filtration.

Another way to demonstrate complex formation was to cross-link the reconstituted Nsp1p/Nup49p/Nup57p complex by the addition of glutaraldehyde and then analyze the cross-linked products using SDS-10% polyacrylamide gels. Addition of increasing amounts of glutaraldehyde to the reconstituted complex resulted in the progressive disappearance of the bands corresponding to Nsp1p, Nup49p, and Nup57p. Concomitant a new somewhat fuzzy band appeared, migrating with an apparent molecular weight of around 150 kDa (Figure 5). This band reacted with the antibodies specific for Nsp1p and Nup57p (our unpublished observations). In contrast, glutaraldehyde-induced cross-linking of the individual proteins did not result in higher molecular weight bands. Cross-linking of Nsp1p yielded faster migrating bands consistent with the generation of intramolecular cross-links. Taken together, the ultracentrifugation and cross-linking experiments suggest that the individual nucleoporins are monomeric in reconstitution buffer, but when combined together spontaneously assemble into a heterotrimeric complex.

Structural Morphology of the Reconstituted Nucleoporin Complex Revealed by Electron Microscopy

The purified individual nucleoporins and the reconstituted complex (i.e., pooled fractions 28–30 from the gel filtration column) were analyzed by electron microscopy after glycerol spraying low-angle rotary metal shadowing (Figure 6). This method has been used to visualize protein complexes by electron microscopy including the p62 complex isolated from rat liver nuclear envelopes (Guan *et al.*, 1995). As shown in Figure 6, the individual nucleoporins appeared as very small particles with no distinct morphology, which is consistent with the observation that they are monomeric. Their small size probably made it impossible to detect the asymmetrical shape, indicated by the small S value to molecular mass ratio. In contrast, the reconstituted complex appeared as distinct particles which, although not completely uniform in shape, often appeared as elongated particles (Figure 6, gallery at 2× magnification). Since the particle size of the reconstituted complex (120–150 kDa) was rather small, further structural details could not be inferred.

First Two Predicted Coiled-Coil Regions within the Carboxyl-Terminal Domain of Nsp1p Are Sufficient for Complex Formation with Nup49p and Nup57p

As displayed in Figure 7 (top panel), prediction of the secondary structure of the Nsp1p carboxyl-terminal domain identifies three distinct regions, each approximately 50–60 amino acids in length, with a high probability to form α-helical coiled-coils. To find out whether all three heptad repeat regions within the carboxyl-terminal domain of Nsp1p are required for the association with Nup49p and Nup57p, heptad repeat regions 1 and 2 (hep1+2) and heptad repeat regions 2 and 3 (hep2+3), each about 100 amino acids in length, were histidine tagged, expressed in *E. coli*,

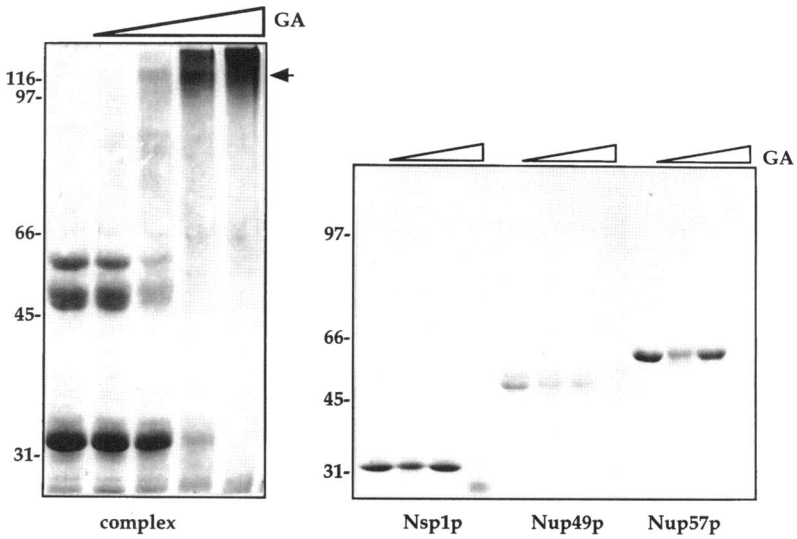

Figure 5. Glutaraldehyde-induced cross-linking of the Nsp1p/Nup49p/Nup57p complex glutaraldehyde (GA) in final concentrations of 0.01, 0.05, and 0.1% was added to the freshly assembled nucleoporin complex and to the individual renatured proteins. The cross-linking was terminated by the addition of Laemmli's sample buffer and boiling. Cross-linked products were separated by SDS-PAGE followed by Coomassie blue staining. The position of the cross-linked 150-kDa band, which is seen in the case of the complex, is indicated by an arrow. Molecular weights in kDa are indicated on the left of the gels.

and purified in the same way as Nsp1p, Nup49p, and Nup57p (see MATERIALS AND METHODS). After in vitro reconstitution and Superdex G200 gel filtration, performed as described above, hep1+2 could still form a complex along with Nup49p and Nup57p. As seen in Figure 7 (lower panels), a significant fraction of hep1+2 shifted from fractions 39–40 to fraction 30, where Nup57p and Nup49p are also found. In contrast, hep2+3 did not show this shift. This demonstrates that the first half of the Nsp1p carboxyl-termi-

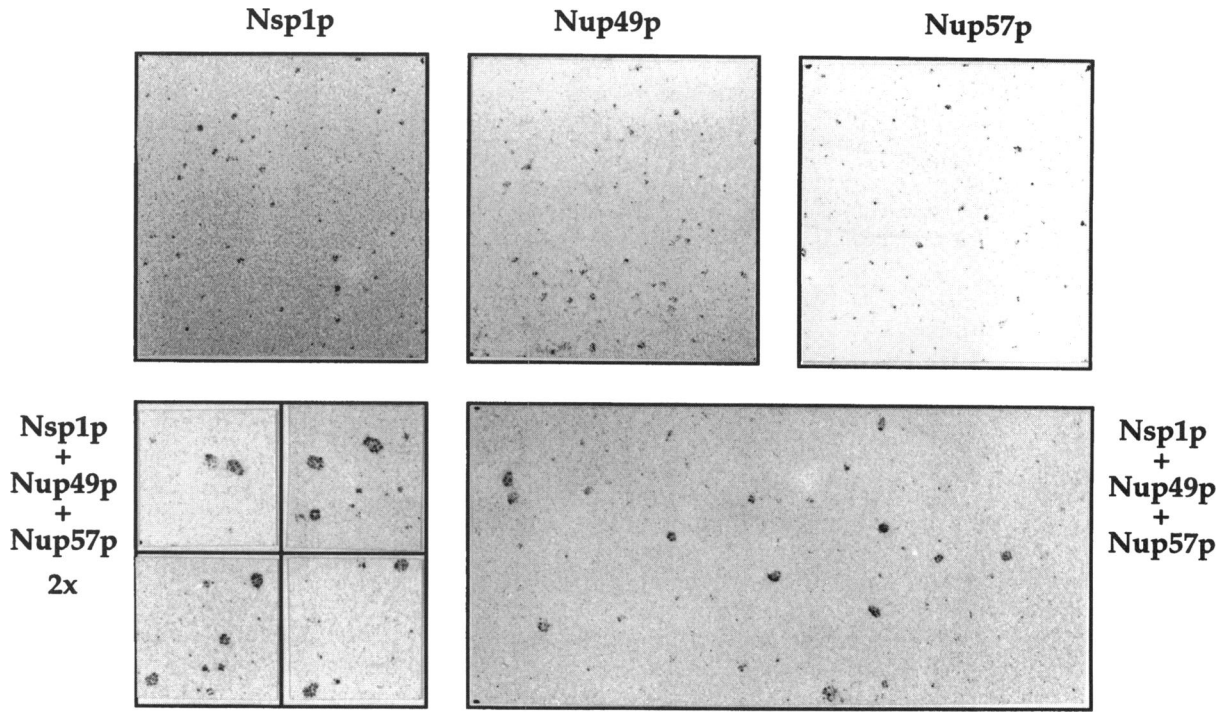

Figure 6. The reconstituted Nup57p/Nup49p/Nsp1p complex reveals an elongated structure in the electron microscope. Glycerol in a final concentration of 30% was added to the individual nucleoporins Nup57p, Nup49p, and Nsp1p as well as to the reconstituted complex. The samples were sprayed onto mica surfaces and prepared for low-angle rotary metal shadowing electron microscopy as described in MATERIALS AND METHODS. A gallery of photographs which are typical views of the reconstituted complex in the electron microscope is shown at 2× magnification. The samples were viewed in a Hitachi 7000 transmission electron microscope. 1 cm = 23.5 nm.

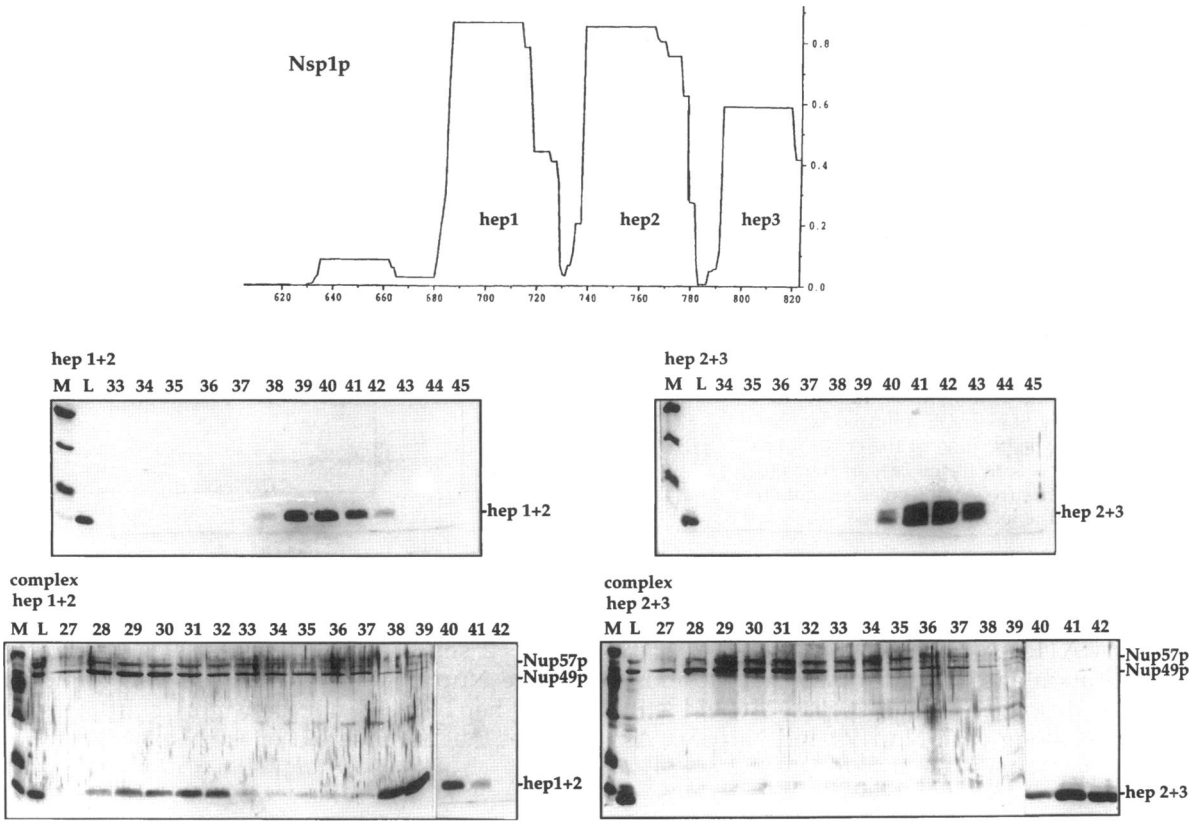

Figure 7. The first two heptad repeats within the Nsp1p carboxyl-terminal domain are sufficient for complex formation. Top panel, prediction of coiled-coil formation in the Nsp1p carboxyl-terminal domain (residues 620–823) using the pepcoil program from the GCG package (University of Wisconsin, Madison, WI). By this prediction, three regions can be identified within the Nsp1p carboxyl-terminal domain (termed hep1, hep2, and hep3) which have a high probability to form coiled-coil interactions. Bottom panels, proteins consisting of regions hep1 and hep2 (hep1+2) or hep2 and hep3 (hep2+3) from the Nsp1p carboxyl-terminal domain were bacterially expressed and purified as described for Nsp1p, Nup49p, and Nup57p (see MATERIALS AND METHODS). The proteins were renatured either as single proteins or together with Nup49p and Nup57p and analyzed by gel filtration. Collected fractions were analyzed by SDS-PAGE and silver staining. Only hep1+2 is shifted to earlier fractions when mixed with Nup49p and Nup57p, indicating complex formation. Positions of the proteins are indicated on the right. L, proteins loaded on gel filtration column; M, protein marker.

nal domain containing the heptad region 1 and 2 contains the signals necessary for complex formation with Nup49p and Nup57p.

Nup57p Binds to both Nsp1p and Nup49p, whereas Nsp1p and Nup49p Do not Directly Interact

To study the direct physical interactions among Nsp1p, Nup49p, and Nup57p, the three nucleoporins were mixed pairwise. Physical associations between renatured Nsp1p plus Nup49p, Nsp1p plus Nup57p, and Nup49p plus Nup57p were tested by gel filtration chromatography (Figure 8A). Whereas Nup57p was able to bind to either Nsp1p (top panel) or Nup49p (middle panel), Nsp1p and Nup49p did not physically associate when mixed together (bottom panel). These heterodimeric Nsp1p/Nup57p and Nup49p/Nup57p complexes were smaller in size as compared with the heterotrimeric Nsp1p/Nup49p/Nup57p complex (as concluded from the fractionation behavior on the Superdex G200 column; compare Figure 2 with Figure 8A).

A qualitatively similar result was independently obtained by blot overlays. In this experiment, Nup57p, Nup49p, Nsp1p, and BSA were separated by SDS-PAGE and then immobilized onto nitrocellulose membranes. Strips of the membrane were incubated with either purified Nup57p or purified and biotinylated Nup49p and Nsp1p. As a control, no recombinant material was added. The ligands bound to the immobilized nucleoporins were detected by either streptavidin conjugated to HRP (for biotinylated Nsp1p and Nup49p) or specific antibodies (for Nup57p). These experiments confirmed that Nup49p as well as Nsp1p can directly bind to Nup57p, but Nsp1p and Nup49p did not associate with each other under the conditions tested (Figure 8B).

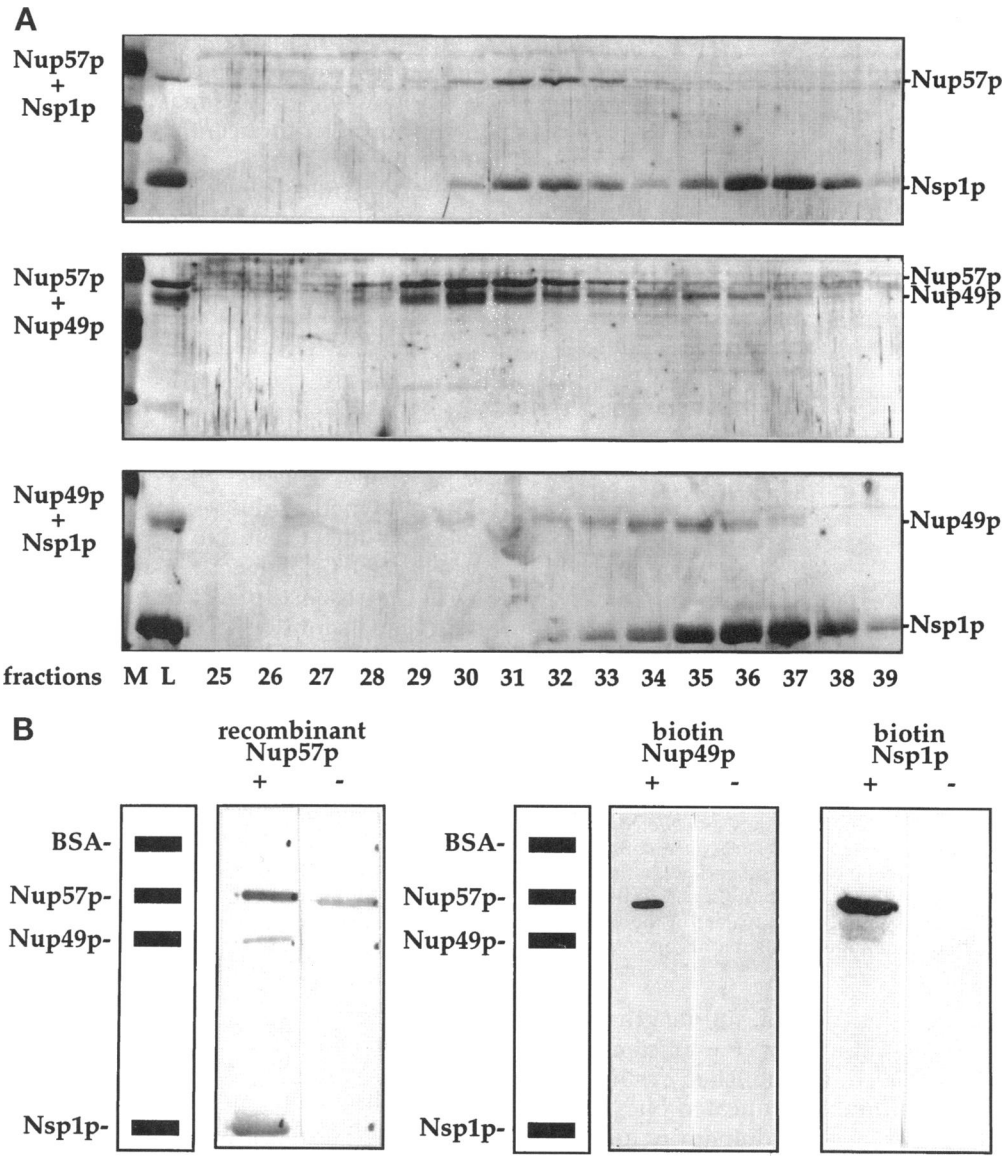

Figure 8. Nup57p can bind individually to Nup49p and Nsp1p. (A) The purified nucleoporins were mixed pairwise Nup57p + Nsp1p, Nup57p + Nup49p and Nup49p + Nsp1p before dialysis and gel filtration was performed as described earlier. Fractions from the gel filtration column were separated by SDS-PAGE and gels were silver stained. Positions of the proteins are indicated on the right. L, proteins loaded on gel filtration column; M, protein marker. (B) Purified Nup57p, Nup49p, and Nsp1p were mixed with BSA, separated by SDS-PAGE, and blotted onto nitrocellulose. Nitrocellulose strips labeled with + were incubated with purified nonlabeled Nup57p or purified and biotinylated Nup49p and Nsp1p; no ligand protein was added to nitrocellulose strips labeled with −. To detect binding of the ligand proteins to the immobilized bands, for Nup57p, a specific antiserum was used followed by a secondary antibody-HRP conjugate. For the detection of the biotinylated ligands Nup49p and Nsp1p, streptavidin-HRP was used. The immobilized proteins were visualized after the transfer to the nitrocellulose with Ponceau S staining, and their positions were marked as indicated by the bars in the scheme.

Reconstituted Nsp1p/Nup49p/Nup57p Complex, but not the Individual Nucleoporins Can Associate with Full-Length Nic96p

To test whether the reconstituted core complex consisting of Nsp1p, Nup49p, and Nup57p exhibits functional properties similar to its native in vivo counterpart, we tested its reassociation with Nic96p. It was previously shown that the association of this core complex with Nic96p in living cells requires the amino-terminal coiled-coil domain of Nic96p and structural integrity of the core complex (Grandi *et al.*, 1995b). To test for such a physical interaction in vitro, freshly assembled core complex and the individual nucleoporins were added to in vitro transcribed and

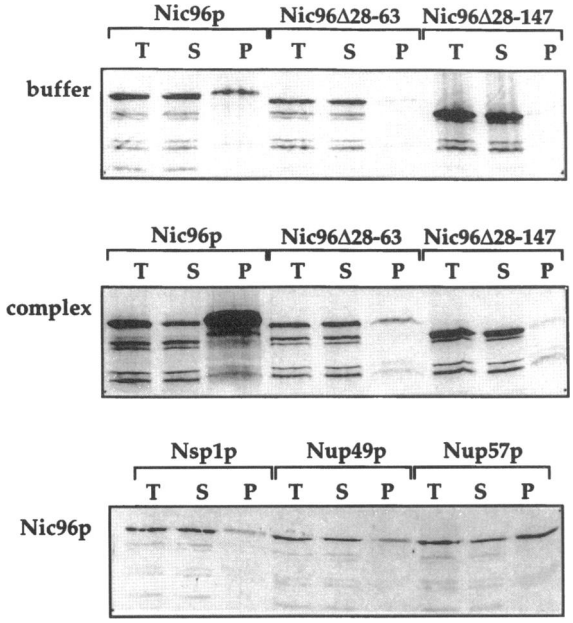

Figure 9. Full-length Nic96p, but not an amino-terminally truncated Nic96p, can associate with the Nsp1p/Nup49p/Nup57p complex. Full-length Nic96p or two amino-terminally truncated mutants of Nic96p 1(Nic96Δ28-63 and Nic96Δ28-147) were in vitro transcribed and translated and [^{35}S]methionine labeled. Dialysis buffer or the indicated renatured recombinant protein samples were added to the translation reaction and after a 30-min incubation Ni^{2+}-agarose was added. The Ni^{2+}-agarose was pelleted, washed, and resuspended in sample buffer. On SDS gels the amount of total input (T) of ^{35}S-labeled protein was compared with that of the labeled protein remaining in the supernatant (S) and to the labeled protein in the pellet (P). An eightfold equivalent of the pellet was loaded as compared with total input and supernatant.

translated [^{35}S]methionine-labeled, full-length Nic96p (recombinant Nic96p could not be efficiently expressed in *E. coli*; Schlaich, unpublished results). For controls, two amino-terminally truncated Nic96p constructs were tested that lack the domain required for interaction with the nucleoporin complex. After incubation with radiolabeled Nic96p proteins, the core complex was pelleted by the addition of Ni^{2+} agarose. As a measure of nonspecific binding of Nic96p to the Ni^{2+} agarose, incubations were also performed in the absence of recombinant proteins. The pellets containing Nic96p coprecipitated with the Ni^{2+} agarose and the supernatants (unbound Nic96p) were compared with total input of Nic96p and assayed by SDS-PAGE and fluorography of the dried gels. Whereas maximally 5% of Nic96p, full-length or mutant forms, bound directly to the Ni^{2+} agarose (Figure 9, buffer), the addition of the reconstituted core complex into the binding assay increased the recovery of wild-type Nic96p in the Ni^{2+} agarose pellet to 20%. In parallel, Nic96p was depleted from the supernatant in comparable amounts (Figure 9, complex). In contrast, only background binding to the core complex was observed for the truncated Nic96p proteins, which lack the amino-terminal coiled-coil domain. If the individual nucleoporins were used in the binding assay, the binding of Nic96p was maximally increased to 9% and therefore not significantly above the 5% background (Figure 9, Nic96p).

These results show that only full-length Nic96p is competent for significant binding to the in vitro reconstituted nucleoporin core complex, whereas Nic96p does not bind to individual Nsp1p, Nup49p, and Nup57p. Thus, the in vitro reconstitution experiments nicely corroborate the in vivo data (Grandi *et al.*, 1995b).

Reconstituted Nucleoporin Complex Is Targeted to the Nuclear Envelope in Semiopen Yeast Cells

To test whether the reconstituted nucleoporin complex was able to bind to nuclear pores, permeabilized yeast cells (Schlenstedt *et al.*, 1993) were incubated with the reconstituted nucleoporin core complex. To visualize its intracellular targeting by fluorescence microscopy, the complex was fluorescently labeled. To stain for nuclear pores, cells were also incubated with the monoclonal antibody mAb414 which recognizes FXFG-containing nucleoporins (Davis and Blobel, 1986) followed by a secondary anti-mouse antibody labeled with Texas Red. Stained cells were viewed using epifluorescence or confocal laser microscopy. This analysis revealed that the reconstituted and fluorescently labeled nucleoporin complex was targeted to the nuclear envelope in semiopen yeast cells. Since the fluorescence signal largely overlapped with the immunostaining seen with the anti-nucleoporin antibody, it appears that some of the reconstituted Nsp1p/Nup49p/Nup57p also associated with the NPCs (Figure 10). A similar staining pattern was also observed in the absence of mAb414, ruling out the possibility that the bivalent antibody, which can bind to Nup57p and Nup49p, directed the complex to the nuclear pores (our unpublished observations). When fluorescently labeled BSA was used as a control instead of the nucleoporin complex, no binding to the nuclear envelope was detected (our unpublished observations).

DISCUSSION

In this work, we have shown for the first time that three bacterially expressed and purified nucleoporins Nsp1p, Nup49p, and Nup57p, which in vivo form an essential complex at the nuclear pores involved in nucleocytoplasmic transport, can self-assemble in vitro. This complex formation occurred in the absence of specific assembly factors. Within this heterotrimeric complex Nup57p accomplishes the role of an organizing center to which Nup49p and Nsp1p can individually bind. This assembly is most likely mediated by

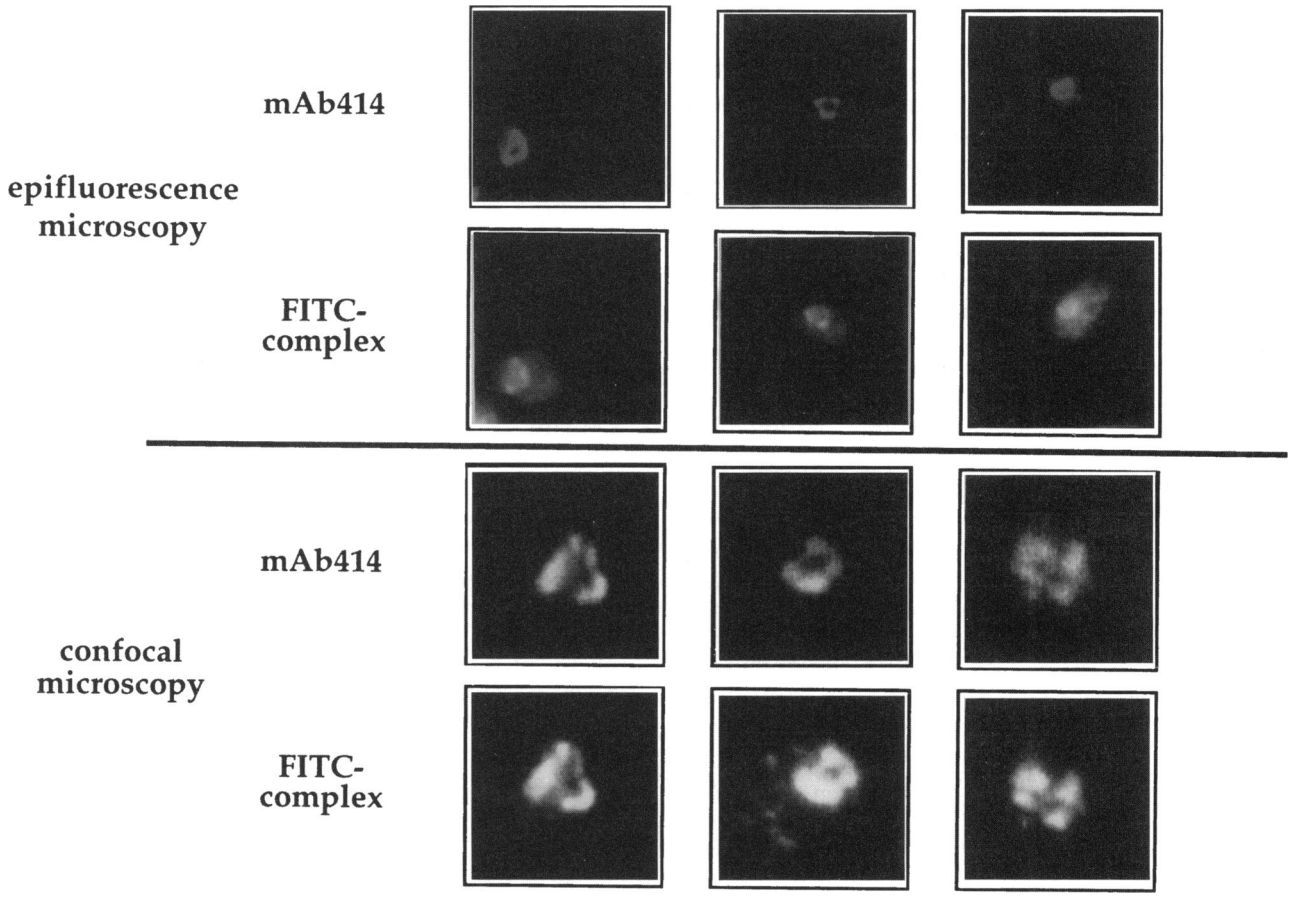

Figure 10. Targeting of the reconstituted Nsp1p/Nup49p/Nup57p complex to the nuclear envelope in permeabilized yeast cells. The reconstituted Nsp1p/Nup49p/Nup57p complex was fluorescein isothiocyanate labeled and added to semiopen yeast cells as described in MATERIALS AND METHODS. Colocalization of the immunofluorescence signal obtained with mAb414 which stains the yeast nuclear pore complex (Davis and Blobel, 1986) and the fluorescently labeled nucleoporins is observed in the epifluorescence (top panels) and confocal microscope (bottom panels).

coiled-coil interactions between the partner proteins consistent with the observation that all three proteins contain in their carboxyl-terminal domains extended sequences with heptad repeat patterns. A high specificity in the coiled-coil interactions between these proteins is indicated by our findings that 1) Nsp1p and Nup49p do not interact with each other, but individually can bind to Nup57p and 2) only heptad repeat domains hep1+2, but not hep2+3 of the Nsp1p carboxyl-terminal domain associate with Nup57p and Nup49p to form a heterotrimeric complex. This result indicates that different heptad repeat regions within a single nucleoporin may be involved in the interaction with other NPC proteins. In the case of Nsp1p, the first heptad repeats are important for binding Nup57p/Nup49p. The third heptad repeat, however, might be used to bind additional nucleoporins. Nup82p, which forms a complex with Nsp1p different from the Nup57p/Nup49p complex (Grandi *et al.*, 1995a), could be bound to Nsp1p by this domain. Taking together our in vivo and in vitro data, one could speculate that some nucleoporins, which fulfill an overlapping function, are arranged into distinct sets at the nuclear pores. These sets might self-assemble after being synthesized into specific hetero-oligomeric subcomplexes before they become incorporated into the nuclear pore complex. This latter interaction may require the presence of other NPC proteins which could serve as docking molecules (e.g., Nic96p for the Nsp1p complex). Other subcomplexes may form in a similar manner and hence nuclear pore complexes could assemble from a relatively small number of modules rather than from its approximately 50–100 different individual proteins (Rout and Blobel, 1993). Recently, another subcomplex of the NPCs, which consists of six proteins, has been isolated from yeast (Siniossoglou *et al.*, 1996). However, no information about the assembly of this Nup84p-containing complex is available so far. Strikingly, this complex contains Sec13p, a protein which is involved in vesicle budding from the endo-

plasmic reticulum and transport of these vesicles to the Golgi apparatus (reviewed in Barlowe 1995). It was speculated that a subpool of Sec13p, which is bound to this nucleoporin subcomplex, might play a role in coordinated nuclear pore and nuclear membrane biogenesis.

It is very likely that the essential Nsp1p/Nup57p/Nup49p subcomplex is structurally and functionally conserved during evolution. One possible candidate counterpart of the Nsp1p complex in the higher eukaryotic system is the p62 complex, which is involved in protein import into the nucleus (Dabauvalle *et al.*, 1988; Finlay *et al.*, 1991), but experimental proof for this is still lacking. In support of this idea, p62 from vertebrate organisms shows a distinct homology to Nsp1p because both consist of an amino-terminal FXFG repeat domain and a carboxyl-terminal coiled-coil domain (Carmo-Fonseca *et al.*, 1991). The p62 complex contains three additional proteins of 58 kDa, 54 kDa, and 45 kDa (Dabauvalle *et al.*, 1990; Finlay *et al.*, 1991, Kita *et al.*, 1993; Buss and Stewart, 1995; Guan *et al.*, 1995), of which the p45 form seems to be a splice variant of p58 (Hu *et al.*, 1996). Thus, the higher eukaryotic complex can be considered a heterotrimeric assembly consisting of p62, p58/p45, and p54 and therefore resembles the Nsp1p/Nup57p/Nup49p complex. Rat p58 and p54, whose genes have been recently cloned and sequenced, contain FG repeat sequences and unique domains with a coiled-coil prediction (Guan *et al.*, 1995; Hu *et al.*, 1996). Interestingly, rat p54 and yeast Nup57p exhibit limited but significant sequence homology (our unpublished observations). When the purified rat liver p62 complex was analyzed by low-angle rotary metal shadow electron microscopy, it also appeared heterogeneous in size and shape (Guan *et al.*, 1995). Thus, both the reconstituted Nsp1p complex and the isolated p62 complex resemble each other and do not form filamentous structures under the conditions tested. However, one cannot exclude that both complexes are part of higher order structures in vivo (e.g., track-like or filamentous arrays) which are not stable during biochemical isolation or do not form during in vitro reconstitution. In this context, it is worth mentioning that the bacterially expressed carboxyl-terminal domain of rat p62 self-assembled into filamentous structures (Buss *et al.*, 1994).

The exact location of the Nsp1p complex at the NPCs is not known. The p62 complex was localized to both sides of the nuclear pore very close to the central channel, where active translocation is believed to occur (Grote *et al.*, 1995; Guan *et al.*, 1995). It was shown that p62 directly interacts with NTF2 (Paschal and Gerace, 1995), a factor involved in nuclear protein import at a stage after docking of the karyophile to the NPCs. Therefore, p62 might be directly involved in transferring karyophile/karyopherin complexes through the NPCs. Accordingly, it was speculated that p62 binds transport substrates at the periphery of the NPCs before delivering them to the central transporter, which suggests that the p62 complex is a mobile structure (Guan *et al.*, 1995). Such a function would require multiple interactions with other NPC proteins. It is worth mentioning that the Nsp1p/Nup57p/Nup49p complex is only loosely associated with Nic96p and that this interaction is sensitive to biochemical or mutational perturbations. In future studies, we will address the question whether Nic96p or other nucleoporins can act as docking proteins at the NPCs, to which the Nsp1p complex could be reversibly bound and released (Grandi *et al.*, 1995b).

Finally, in vitro assembled nuclear pore subcomplexes, consisting of nucleoporins with repeat sequences, might be useful in the future for the subsequent, more sophisticated steps of in vitro reconstitution of the nuclear protein import machinery. Since repeat sequences of certain nucleoporins can bind to the purified NLS receptor complex in vitro, it was proposed that a reiterative cycle of binding and release of the karyophile/karyopherin complex from the immobilized repetitive domains of the nucleoporins allows translocation of substrate through the NPCs (Radu *et al.*, 1995a,b; Rexach and Blobel 1995). Attaching different sets of in vitro reconstituted NPCs subcomplexes on a solid support might create a track on which a fluorescently labeled karyophilic substrate bound to its soluble transport factors could migrate.

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