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Purification of NSP1 reveals complex formation with 'GLFG' nucleoporins and a novel nuclear pore protein NIC96

Paola Grandi, Valérie Doye and Eduard C.Hurt

EMBL, Postfach 1022.09, Meyerhofstrasse 1, D-6900 Heidelberg, Germany

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The essential C-terminal domain of NSP1 mediates assembly into the nuclear pore complex (NPC). To identify components which interact physically with this yeast nucleoporin, the tagged C-terminal domain of NSP1 (ProtA-NSP1) was isolated by affinity chromatography under non-denaturing conditions. The purified complex contains ProtA-NSP1, two previously identified 'GLFG' nucleoporins, NUP49 (NSP49) and p54 and a novel protein designated NIC96 (for Nucleoporin-Interacting Component of 96 kDa). Conversely, affinity purification of tagged NSP49 enriches for NSP1, the p54 and the NIC96 component. The *NIC96* gene was cloned; it encodes a novel 839 amino acid protein essential for cell growth. By immunofluorescence, protein A-tagged NIC96 exhibits a punctate nuclear membrane staining indicative of nuclear pore location. Therefore, affinity purification of tagged nucleoporins has allowed the definition of a subcomplex of the NPC and analysis of physical interactions between nuclear pore proteins.

Key words: affinity purification/IgG binding domain/nuclear membrane/nuclear pore complex/nucleoporin/yeast

Introduction

One of the largest supramolecular structures in eukaryotic cells are the nuclear pores. It is widely accepted that the nuclear pores, embedded in the nuclear membrane, mediate the trafficking of molecules between the nucleus and the cytoplasm (Feldherr *et al.*, 1984; Bossie and Silver, 1992; Forbes, 1992). The nuclear pore complex (NPC) has an overall diameter of 120 nm, a mass of 125 MDa and exhibits octagonal symmetry (Unwin and Milligan, 1982; Reichelt *et al.*, 1990). EM analysis of NPC structure allowed the identification of a cytoplasmic and a nuclear ring, eight spokes connecting these two rings (Unwin and Milligan, 1982; Akey, 1989; Jarnik and Aebi, 1991) and in the centre a particle referred to as the 'plug' (Unwin and Milligan, 1982) or 'transporter' (Akey, 1990). It has been proposed that this central particle might represent the site of active (ATP-dependent and signal-mediated) nuclear transport of macromolecules (proteins, snRNPs, mRNAs) (Akey, 1990) while passive diffusion could occur through peripheral channels between the spokes (Hinshaw *et al.*, 1992). More recently, a filamentous cage-like structure protruding from the nuclear ring towards the nucleoplasm and filaments attached to the cytoplasmic ring were visualized (Ris, 1989; Jarnik and Aebi, 1991; Goldberg and Allen, 1992).

To understand the structure and function of the NPC, a first step is to clone and characterize the proteins that build up this complex. Numerous efforts have been made to isolate NPCs (Franke and Scheer, 1974; Dwyer and Blobel, 1976; Krohne *et al.*, 1978; Fisher *et al.*, 1982; Gerace *et al.*, 1982; Snow *et al.*, 1987; Scheer *et al.*, 1988; Allen and Douglas, 1989). Such studies have been complicated by the fact that NPCs are tightly connected to other macromolecular structures such as the nuclear lamina (Dwyer and Blobel, 1976) and cytoskeletal elements (Carmo-Fonseca *et al.*, 1987; Allen and Douglas, 1989). Besides, certain substructures of the NPC are poorly preserved during isolation procedures (Allen and Douglas, 1989; Jarnik and Aebi, 1991) or can be lost as in the case of the 'transporter' (Jarnik and Aebi, 1991; Hinshaw *et al.*, 1992). However, a large scale purification method for yeast NPCs devoid of filamentous structures has recently been reported (Rout and Blobel, 1991).

One of the first pore proteins to be identified was gp210 (Gerace *et al.*, 1982). Gp210 is a transmembrane glycoprotein involved both in anchoring the pore to the nuclear membrane (Wozniak *et al.*, 1989; Greber *et al.*, 1990) and in nuclear transport (Greber and Gerace, 1992). Apart from gp210, all the nuclear pore proteins cloned so far belong to two families of related polypeptides termed nucleoporins and contain antigenic, tandemly repeated motifs in their primary amino acid sequences. The term 'nucleoporin' was first introduced for higher eukaryotic pore proteins carrying *N*-acetyl glucosamine sugars and being reactive with wheat germ agglutinin or certain monoclonal antibodies (Davis and Blobel, 1986, 1987; Park *et al.*, 1987; Snow *et al.*, 1987). Higher eukaryotic p62 (Starr *et al.*, 1990) and NUP153 (Sukegawa and Blobel, 1993) as well as *Saccharomyces cerevisiae* NSP1 (Nehrbass *et al.*, 1990) and NUP1 (Davis and Fink, 1990) belong to the first family of nucleoporins, characterized by multiple 'FSFG' sequences. NSP1 appears to be the homologue of mammalian p62 since the C-terminal domains of the two proteins show 50% similarity (Carmo-Fonseca *et al.*, 1991; Cordes *et al.*, 1991; Starr and Hanover, 1991). NSP1 is an essential protein (Hurt, 1988); although evolutionarily conserved, its 'FSFG' repeats are dispensable for cell growth while the C-terminal domain is both necessary and sufficient for viability (Nehrbass *et al.*, 1990). *In vitro* and *in vivo* experiments demonstrated that p62 and NSP1 are involved in nuclear transport and possibly in nuclear pore biogenesis (Finlay *et al.*, 1987; Featherstone *et al.*, 1988; Dabauvalle *et al.*, 1990; Finlay and Forbes, 1990; Finlay *et al.*, 1991; Mutvei *et al.*, 1992). Affinity purification of rat p62 has led to co-isolation of two other proteins, p58 and p54, which are organized in a stable complex and essential for nuclear transport (Finlay *et al.*, 1991). However, further biochemical analysis of this NPC subcomplex and the molecular characterization of p58 or p54 have not been reported.

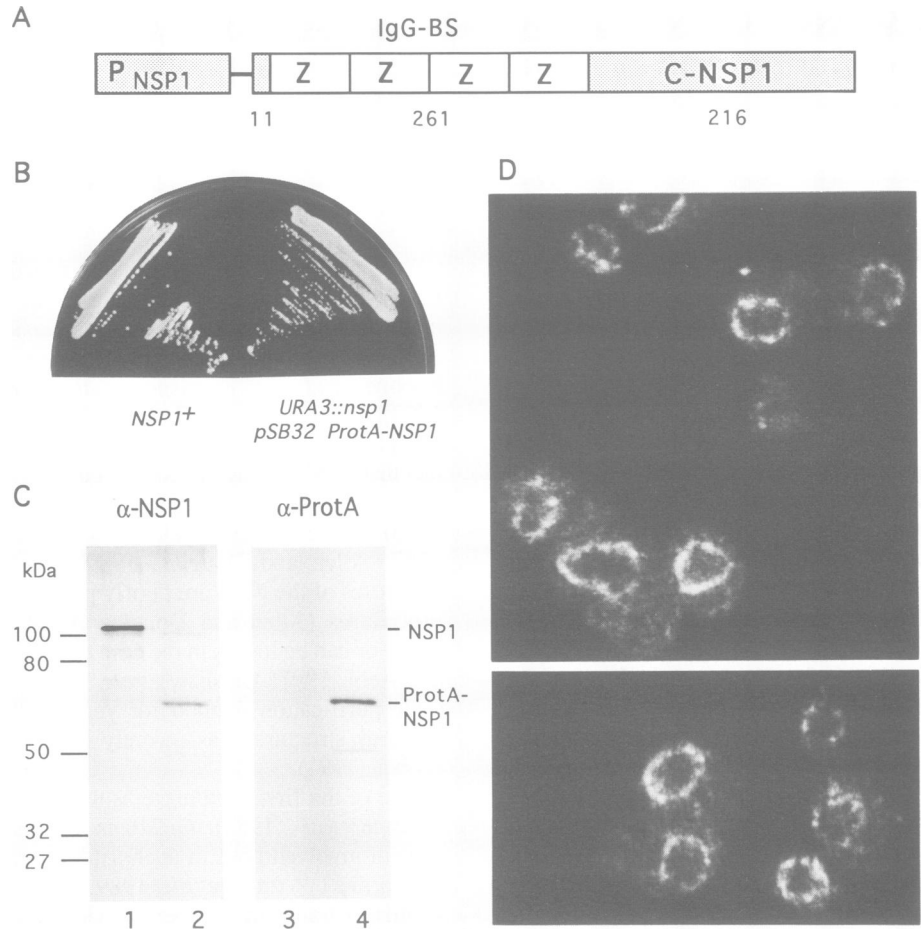

Fig. 1. Expression and location of NSP1 tagged with IgG binding domains from *S.aureus* protein A in yeast. (**A**) Schematic drawing of the fusion protein consisting of four IgG binding domains (Z or IgG-BS, IgG binding sequence) derived from *S.aureus* protein A and the essential NSP1 C-terminal domain. The number of amino acids for the individual domains is given below the graph. (**B**) Growth of yeast strains NSP1$^+$ and ProtA−NSP1 (*URA3::nsp1/pSB32-ProtA−NSP1*). Cells were incubated on YPD plate for 3 days at 30°C. (**C**) Immunoblot analysis. Equivalent amounts of whole cell lysates from yeast strains NSP1$^+$ (lanes 1 and 3) and ProtA−NSP1 (*URA3::nsp1/pSB32-ProtA−NSP1*) (lanes 2 and 4) were analysed by SDS−PAGE and immunoblotting using an IgG-HRP conjugate to detect ProtA−NSP1 (lanes 3 and 4) and anti-NSP1 antibodies (immune serum EC7-1) to detect both authentic NSP1 and ProtA−NSP1 (lanes 1 and 2). The positions of the ProtA−NSP1 fusion protein and full-length NSP1 are indicated. The weak band around 100 kDa in lane 2 corresponds to NSP2 which cross-reacts with anti-NSP1 antibodies (Nehrbass *et al.*, 1990). (**D**) Confocal immunofluorescence microscopy. The subcellular localization of ProtA−NSP1 in haploid yeast cells with disrupted *nsp1* was analysed by immunofluorescence using rabbit anti-chicken IgG as first antibody followed by goat anti-rabbit IgG coupled to Texas Red. A punctate staining of the nuclear envelope is observed typical of nuclear pore labelling. Nuclear staining using Hoechst 33258 could only be viewed in the conventional microscope.

A second family of nucleoporins was recently identified in yeast with the help of a monoclonal antibody (mAB192) (Davis and Blobel, 1986; Davis and Fink, 1990; Wente *et al.*, 1992) reactive towards the 'GLFG' repeated motif shared by the members of this family. This mAB recognizes at least five different proteins among which NUP116, NUP100 and NUP49 have already been cloned (Wente *et al.*, 1992). Interestingly, two of these nucleoporins, NUP116/NSP116 and NUP49/NSP49, were also identified in a genetic screen for synthetic lethal mutants of the nuclear pore protein NSP1, thereby suggesting a functional interaction between NSP1 and nucleoporins of the 'GLFG' family (Wimmer *et al.*, 1992).

Considering the increasing number of cloned nuclear pore proteins, the analysis of their interaction within the NPC becomes crucial. The combination of biochemical and molecular genetic approaches may facilitate such a structure−function analysis. We therefore decided to affinity purify NSP1 from the yeast *S.cerevisiae* and to characterize co-purifying components. For this purpose, we tagged the essential C-terminal domain of NSP1 with IgG binding sequences from *Staphylococcus aureus* protein A. In this report, we show that several proteins co-purify with NSP1, including 'GLFG' nucleoporins and a novel pore protein, NIC96. This subcomplex could represent an essential unit of the NPC, involved in pore structure and/or function.

Results

NSP1 tagged with IgG binding epitopes assembles into the nuclear pores

In the past, sequences derived from *S.aureus* protein A which bind IgG with high affinity were successfully used as a tag to purify bacterially expressed fusion proteins by IgG-Sepharose chromatography (Moks *et al.*, 1987). Furthermore, protein A was used in yeast to purify ProtA-tagged calmodulin (Stirling *et al.*, 1992). For efficient affinity

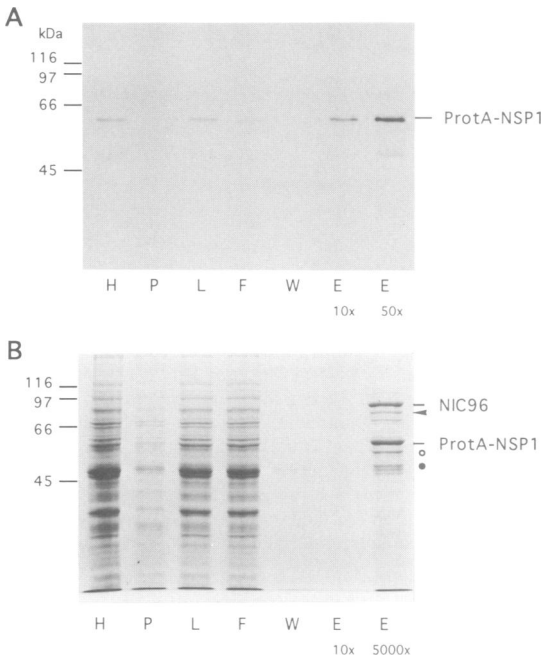

Fig. 2. One-step purification of ProtA−NSP1 by IgG-Sepharose affinity chromatography. Expression of ProtA−NSP1 in a nsp1$^-$ strain, lysis of spheroplasted cells under non-denaturing conditions in Triton X-100-containing buffer and purification of the ProtA−NSP1 fusion protein by IgG-Sepharose chromatography was done as described in Materials and methods. Aliquots of the various fractions were analysed by SDS−PAGE followed by immunoblotting using an IgG−HRP conjugate to detect ProtA−NSP1 (**A**) or by Coomassie staining (**B**). Homogenate, H (1×equivalent); insoluble pellet, P (1×); load fraction, L (1×); flow through, F (1×); wash, W (1×); eluate, E (10×, 50×, 5000×). The bands representing NIC96 and ProtA−NSP1 are indicated, as well as the position of an 80 kDa protein (arrowhead), p54 (open circle) and NUP49 (closed circle) (see also Figure 5). The position of a molecular weight protein standard is given.

purification of NSP1 and its associated components, we tagged the essential NSP1 C-terminal domain with four IgG binding sequences derived from protein A (Figure 1A). The fusion gene with the authentic *NSP1* promoter, was inserted in a single copy number ARS/CEN plasmid and transformed into a diploid yeast strain in which one gene copy of *NSP1* was disrupted. After sporulation and tetrad analysis, four viable spores could be recovered, two of them being *URA3::nsp1* and always carrying the plasmid-borne *ProtA−NSP1* gene. This demonstrates that ProtA−NSP1 is functional in yeast and can complement an otherwise non-viable nsp1$^-$ mutant. The ProtA−NSP1 complemented strain grows only slightly more slowly (t_2 of 2.5 h) as compared with the NSP1$^+$ strain (t_2 of 2 h) (Figure 1B).

To check for expression of ProtA−NSP1 in yeast, total cell lysates were analysed by SDS−PAGE and Western blotting; the blot was directly probed with IgG coupled to horseradish peroxidase which specifically binds to the protein A moiety of the fusion protein. In a control NSP1$^+$ strain, no cross-reaction with endogenous yeast proteins is seen (Figure 1C, lane 3). In the ProtA−NSP1 expressing strain, a 62 kDa band (which is the expected size for the fusion protein), plus some minor breakdown products, are visible (Figure 1C, lane 4). Authentic NSP1 is detected only in NSP1$^+$ cells, but not in the *URA3::nsp1* null mutant complemented by ProtA−NSP1 (Figure 1C, compare lanes 1 and 2), while cross-reaction with nucleoporin NSP2 [which migrates slightly faster than NSP1 (Nehrbass *et al.*, 1990)] is seen in both strains.

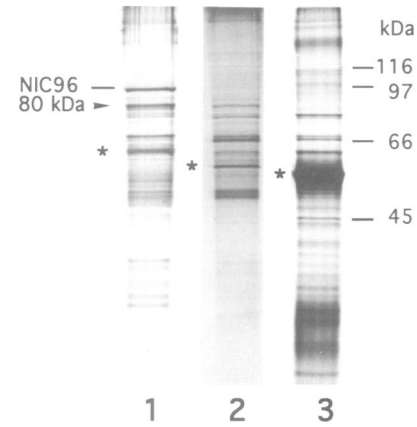

Fig. 3. NIC96 no longer binds to a mutant form of NSP1. Affinity purification of ProtA−NSP1, ProtA−ala6-nsp1 and ProtA−NOP1 fusion proteins by IgG-Sepharose chromatography was done as described in Materials and methods. Aliquots of purified ProtA−NSP1 (1), ProtA−ala6-nsp1 (2) and ProtA−NOP1 (3) were analysed by SDS−PAGE followed by silver staining. The position of NIC96, the 80 kDa protein and the three different fusion proteins carrying the protein A tag (*) are indicated. Note that ProtA−ala6-nsp1 migrates faster on the SDS−polyacrylamide gel as compared with ProtA−NSP1 which is due to the change of six negatively charged residues into neutral alanines. The doublet band which migrates at 66 kDa is particularly well stained by silver, but is a contaminant since it is seen in all three lanes.

The subcellular distribution of the ProtA−NSP1 fusion protein was determined by immunofluorescence confocal microscopy in strain ProtA−NSP1 (*URA3::nsp1/pSB32 ProtA−NSP1*) (Figure 1D). NSP1 tagged with ProtA shows a punctate staining at the nuclear periphery similar to authentic NSP1 immunolabelling and typical for a nuclear pore distribution (Nehrbass *et al.*, 1990). In contrast, dihydrofolate reductase from mouse tagged with ProtA gave cytoplasmic immunolabelling when expressed in *S.cerevisiae* (E.Fabre, unpublished results).

Affinity purification of ProtA−NSP1 by IgG-Sepharose chromatography

Since ProtA−NSP1 appeared to assemble into nuclear pores, we sought to purify the fusion protein under non-denaturing conditions by affinity chromatography and to analyse the co-purifying components. Briefly, yeast cells expressing ProtA−NSP1 were spheroplasted and lysed in buffer containing 2% Triton X-100. Under these conditions, ProtA−NSP1 was released and mostly recovered in the supernatant after high speed centrifugation of the cell lysate (Figure 2A). This supernatant was subsequently applied to an IgG-Sepharose column to which >50% of the fusion protein bound. ProtA−NSP1 was eluted from the column by lowering the pH to 3.4. A 10-fold volume equivalent of the eluate was analysed by Western blotting demonstrating enrichment of the ProtA−NSP1 band (Figure 2A).

The Coomassie stained SDS−polyacrylamide gel of the various fractions obtained during ProtA−NSP1 purification showed that the bulk of the proteins is not bound to the column and that in the eluate only a few bands are enriched (Figure 2B, E 5000×). The two most prominent bands correspond to the ProtA−NSP1 fusion protein (62 kDa) and a 90 kDa protein (termed NIC96, see below). The relevance

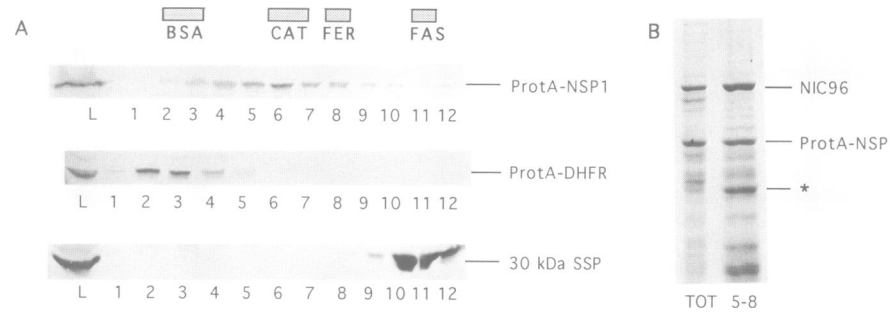

Fig. 4. Sedimentation of ProtA−NSP1 and ProtA−DHFR on sucrose gradients. **(A)** Cell lysates from strains ProtA−NSP1 and ProtA−DHFR, as well as a protein standard (bovine serum albumin: BSA, 66 kDa; catalase: CAT, 240 kDa; ferritin: FER, 450 kDa; yeast fatty acid synthetase: FAS, 2400 kDa) were loaded on a 7−40% sucrose density gradient and centrifuged for 17 h at 34 000 r.p.m. Each gradient was collected in 12 fractions and aliquots of the load (L) and fractions 1−12 were analysed by SDS−PAGE and Western blotting. Only the relevant area of the immunoblot containing the different fusion proteins is shown. The sedimentation of the protein standards on the gradient is indicated above the figure. In addition, the immunoblot was redecorated with an antibody recognizing a 30 kDa ribosomal protein of the small subunit (30 kDa SSP) to reveal the sedimentation of ribosomes on the sucrose gradient. **(B)** ProtA−NSP1 containing fractions 5−8 from the sucrose gradient were pooled and used to purify the ProtA−NSP1 fusion protein by IgG-Sepharose chromatography as described in Materials and methods. ProtA−NSP1 purified either from a whole cell lysate (TOT) or from pooled fractions 5−8 (5−8) were analysed by SDS−PAGE and Coomassie staining. The positions of NIC96, ProtA−NSP1 and a major breakdown product of ProtA−NSP1 (*) are indicated.

of some of the less prominent bands will be discussed later. In a large scale purification of the ProtA−NSP1 fusion protein from a 5 l yeast culture, a total yield of 50−100 μg of NIC96 and ProtA−NSP1 bands, which generally appear in stoichiometric amounts, was achieved. The overall enrichment of ProtA−NSP1 in this one-step purification procedure was ~1000- to 2000-fold (see also Figure 2).

When an NSP1$^+$ strain not harbouring the *ProtA−NSP1* fusion gene was subjected to the same purification procedure, no protein bound specifically to the IgG-Sepharose column (data not shown). Furthermore, upon affinity purification of other ProtA fusion proteins such as ProtA−NOP1 (Figure 3, lane 3) or ProtA−DHFR (data not shown), NIC96 and the other indicated polypeptides detected in the ProtA−NSP1 eluate did not copurify.

Finally, a ts nsp1 mutant was previously generated by changing six negatively charged amino acids within a predicted amphipathic α-helix of the NSP1 C-terminal domain into alanines (Wimmer *et al.*, 1993). When this ala6-nsp1 mutant protein was tagged with ProtA, expressed in yeast and then isolated by IgG-Sepharose chromatography, ProtA−ala6-nsp1 was enriched on the column, but NIC96 was no longer present, as revealed by silver staining of the SDS−polyacrylamide gel (Figure 3, compare lanes 1 and 2); however, an 80 kDa species which co-migrates with the 80 kDa band seen with intact ProtA−NSP1 still appears in the eluate fraction of ProtA−ala6-nsp1 (see also Figure 2). Neither NIC96 nor the 80 kDa protein is seen if ProtA−NOP1 is subjected to affinity purification (Figure 3, lane 3). This suggests that a functional NSP1 C-terminal domain is required for co-isolation of the NIC96 protein.

ProtA−NSP1 and NIC96 are part of a complex as revealed by sucrose gradient centrifugation

To find out about the size of the ProtA−NSP1 complex, spheroplasts expressing ProtA−NSP1 were lysed in 2% Triton X-100 buffer as described above, centrifuged and the supernatant was loaded on to a 7−40% sucrose density gradient. In a similar way, a lysate from a strain expressing ProtA−DHFR was analysed by density gradient centrifugation. While ProtA−DHFR behaves like a monomeric protein and stays on the top of the gradient, ProtA−NSP1 migrates deeper into the gradient with a peak in fraction 6 (Figure 4A). A small part of ProtA−NSP1 also sediments in the lower part of the gradient where ribosomes migrate. From the molecular weight markers used, the distribution of catalase with a native particle size of 240 kDa reveals the best overlap with the ProtA−NSP1 peak fractions. When the peak fractions containing ProtA−NSP1 were pooled (Figure 4A, fractions 5−8) and subjected to IgG-Sepharose affinity chromatography, the eluate was strongly enriched in ProtA−NSP1 and the NIC96 protein (Figure 4B). These data further confirm that NSP1 and NIC96 are held together in a native complex, most likely in a 1:1 molar ratio.

Nucleoporins NUP49 (NSP49) and p54 co-purify with ProtA−NSP1 during affinity purification

In order to check whether other known pore proteins are present in the eluate, the purified ProtA−NSP1 fraction was probed with the monoclonal antibody mAB192 which reacts with the previously identified pore proteins, NUP116, NUP100, p65, p54 and NUP49 (Wente *et al.*, 1992). Here, the mAB192 was first tested on a whole yeast cell extract; it reacted with all five 'GLFG' nucleoporins, NUP100 giving the strongest signal (Figure 5A, H). If NSP49 [identical to NUP49 (Wimmer *et al.*, 1992)] was overexpressed, an increased immunostaining of this nucleoporin was observed with mAB192 (Figure 5A, H*). In the purified ProtA−NSP1 fraction only two 'GLFG' nucleoporins, NUP49 and p54, were found (Figure 5A, Eluate mAB192). These two nucleoporins are clearly enriched in the eluate and can be seen as distinct bands of similar intensity on the Coomassie stained SDS−polyacrylamide gel (Figure 5A, Coomassie; see also Figure 2B).

To find out whether affinity purification of a similar complex can be achieved by tagging another component of the purified fraction, we used a yeast strain expressing the protein A-tagged NSP49. ProtA−NSP49 was shown to be functional in yeast and to assemble into the nuclear pores (Wimmer *et al.*, 1992). The full-length NSP49 protein fused to the ProtA tag was more sensitive to proteolysis during purification than the ProtA-tagged C-terminal domain of NSP1, possibly due to the presence of the 'GLFG' repeat

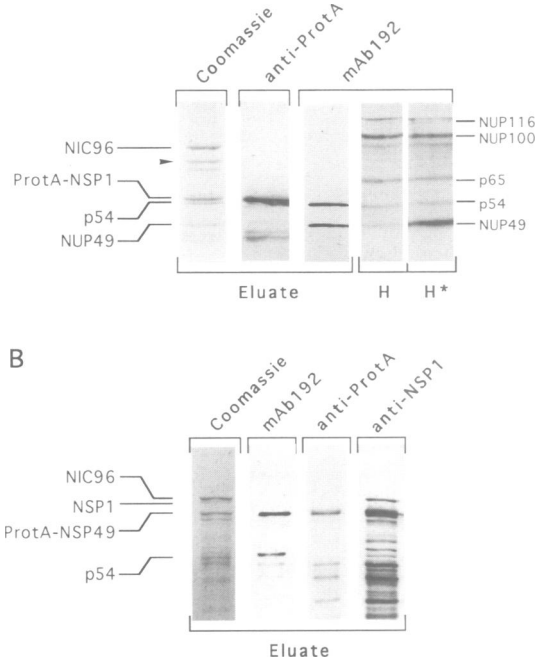

Fig. 5. Affinity purification of ProtA−NSP1 reveals association with 'GLFG' nucleoporins NUP49/p54 and NIC96. Affinity-isolation of ProtA−NSP1 and ProtA−NSP49 was carried out as described in Materials and methods. Aliquots of purified ProtA−NSP1 (**A**) and ProtA−NSP49 (**B**) (Eluate), a whole cell lysate from a control strain (H) and a NSP49 overproducing strain (H*) were analysed by SDS−PAGE followed by Coomassie staining or Western blotting to reveal the ProtA−NSP1/ProtA−NSP49 fusion proteins (anti-ProtA), 'GLFG' nucleoporins (mAB192) and 'FSFG' nucleoporins (anti-NSP1). Note that the anti-NSP1 antibodies, like any rabbit IgG also react with the IgG binding sequences of ProtA−NSP49. The positions of NIC96, ProtA−NSP1, ProtA−NSP49 and the GLFG nucleoporins NUP116, NUP100, p65, p54 and NUP49 are indicated. The position of the 80 kDa protein is marked by an arrowhead.

sequences within the ProtA−NSP49 chimeric protein. Nevertheless, Coomassie staining of the purified fraction eluted from the corresponding IgG column reveals discrete bands (Figure 5B) including ProtA−NSP49 (apparent mol. wt of 79 kDa) and the NIC96 protein (as verified by comparative 2D gel electrophoresis; unpublished results). Apart from the ProtA−NSP49 fusion protein, the mAB192 also detected p54 in the eluate. Furthermore, anti-NSP1 antibodies reveal the presence of NSP1 in this eluate (and its many breakdown products) which was also seen by Coomassie staining (Figure 5B).

NSP1, NSP49, p54 and NIC96 therefore appear to be held together in a NPC subcomplex which can be purified with the help of a protein A-tagged nucleoporin, either NSP1 or NSP49.

NIC96 is a novel protein essential for cell growth

As shown above, NIC96 is a specific nucleoporin-interacting component which does not react with anti-nucleoporin antibodies (see Figure 5). To clone its gene, peptide sequence data were obtained from the purified protein and derived degenerate oligonucleotide primers were used to amplify a corresponding DNA fragment of the *NIC96* gene by PCR. A complete genomic clone was finally isolated from a yeast genomic library by colony hybridization. As deduced from the DNA sequence, a single open reading frame was found within the genomic clone, capable of encoding a protein of 839 amino acids or 96 kDa molecular weight (Figure 6). The gene was therefore designated *NIC96* (for Nucleoporin-Interacting Component of 96 kDa). The peptides obtained from microsequencing of the purified protein, including the *N*-terminal peptide, were found in the NIC96 open reading frame. Comparison of the NIC96 amino acid sequence with known protein sequences present in the EMBL database did not reveal any striking homology. Furthermore, NIC96 does not contain repeat sequences of the 'FSFG' or 'GLFG' type as found in other nucleoporins. No sequence homology exists between NSP1 and NIC96 except a 50% identity within a stretch of 20 residues found in the NSP1 C-terminal domain (residues 753−772) and the N-terminal end of NIC96 (30−49). Interestingly, both sequences show a heptad repeat pattern with hydrophobic amino acids at positions 1 and 4 that have the probability to form coiled-coil interactions (Steinert and Roop, 1988). A second putative domain for coiled-coil interactions is predicted for residues 109−141 within the N-terminal region of NIC96. Finally, a continuous stretch of 20 uncharged and apolar amino acids is found between residues 321 and 342 of the NIC96 amino acid sequence which could be involved in interaction with the nuclear membrane.

Heterozygous diploids have been generated by homologous recombination in which one *NIC96* gene copy was replaced by *HIS3::nic96*. Sporulation of *HIS3::nic96* heterozygous integrants and tetrad analysis gave a 2:2 segregation for viability, the two growing haploid progeny being always his^- (data not shown). The lethal phenotype of the *HIS3::nic96* disruption mutant was complemented by the cloned *NIC96* gene present on a single-copy ARS/CEN plasmid (data not shown). These data show that *NIC96* is essential for cell viability. Microscopic inspection of haploid cells bearing *HIS3::nic96* revealed that the spores germinated but stopped growth at the 2−4 cell stage.

Immunolabelling of tagged NIC96 reveals nuclear pore location

In order to determine its location within the yeast cell, the NIC96 protein was tagged at its N-terminal end with two protein A-derived IgG binding sequences. The authentic *NIC96* gene was replaced by a *ProtA−NIC96* fusion gene in a diploid strain by homologous recombination (Figure 7A). After sporulation, haploid yeast expressing only the ProtA−NIC96 fusion protein grew with no apparent growth difference as compared with $NIC96^+$ cells (Figure 7B). This shows that the ProtA−NIC96 fusion protein can fully replace authentic NIC96. Expression of ProtA−NIC96 was followed by Western blotting which reveals a major band of 105 kDa not seen in $NIC96^+$ strains (Figure 7C, lanes 1 and 2). Subtraction of the ProtA moiety from the fusion protein allows a molecular weight of 91 kDa for authentic NIC96 to be calculated. This is close to the apparent molecular weight of NIC96 present in the eluate (Figure 2B).

Subcellular fractionation of yeast cells expressing ProtA−NIC96 was performed to obtain a nuclear and a post-nuclear supernatant fraction (Figure 7D, lanes 1−5). The NIC96 fusion protein, as well as other nuclear marker proteins such as NOP1, were enriched in the nuclear pellet and largely depleted from the post-nuclear supernatant (Figure 7D) in which cytoplasmic hexokinase was recovered (Hurt *et al.*, 1988).

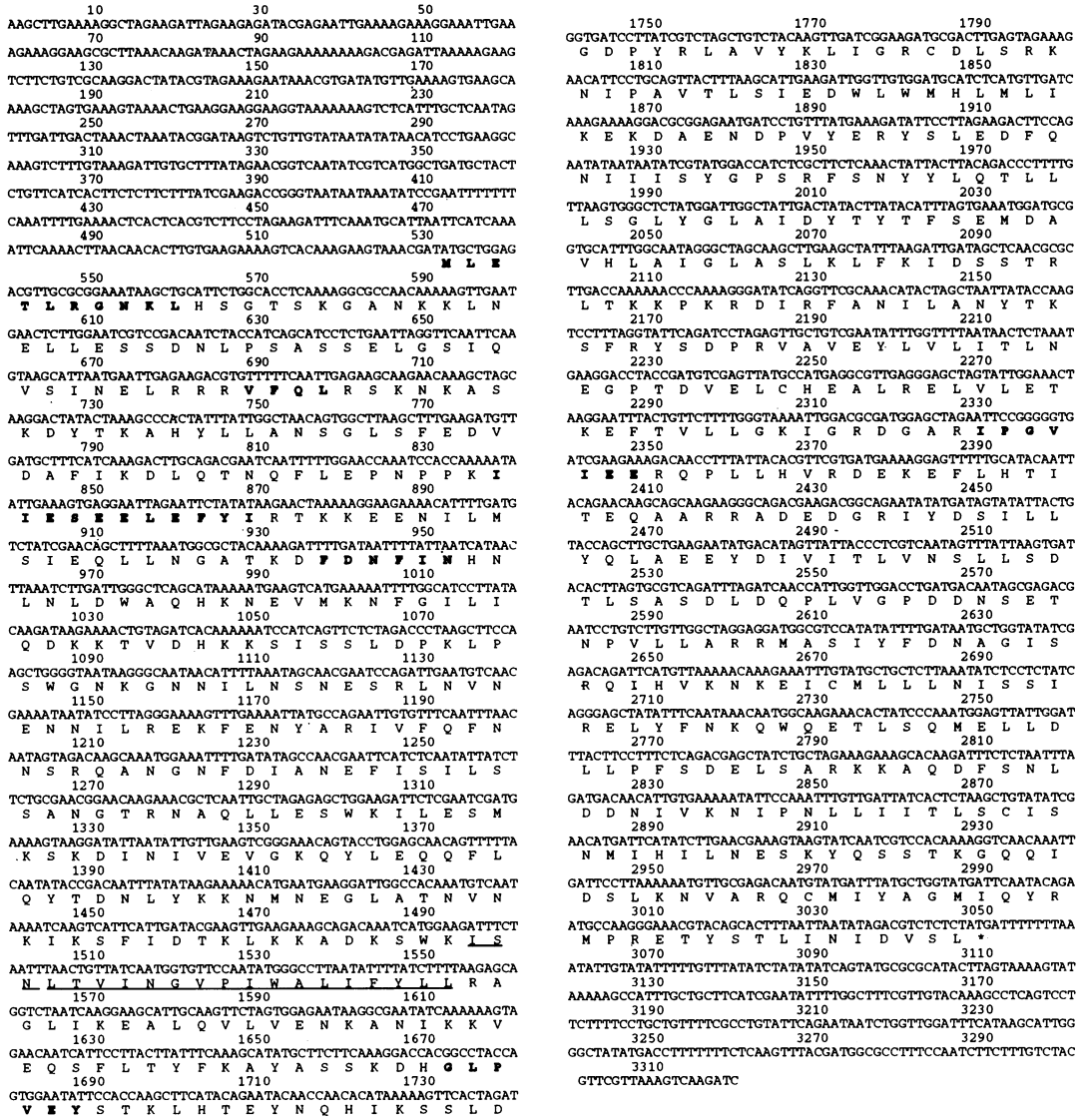

Fig. 6. DNA and deduced amino acid sequence of NIC96. Cloning of the genomic DNA encoding NIC96 and DNA sequence analysis are described in Materials and methods. The peptide sequences obtained from microsequencing of the purified and proteolytically cleaved NIC96 protein are marked in bold. Note that a continuous stretch of 20 uncharged and apolar amino acids is found between residues 321 and 342 of the NIC96 amino acid sequence (sequence underlined).

The *in vivo* location of ProtA−NIC96 was analysed by indirect immunofluorescence both in the conventional (Figure 8A) and confocal fluorescence microscope (Figure 8B). ProtA−NIC96 is localized at the nuclear envelope revealing a distinct ring-like and punctate staining. The observed immunolabelling highly resembles the immunostaining of other nucleoporins (see also Figure 1D) suggesting that NIC96 is a nuclear pore protein.

Discussion

Here, we report on the isolation of a NPC subcomplex composed of distinct members of known nucleoporins and a novel nuclear pore protein, NIC96. The purified nucleoporin complex contains members of both the 'GLFG' and the 'FSFG' nucleoporin subfamilies (Wente *et al.*, 1992; Wimmer *et al.*, 1992).

A biochemical approach is important to understand how nuclear pore proteins physically interact with each other, how they assemble into higher order complexes and how they become incorporated into nuclear pores. Although genetic studies have led to the identification of new pore proteins which functionally interact with NSP1 (Wimmer *et al.*, 1992), nearest neighbour relationships between the various components, their mode of physical interaction and their stoichiometric relationship can only be examined by *in vitro* approaches. The availability of a relatively simple nucleoporin subcomplex preparation now enables us to address these questions. The tagging of nucleoporins with protein A IgG binding sequences turned out to be a powerful approach to obtain larger quantities of pore proteins by a one-step affinity purification. The minimal IgG binding unit is relatively small (~50 amino acids, Moks *et al.*, 1987); the tandem arrangement of several of these binding units seems to allow a better exposure of the tag, thereby increasing the efficiency of IgG binding. Indeed when NSP1 was tagged with two (instead of four) IgG binding units the resulting chimeric protein gave only a weak signal when analysed by indirect immunofluorescence. The enrichment

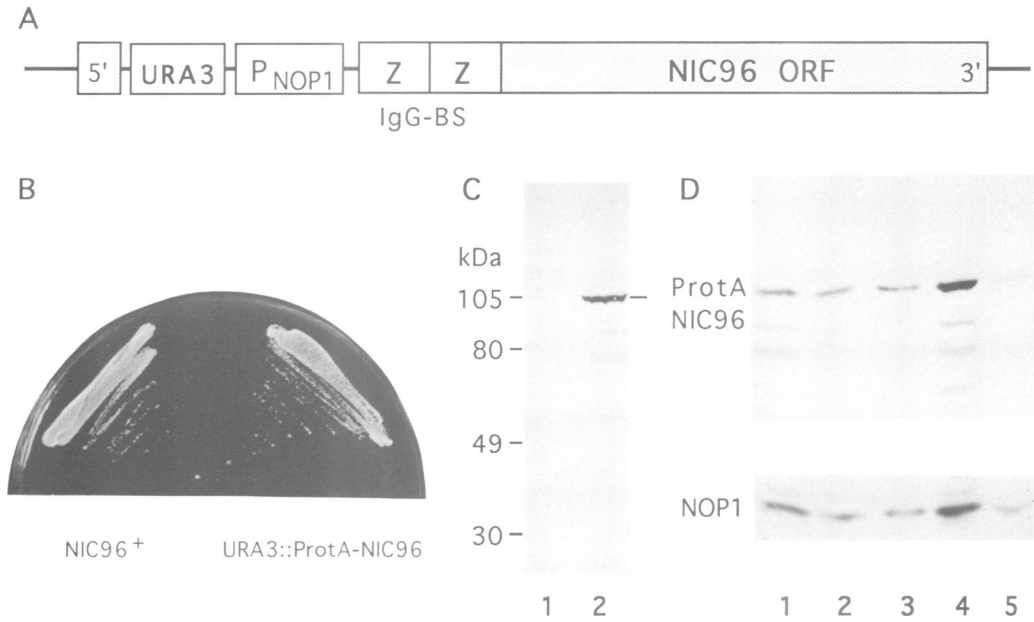

Fig. 7. Tagging of NIC96 with IgG binding sequences. (**A**) Schematic drawing of the fusion gene between two IgG binding domains (Z; IgG-BS) derived from *S.aureus* protein A and the NIC96 open reading frame joined at the ATG start codon. The fusion gene was placed under the control of the *NOP1* promoter and integrated into the *NIC96* gene locus by homologous recombination using the *URA3* gene as a selectable marker. (**B**) Growth of yeast strains NIC96$^+$ and ProtA−NIC96. Cells were incubated on YPD plate for 2 days at 30°C. (**C**) Immunoblot analysis. The same amount of whole cell extracts from yeast strains NIC96$^+$ (lane 1) and ProtA−NIC96 (lane 2) were analysed by SDS−PAGE and immunoblotting using IgG coupled to horseradish peroxidase to detect ProtA−NIC96. The position of ProtA−NIC96 and a molecular weight protein standard is indicated. (**D**) Distribution of NIC96 during subcellular fractionation. Aliquots of the cell lysate (lane 1), the homogenate supernatant (lane 2), a 1-fold (lane 3) and 10-fold crude nuclear pellet (lane 4) and post-nuclear supernatant (lane 5) were analysed by SDS−PAGE and immunoblotting using IgG coupled to horseradish peroxidase (upper part) and anti-NOP1 antibodies (lower part) to detect ProtA−NIC96 and the nuclear marker NOP1.

of ProtA−NSP1 isolated by IgG-Sepharose affinity chromatography under non-denaturing conditions is 1000- to 2000-fold, and accordingly, we could estimate that ~10−20 copies of NSP1 are present per nuclear pore complex; this comes close to the value determined for p62 and other nucleoporins in higher eukaryotic NPCs (Snow *et al.*, 1987). Besides affinity purification, the relatively simple detection of protein A-carrying fusion proteins by Western blotting or immunofluorescence (using reagents commercially available) allows valuable information on the subcellular location of the protein of interest to be detected.

Apart from NIC96 two known nucleoporins (NSP49 and p54) co-purified during affinity isolation of ProtA−NSP1. Conversely, NSP1 was enriched together with these three proteins during affinity purification of ProtA−NSP49. Thus, this biochemical analysis corroborates recent genetic data in which NSP49 was identified as a component in functional interaction with NSP1 in living cells (Wimmer *et al.*, 1992). Here, we show that NSP1 and NSP49 are both present within a multimeric complex. However, NSP49 and p54 were never found to be present in stoichiometric quantities as compared with NIC96 and ProtA−NSP1. This could be due to an increased proteolysis of full-length nucleoporins during affinity purification or gradient centrifugation since 'GLFG' or 'FSFG' repeat sequences are very susceptible to proteolysis (Nehrbass *et al.*, 1990; Wimmer *et al.*, 1992).

Although NSP116 was isolated several times in the genetic screen for synthetic lethals of NSP1 (Wimmer *et al.*, 1992), it could not be detected in significant amounts in the purified complexes. This could mean that NSP116 (i) is not part of this nucleoporin complex, (ii) is only loosely attached to the complex and therefore released during biochemical purification or (iii) is present, but preferentially degraded during purification. Affinity isolation of ProtA−NSP116 may help to clarify this issue.

What could be the molecular interaction by which NIC96, NSP1 and NSP49 are held together in a complex? Using a prediction programme for 'coiled-coils' in protein sequences (Lupas *et al.*, 1991), NSP1, NSP49 and, to a lesser extent, NIC96 show the potential to form coiled-coil interactions (Steinert and Roop, 1988). Resulting homodimers or heterodimers could account for the formation of this complex. It will be interesting to see whether heptad repeats are also found within the sequence of the fourth component of this complex, p54, which has not yet been cloned. Cross-linking experiments are under way and these should help to characterize further the interaction between these four components.

It is so far not clear where assembly of NSP1, NSP49, p54 and NIC96 takes place. If this occurs in the cytoplasm, a mutation in one of the four components might already block assembly, thereby impairing targeting of the other proteins to the NPC. Alternatively, each member of the purified complex may assemble independently into the NPC. In view of the presence of a continuous stretch of 20 uncharged and apolar amino acids in the NIC96 protein sequence, one attractive speculation is that NIC96 could make a physical link between repeat-containing nucleoporins and the 'nuclear pore membrane'. Preliminary data suggest that NIC96 is not an integral membrane protein, but it may be peripherally membrane associated (P.Grandi, unpublished data). Interestingly, NIC96 is the first characterized nuclear pore protein in yeast which lacks the diagnostic 'nucleoporin repeats'. It is not clear whether this feature of NIC96 will

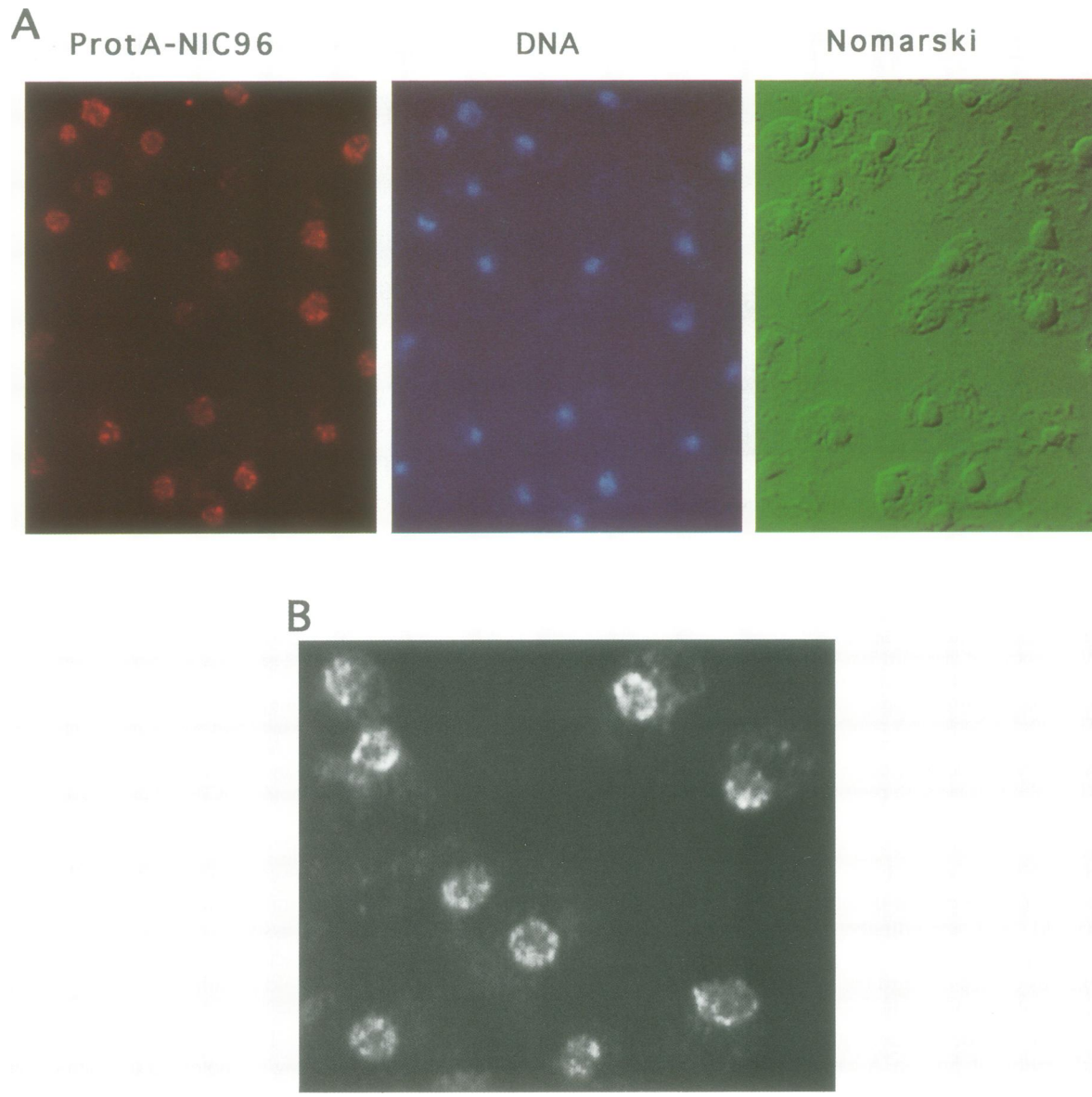

Fig. 8. Subcellular location of ProtA−NIC96 as revealed by immunofluorescence microscopy. (**A**) Conventional immunofluorescence microscopy. The subcellular location ProtA−NIC96 was analysed by immunofluorescence using rabbit anti-chicken IgG as first antibody followed by goat anti-rabbit IgG coupled to Texas Red. Cells were also stained for DNA using Hoechst 33258 and viewed by Nomarski optics. A ring-like staining of the nuclear periphery is observed with no apparent staining in the cytoplasm. (**B**) Confocal immunofluorescence microscopy. Cells were labelled for immunofluorescence as described for (A) and viewed in the confocal fluorescence microscope. A typical punctate nuclear pore labelling is seen.

Table I. Yeast strains

Strain	*Genotype*
RS453	**a**/α, *ade2/ade2, his3/his3, trp1/trp1, leu2/leu2, ura3/ura3*
JU4-2×JR26-19B	**a**/α, *ade2/ade2, ade8/ADE8, can1-100/can1-100, his4/HIS4, his3/HIS3, leu2/leu2, lys1/lys1, ura3/URA3*
TF2	**a**/α, *ade2/ade2, ade8/ADE8, can1-100/can1-100, his4/HIS4, his3/HIS3, leu2/leu2, lys1/lys1, ura3/URA3::nsp1/NSP1* (derived from JU4-2×JR26-19B)
VD1	**a**/α, *ade2/ade2, his3/his3, leu2/leu2, ura3/ura3, trp1/TRP1::nsp49/NSP49* (derived from RS453; see also Wimmer *et al.*, 1992)
PG1	**a**/α, *ade2/ade2, trp1/trp1, leu2/leu2, ura3/ura3, his3/HIS3::nic96/NIC96* (derived from RS453).
PG2	**a**/α, *ade2/ade2, his3/his3, trp1/trp1, leu2/leu2, ura3/URA3::ProtA−NIC96/NIC96* (derived from RS453)
ProtA−NSP1	α, *ade2, ade8, can1-100, leu2, lys1, URA3::nsp1, his*$^-$, pSB32-ProtA-NSP1 (derived from TF2)
ProtA−NSP49	**a**, *ade2, his3, trp1::nsp49, leu2, ura3*, pUN100-ProtA-NSP49 (derived from VD1)
ProtA−NIC96	α, *ade2, his3, trp1, leu2, URA3::ProtA-NIC96* (derived from PG2)
ProtA−DHFR	**a**/α, *ade2/ade2, ade8/ADE8, can1-100/can1-100, his4/HIS4, his3/HIS3, leu2/leu2, lys1/lys1, ura3/URA3*, YEp13-ProtA-DHFR

Table II. Plasmids

pSB32: ARS1/CEN4 plasmid with the LEU2 marker
pUN100: ARS1/CEN4 plasmid with the LEU2 marker
YEP13: 2μ high copy number plasmid containing the LEU2 marker
pSB32-ProtA-NSP1: containing the fusion gene between four IgG binding units of protein A and the gene encoding the NSP1 C-terminal domain
pUN100-NIC96: pUN100 containing a *Sna*BI–*Sph*I restriction fragment encoding the complete *NIC96* gene
YEP13-NIC96: YEP13 containing a 7 kb genomic insert that includes the complete *NIC96* gene
pUN100-ProtA-NSP49: containing the fusion gene between four IgG binding units of protein A and the complete *NSP49* gene
YEp13-ProtA-DHFR: containing the fusion gene between two IgG binding units of protein A and the complete *DHFR* gene from mouse under the control of the *NOP1* promoter (Wimmer *et al.*, 1992)

imply a fundamentally different role for this protein from that performed by NSP1 or NSP49.

So far, all the identified proteins found in this NPC subcomplex are encoded by essential genes. This shows that these pore proteins do not perform redundant functions and that each individual member is absolutely required for the function of the whole complex. What could be the function of this complex? A nucleoporin subcomplex was previously isolated from rat liver cells consisting of p62 [which is the mammalian homologue of yeast NSP1 (Carmo-Fonseca *et al.*, 1991)] and two other pore proteins, p58 and p54, which are in strong physical interaction and form a multimeric complex of 550–600 kDa (Finlay *et al.*, 1991). Nuclei lacking p62/p58/p54 are defective for nuclear transport. We have recently shown that NSP1 is also involved in nuclear transport reactions and pore biogenesis (Mutvei *et al.*, 1992). This suggests that NSP1/p62 and their interacting components are indispensable for pore function. However, their specific role with the NPC still remains to be determined. Although the function and organisation of these complexes may have been conserved during evolution, we do not have evidence so far that the NSP1 complex described in this study bears any resemblance to the p62 complex (see also above).

In the long term, isolation of other NPC subcomplexes may allow reconstitution of pore complexes *in vitro*. Determination of the exact location of the different proteins of the NPC will be crucial for generating topological maps of nuclear pore substructures.

Materials and methods

Yeast strains and plasmids

The yeast strains and plasmids used in this study are listed in Tables I and II, respectively. Microbiological techniques including yeast growth on minimal and rich YPD medium, plasmid transformation, gene disruption, sporulation of diploid cells and tetrad analysis were performed essentially as described in Wimmer *et al.* (1992).

Construction of ProtA–NSP1 and ProtA–NIC96 fusion genes and detection of the fusion proteins in yeast

The DNA encoding two synthetic IgG binding domains (designated Z) from protein A present in plasmid p28NZZtrc (kindly provided by Dr M.Uhlén) was amplified by PCR using primers which generated *Sac*II restriction sites at both the 5′ and 3′ ends. Two of these DNA fragments, each 360 bp in length, were tandemly inserted in-frame at a unique *Sac*II restriction site previously generated at the 5′ end of the gene encoding the NSP1 C-terminal domain. By this manipulation, the authentic *NSP1* promoter and the NSP1 intron adjacent to the fusion gene were not altered (Hurt, 1988). The derived amino acid sequence of the 488 residue ProtA–NSP1 fusion protein around the fusion sites was

(i) NSP1 (N-terminal residues Met1–Thr11),
(ii) linker sequence (Ala-Ala),
(iii) two IgG binding sequences (126 amino acids),
(iv) linker sequence (Asn-Ser-Ala-Ala),
(v) two IgG binding sequences (126 amino acids),
(vi) linker sequence (Asn-Ser-Ala),
(vii) NSP1 (C-terminal residues Gly607–Lys823).

The *ProtA–NSP1* fusion gene was inserted into the single-copy ARS/CEN plasmid pSB32 and transformed into diploid strain TF2 heterozygous for NSP1 by selection for LEU^+ transformants. Diploid transformants were sporulated and after tetrad dissection haploid progeny was selected which were *URA3::nsp1* and complemented by pSB32-ProtA–NSP1.

To construct the *ProtA–NIC96* fusion gene, the DNA encoding two synthetic IgG binding domains from protein A (see above) under control of the *NOP1* promoter (Wimmer *et al.*, 1992) was fused in-frame to the ATG start codon of the NIC96 ORF. The deduced amino acid sequence of the *ProtA–NIC96* fusion gene around the junction site is

(i) start sequence (Met-Ser-Ala-Cys),
(ii) two IgG binding sequences (126 amino acids),
(iii) linker sequence (Ala-Asn-Ser-Ser-Ser-Val-Pro),
(iv) NIC96 (Met1–Leu839).

Subsequently, a 130 bp long *Hin*dIII–*Sna*bI restriction fragment derived from the 5′ non-coding region of the *NIC96* gene and the *URA3* gene were joined to the 5′ end of the *NOP1* promoter, yielding 5′-NIC96 (non-coding sequence)-URA3-P_{NOP1}-ProtA–NIC96-3′ (coding sequence). This construct was excised from plasmid pBluescript and the linear DNA was used for homologous integration at the *NIC96* gene locus by transforming diploid RS453 and selection for URA^+ transformants. Heterozygous diploids were sporulated and haploid progeny selected which were *URA3::ProtA–NIC96*. Construction of the *ProtA–NSP49* fusion gene and expression in a *nsp49*$^-$ mutant were described earlier (Wimmer *et al.*, 1992). The *ProtA–ala6-nsp1* fusion gene was identical to authentic *ProtA–NSP1* except that six negatively charged amino acids (residue 701, 705, 706, 708, 712 and 715) within the essential C-terminal domain were changed into neutral alanines (Wimmer *et al.*, 1993). Haploid strains ProtA–NSP1, ProtA–NSP49, ProtA–NIC96 and ProtA–ala6-nsp1 were grown in YPD or SD –leu medium and whole cell extracts or the purified fusion proteins were analysed by SDS–PAGE and immunoblotting using IgG coupled to horseradish peroxidase (Dakopatts, Denmark). Monoclonal antibody mAB192 (kindly provided by Dr S.Wente) recognizing 'GLFG' nucleoporins (Wente *et al.*, 1992) was used in a 1:50 dilution for Western blotting.

To immunolocalize ProtA fusion proteins *in vivo*, yeast cells expressing the corresponding tagged protein were fixed in 3.7% formaldehyde for 1 h, converted into spheroplasts using 0.5 mg/ml zymolyase 100.000 T, processed for immunofluorescence and analysed in the conventional and confocal fluorescence microscope as described earlier (Wimmer *et al.*, 1992). Rabbit anti-chicken IgG (Medac, Hamburg, Germany) which binds to the protein A moiety was used as first antibody (1:50 dilution) followed by goat anti-rabbit IgG coupled to Texas Red (1:50 dilution).

Purification of the ProtA–NSP1 and ProtA–NSP49 fusion proteins under non-denaturing conditions

Strain ProtA–NSP1 was grown in 5 l YPD medium at 30°C for 15 h. Cells were harvested at OD_{600} 1–2, converted into spheroplasts and lysed in 300 ml lysis buffer (2% Triton X-100, 20 mM NaCl, 0.2 mM $MgCl_2$, 20 mM Tris–HCl, pH 8.0) by performing several strokes with a Dounce homogenizer. The solubilized fraction was centrifuged for 10 min at 15 000 r.p.m. in a SS34 rotor and the supernatant was loaded onto a 30 mm diameter column packed with 5 ml IgG-Sepharose (Pharmacia, Germany) and equilibrated with washing buffer (0.05% Tween 20, 150 mM NaCl, 20 mM Tris–HCl, pH 7.4). The flow rate during the loading step was 10 ml/min. The flow-through was applied a second time to the column. It was washed with 100 ml washing buffer, followed by an additional wash with 20 ml 5 mM NH_4Ac, pH 5.0. The bound ProtA fusion protein was finally eluted with 5 ml 0.5 M acetic acid, pH 3.4, concentrated by lyophilization and resuspended in SDS-sample buffer. Aliquots of the homogenate, the flow-through, the wash and the eluted fraction were analysed by SDS–PAGE and Western blotting as described above. The purification of ProtA–NSP49 and ProtA–ala6-nsp1 was performed in a similar way.

Sucrose density gradient centrifugation

Strains ProtA−NSP1 and ProtA−DHFR were inoculated in 500 ml of, respectively, YPD and SD −leu medium, and incubated at 30°C for 15 h. Cells grown to OD_{600} 1−2 were centrifuged, spheroplasted and lysed in 10 ml lysis buffer (2% Triton X-100, 20 mM NaCl, 0.2 mM $MgCl_2$, 20 mM Tris−HCl, pH 8.0) as described above. After centrifugation for 10 min at 15 000 r.p.m. in a SS34 rotor, 1.5 ml supernatant were loaded on a 7−40% sucrose density gradient prepared in buffer 0.1% Triton X-100, 20 mM NaCl, 0.2 mM $MgCl_2$, 20 mM Tris−HCl, pH 8.0. It was centrifuged for 17 h at 34 000 r.p.m. in a SW40 rotor. The whole gradient was collected in 12 fractions and an aliquot of each fraction was analysed by SDS−PAGE and Western blotting. For calibration of the sucrose density gradient, BSA (66 kDa; Pharmacia), catalase (240 kDa; Pharmacia) and ferritin (450 kDa; Pharmacia), 2 mg each per SW40 tube, were used.

Pooled fractions 5−8 from the sucrose gradient, derived from strain ProtA−NSP1, were used to isolate the ProtA−NSP1 fusion protein by IgG-Sepharose chromatography followed by SDS−PAGE and Coomassie staining as described above.

Cloning, DNA sequencing and gene disruption of the NIC96 gene

Internal peptide sequences derived from the purified and proteolytically cleaved NIC96 protein and the N-terminal peptide MLETLRGNKL were obtained by microsequencing. Degenerate oligonucleotides were synthesized for the N-terminal peptide and the internal peptide IIESEELEFYI and used to clone a corresponding 338 bp DNA fragment from total yeast genomic DNA by PCR using conditions described earlier (Carmo-Fonseca *et al.*, 1991). The ^{32}P-labelled 338 bp PCR fragment (Megaprime DNA labelling, Amersham) was used in a colony hybridization assay to clone the complete *NIC96* gene from a yeast genomic library. From plasmid YEp13-NIC96 which contained a 7 kb insert, a 3.36 kb *Sna*BI−*Sph*I restriction fragment was excised which contained the entire *NIC96* gene including a 187 bp fragment derived from the tetracycline gene which is located at the 3′ end. This *Sna*B−*Sph*I fragment was inserted into plasmid pUN100 yielding pUN100-NIC96. Both DNA strands of the complete *Sna*BI−*Sph*I fragment were sequenced by the dideoxy chain termination method (Sanger *et al.*, 1977). Homology of the NIC96 protein sequence to known protein sequences were analysed by searching the EMBL database using the FASTA program.

Gene eviction was done by the method of Rothstein (1983). For disruption of the *NIC96* gene, the selectable *HIS3* marker, isolated as a 1.15 kb *Bam*HI fragment from plasmid YDpH, was used to replace an internal 1.3 kb *Nhe*I fragment thereby releasing most of the NIC96 ORF. A linear *HIS3::nic96* DNA fragment was excised from the recombinant plasmid with restriction enzymes *Sna*BI−*Aat*II and transformed into diploid strain RS453 by selection for HIS^+ transformants. One diploid transformant, PG1, was sporulated and tetrads were dissected on YPD plates. In order to test whether the strain harbouring the disrupted *HIS::nic96* construct is complemented by the cloned *NIC96* gene, PG1 was transformed with pUN100-NIC96 followed by sporulation and tetrad analysis.

Miscellaneous

Subcellular fractionation including purification of nuclei was done as described earlier (Hurt *et al.*, 1988). Isolation of total DNA and Southern analysis was done essentially as described (Sherman, 1990). DNA manipulations including restriction analysis, fill-in reactions with Klenow and T4 DNA polymerase and ligations were done essentially according to Maniatis *et al.* (1982).

GenBank accession number

The accession number for the NIC96 sequence reported in this paper is X72923.

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