

NAP57, a Mammalian Nucleolar Protein with a Putative Homolog in Yeast and Bacteria

U. Thomas Meier and Günter Blobel*

Department of Anatomy and Structural Biology, Albert Einstein College of Medicine, Bronx, New York 10461; and *Laboratory of Cell Biology, Howard Hughes Medical Institute, The Rockefeller University, New York 10021

Abstract. We report the identification and molecular characterization of a novel nucleolar protein of rat liver. As shown by coimmunoprecipitation this protein is associated with a previously identified nucleolar protein, Nopp140, in an apparently stoichiometric complex and has therefore been termed NAP57 (Nopp140-associated protein of 57 kD). Immunofluorescence and immunogold electron microscopy with NAP57 specific antibodies show colocalization with Nopp140 to the dense fibrillar component of the nucleolus, to coiled bodies, and to the nucleoplasm. Immunogold staining in the nucleoplasm is occasionally seen in the form of curvilinear tracks between the nucleolus and the nuclear envelope, similar to those previously reported for Nopp140. These data suggest that Nopp140 and NAP57 are indeed associated with each other in these nuclear structures.

The cDNA deduced primary structure of NAP57 shows a protein of a calculated molecular mass of 52,070 that contains a putative nuclear localization signal near its amino and carboxy terminus and a hydrophobic amino acid repeat motif extending across 84 residues. Like Nopp140, NAP57 lacks any of the known consensus sequences for RNA binding which are characteristic for many nucleolar proteins. Data bank searches revealed that NAP57 is a highly conserved protein. A putative yeast (*S. cerevisiae*) homolog is 71% identical. Most strikingly, there also appears to be a smaller prokaryotic (*E. coli* and *B. subtilis*) homolog that is nearly 50% identical to NAP57. This indicates that NAP57 and its putative homologs might serve a highly conserved function in both pro- and eukaryotes such as chaperoning of ribosomal proteins and/or of preribosome assembly.

FUNCTIONAL ribosomal subunits of *E. coli* have been reconstituted in vitro from their RNA and protein components some time ago (Traub and Nomura, 1968). Reconstitution was observed at empirically defined windows of ionic strength and temperature (unphysiologically high) and proceeded unassisted by nonribosomal proteins. Little is known how ribosomal subunit assembly occurs in bacteria in vivo. What, for example, is the order in which ribosomal proteins associate with the ribosomal RNAs co- and posttranscriptionally? To what extent is ribosomal subunit assembly assisted by nonribosomal proteins?

In eukaryotes, ribosomal subunit assembly occurs in the nucleolus. Unlike in bacteria where ribosomal protein and RNA synthesis take place in one compartment, the bacterial cytosol in eukaryotes the two processes occur in two compartments, the cytosol and the nucleolus. For ribosomal subunit assembly, ribosomal proteins therefore need to be

transported into the nucleolus (Warner and Soeiro, 1967). The assembled subunits are then transported into the cytosol to function in protein biosynthesis. Little is known either about how ribosomal proteins are transported into the nucleolus or their order of assembly with the ribosomal RNAs co- or posttranscriptionally. Not all of the ribosomal proteins may be transported into the nucleolus as indicated by the differences in protein composition between nucleolar and cytoplasmic (pre)ribosomal particles (Kumar and Warner, 1972; Tsurugi et al., 1973; Prestayko et al., 1974; Kumar and Subramanian, 1975) and by the in vivo exchange of certain proteins between ribosome associated and soluble proteins (Warner, 1966; Zinker and Warner, 1976). Does, therefore, a distinct cytoplasmic phase of ribosomal subunit assembly yield functionally active cytoplasmic subunits (as opposed to functionally inactive nuclear subunits)? To what extent are nonribosomal proteins involved in assembly and subsequent nucleocytoplasmic transport of ribosomal subunits?

A number of nonribosomal nucleolar proteins, largely of unknown function, have now been identified. Some of these proteins could represent structural proteins of a nucleolar skeleton (Franke et al., 1981). Others, such as fibrillarin, are

Please address all correspondence to Dr. U. Thomas Meier, Department of Anatomy and Structural Biology, Albert Einstein College of Medicine, 1300 Morris Park Avenue, Bronx, NY 10461. Tel.: (718) 430-3294. Fax: (718) 518-7236.

involved in processing of ribosomal RNA (Tollervey et al., 1991). These two groups of proteins can be considered resident nucleolar proteins. A third group of nucleolar proteins appears to shuttle between the nucleolus and the cytoplasm (Borer et al., 1989; Meier and Blobel, 1992). It is likely that these proteins function in ribosomal subunit assembly and in nucleocytoplasmic transport.

Noppl40, a nucleolar phosphoprotein of 140 kD, is a representative of the shuttling variety of nucleolar proteins (Meier and Blobel, 1992). Immunogold electron microscopy localized Noppl40 primarily to the dense fibrillar component (DFC)¹ of the nucleolus. In addition, Noppl40 could be seen in the nucleoplasm and occasionally was found in curvilinear tracks that extended for microns across the nucleoplasm from the DFC of the nucleolus to nuclear pore complexes (Meier and Blobel, 1992). Similar tracks have now been observed with antibodies to the ribosomal protein S1 (Raska et al., 1992) and to the human immunodeficiency virus type 1 Nef protein (Murti et al., 1993). These localization data are consistent with Noppl40 shuttling between the nucleolus and the cytoplasm. It is not known, however, why and with whom Noppl40 shuttles. Noppl40 was identified as a protein that binds nuclear localization signals (NLS) *in vitro* (Meier and Blobel, 1990). If NLS binding were its physiological function (or one of its functions), Noppl40 might serve in protein import and therefore be associated with proteins to be imported. In addition or alternatively, the repetitive acidic, serine-rich motifs of Noppl40 and the basic stretches separating them (Meier and Blobel, 1992) could serve a chaperone function in ribosomal subunit assembly by interacting with regions of opposite charge (ribosomal RNA, ribosomal proteins) displayed by ribosomal subunit assembly intermediates. In this scenario Noppl40 might remain associated with a preribosomal particle that is transported from the nucleolus to the cytoplasm.

In an attempt to elucidate the basis of the Noppl40 tracks and the Noppl40 function, we present here the identification and molecular characterization of the Noppl40 associated protein, NAP57. We show that NAP57 coprecipitates and colocalizes with Noppl40 and that it is evolutionarily highly conserved. Possible NAP57/(Noppl40) functions are discussed in this context.

Materials and Methods

Immunoprecipitation

Rat liver nuclei were prepared and repeatedly extracted with low ionic strength buffer (25 mM Tris-HCl, pH 8.1) exactly as described previously (Meier and Blobel, 1990). Only the second extract containing most of the Noppl40 was used in these experiments. Various volumes (routinely 0.5 ml) of nuclear extracts were diluted 1:1 either directly or after denaturation with 0.4% SDS at 90°C for 10 min. to reach the final precipitation conditions of 1% Triton X-100, 0.2% SDS, 150 mM NaCl, 50 mM Tris-HCl (pH 8.1). Affinity purified IgGs (5 µg/ml) were added to the diluted extracts, incubated for 2 h at 25°C, precipitated with protein A-Sepharose beads (5 µl of packed beads, Pharmacia Fine Chemicals, Piscataway, NJ), and the precipitate washed three times with 1 ml of 0.1% Triton X-100, 0.02% SDS, 5 mM EDTA, 150 mM NaCl, 50 mM Tris-HCl (pH 8.1) and once with 1

related control peptide were present resulting in approximately a 100-fold molar excess over the IgGs. Proteins were eluted with 40 µl SDS sample buffer containing 0.1 M DTT for 5 min at room temperature and analyzed directly by SDS-PAGE (9%, Laemmli, 1970) and 0.2% Coomassie blue R250 and subsequent silver stain (Merril et al., 1984). For immunoblot analysis (Meier and Blobel, 1990), SDS-PAGE-separated proteins were transferred to nitrocellulose (Towbin et al., 1979). Molecular weight marker proteins were from Sigma Chemical Co. (St. Louis, MO).

Protein Sequencing and Antibody Production

To obtain protein sequence of NAP57, the coimmunoprecipitation procedure was scaled up 10-fold, and the precipitated proteins separated by SDS-PAGE and transferred to polyvinylidene difluoride membrane (immobilon PVDF, Millipore Continental Water Systems, Bedford, MA) as described previously (Meier and Blobel, 1992). Amino terminal and internal amino acid sequencing of NAP57 after digestion with endoproteinase Lys-C was then performed at the Rockefeller University Biopolymer Facility (New York, NY) (Fernandez et al., 1992).

Antibodies were raised in rabbits by Rockland Inc. (Gilbertville, PA) against a synthetic peptide (see Fig. 5) corresponding to residues 21–36 of NAP57, with the exceptions of a serine instead of a histidine in position 33 and of an additional amino terminal cysteine for cross-linking purposes. Anti-NAP57 peptide IgGs were affinity purified on peptide columns as described (Meier and Blobel, 1992). The use of serine instead of the authentic histidine was because the amino acid peptide sequence in that position could not be identified with certainty and was wrongly guessed to be serine. Peptide synthesis and antibody production was completed before the misidentification of serine in that position became evident from the cDNA deduced primary structure of NAP57. Nevertheless, the immunoprecipitation and Western blot data shown in Fig. 2 demonstrate that this single mismatch did not interfere with the production of monospecific anti-NAP57 antibodies.

Immunochemical Methods

Immunoblots were performed exactly as described previously (Meier and Blobel, 1990) using the affinity purified IgGs at 1 µg/ml and a 100-fold molar excess of free synthetic peptide where indicated.

Indirect immunofluorescence experiments were carried out essentially as described (Meier and Blobel, 1990). Paraformaldehyde fixed and Triton X-100 permeabilized buffalo rat liver (BRL) cells were incubated in PBS containing 0.1% bovine serum albumin and antibodies (anti-NAP57 peptide at 2 µg/ml, anti-Noppl40 peptide at 1 µg/ml, and anti-p80 coilin serum at 1:200). Secondary antibodies were fluorescein-conjugated donkey anti-human and Texas red-conjugated donkey anti-rabbit IgGs affinity purified and mutually preadsorbed against IgGs from other species for minimal cross-reactivity (30 µg/ml, Jackson ImmunoResearch Laboratories, Inc., West Grove, PA). Immunofluorescence was observed on two wavelengths simultaneously using a MRC-600 confocal laser scanning microscope (Bio-Rad Laboratories, Hercules, CA). Pictures were photographed using a computer image recorder (Datagraf, Inc., Wheeling, IL) and Kodak T-max 400 film. In double immunofluorescence experiments pictures were recorded in pseudo color and superimposed. In addition, each antibody was incubated separately and the fluorescence signal analyzed independently to ensure the identity of each signal. Anti-p80 coilin antibodies correspond to human autoimmune serum "Sh" (Raska et al., 1990; Andrade et al., 1991; Raska et al., 1991). Immunoelectron microscopy was performed exactly as described (Meier and Blobel, 1992). Cryosections or sections of L. R. White resin (EM Sciences, Fort Washington, PA) embedded BRL cells were incubated with affinity purified anti-NAP57 peptide IgGs at 0.5 µg/ml.

Cloning and Analysis of the NAP57 cDNA

Degenerate oligonucleotides were designed corresponding to amino acid residues 27–32 and 83–88 (see Fig. 5) obtained from peptide sequencing and used in a polymerase chain reaction with rat cDNA as template (Meier and Blobel, 1992) to amplify 181 nucleotides of NAP57 cDNA. This NAP57 specific DNA probe was labeled with [³²P]dCTP by the random prime method (Feinberg and Vogelstein, 1983) and used to screen an amplified λ ZAP II rat cDNA library (Stratagene, La Jolla, CA). Six positive clones out of 600,000 phages screened were directly *in vivo* excised according to the supplier's protocol and determined by dideoxy nucleotide sequencing (Sanger et al., 1977; Schuurman and Keulen, 1991) to be overlapping but lacking the 5'-end. One of these partial NAP57 cDNAs was used as probe to screen 150,000 independent phages of an unamplified λgt10 rat

1. *Abbreviations used in this paper:* BRL, buffalo rat liver; DFC, dense fibrillar component; FC, fibrillar centers; GC, granular component; NLS, nuclear localization signal.

cDNA library (Sukegawa and Blobel, 1993). Out of sixteen positive clones eight were isolated, the inserts analyzed by size and restriction digests, and the five longest ones subcloned into pBluescript II SK⁺ (Stratagene) for DNA sequencing. One of these, pTM575, contained the complete open reading frame of NAP57 (see Fig. 5). DNA analysis was performed with DNASTAR software program (DNASTAR, Madison, WI) and homology searches in GenBank by the BLAST algorithm (Altschul et al., 1990). Northern blot analysis was carried out on poly(A)⁺ RNA isolated from BRL cells exactly as published (Meier and Blobel, 1992). Probes representing the whole pTM575 sequence (not shown) or a fragment corresponding to the first 1337 nucleotides of the NAP57 cDNA (Fig. 6 A) hybridized to a single mRNA species of identical mobility.

For in vitro transcription/translation assays (Meier and Blobel, 1992), pTM575 was linearized using XbaI restriction enzyme (Boehringer Mannheim Biochemicals, Indianapolis, IN). Between 1 and 6 μ g pTM575 RNA was used for in vitro translation in rabbit reticulocyte lysate (Promega, Madison, WI). Analysis of translation products was as previously described (Meier and Blobel, 1992).

Results

Noppl40 Is Associated with a 57-kD Protein

We showed previously (Meier and Blobel, 1990) that most of Noppl40 can be extracted from isolated rat liver nuclei by 25 mM Tris, pH 8.1. To determine whether the extracted Noppl40 was associated with other protein(s), we subjected the nuclear extract to immunoprecipitation with affinity purified IgGs prepared from a previously characterized rabbit antiserum against a Noppl40 peptide (Meier and Blobel, 1992). Indeed, under nondenaturing conditions a 57-kD protein was coimmunoprecipitated with Noppl40 (Fig. 1, lane 5). The relative Coomassie blue and silver staining intensity of the coprecipitated 57-kD protein to that of Noppl40, which did not vary when increasing amounts of Noppl40 were precipitated (not shown), suggested that the 57-kD protein and Noppl40 occurred in a stoichiometric complex. We therefore named this protein NAP57, Noppl40-associated protein of 57 kD. As expected, NAP57 was not coimmunoprecipitated with Noppl40 when the nuclear extract was preincubated with 0.4% SDS at 90°C (Fig. 1, lane 3) or when immunoprecipitation was carried out in the presence of the competing Noppl40 peptide against which the antibodies were raised (Fig. 1, lanes 4 and 6).

In addition to Noppl40, we had previously identified a 55-kD NLS-binding protein (Meier and Blobel, 1990). Because of its similar mobility to NAP57 on SDS-PAGE, we tested the ability of immunoprecipitated NAP57 to bind NLS peptide conjugates by ligand blotting (Meier and Blobel, 1990). NAP57 bound neither wild-type nor mutant NLS peptide conjugates and was thus distinct from the originally identified 55-kD protein (data not shown).

To obtain amino acid sequence of NAP57, the coimmunoprecipitation procedure was scaled up and the proteins transferred to immobilon membranes for protein sequencing. While the amino terminus appeared to be blocked, we obtained amino terminal sequences from three internal peptides after proteolytic digestion of NAP57 with endoproteinase Lys-C (see Fig. 5, Fernandez et al., 1992). Antibodies were raised in rabbits against a synthetic NAP57 peptide and affinity purified on a peptide column. These affinity purified anti-NAP57 peptide antibodies recognized a single protein of 57 kD on Western blots of whole rat liver nuclei in the absence (Fig. 2 A, strip 1) but not in the presence of

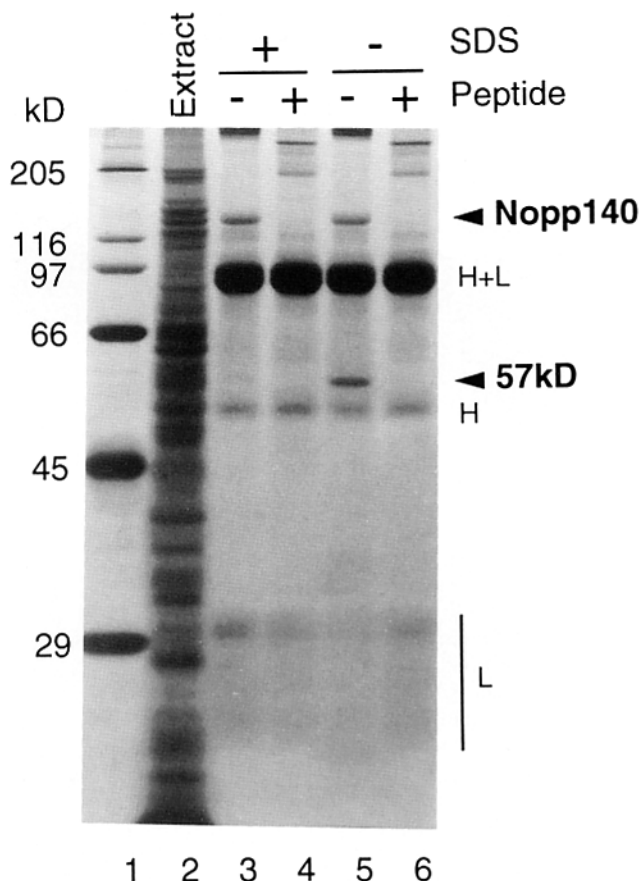


Figure 1. Identification of NAP57 by coimmunoprecipitation with Noppl40. Silver stain of immunoprecipitates from rat liver nuclear extracts (lane 2, 1/20 equivalent of lanes 3–6) by affinity purified anti-Noppl40 peptide antibodies (lanes 3–6) after separation by SDS-PAGE. Noppl40 was precipitated either from extracts that were previously denatured by SDS (lanes 3 and 4) or from native extracts (lanes 5 and 6) in the presence of synthetic control peptide from NAP57 (residues 21–36; lanes 3 and 5) or of free competing Noppl40 peptide (lanes 4 and 6) against which the antibodies had been raised (Meier and Blobel, 1992). Lane 1 contained molecular mass marker proteins, the mass of which is indicated on the left in kD. The mobility of Noppl40, the coprecipitated 57-kD protein and the IgG heavy (H) and light (L) chains are indicated on the right. On some occasions, the presence of free competing peptides caused the high molecular mass aggregates caught at the interface between stacking and separating gel (top of lanes 3 and 5) to increase their mobility (top of lanes 4 and 6).

free competing peptide (Fig. 2 A, strip 2). In addition, they recognized on Western blots the 57-kD protein that was coprecipitated with Noppl40 (not shown) demonstrating that they were specific for NAP57.

The anti-NAP57 peptide antibodies were also used to immunoprecipitate NAP57 from rat liver nuclear extracts as described above for the anti-Noppl40 peptide antibodies. SDS-PAGE separation of the immunoprecipitate, transfer of the proteins to nitrocellulose membrane, and staining with amido black showed that anti-NAP57 peptide antibodies coprecipitated a protein of 140 kD (Fig. 2 B, lane 1). This protein corresponded to Noppl40 as it reacted with anti-Noppl40 antibodies on Western blots (Fig. 2 C, lane 1).

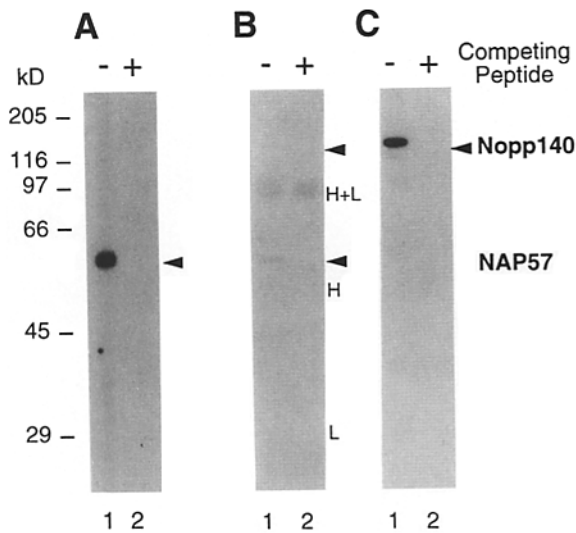


Figure 2. Characterization of affinity purified anti-NAP57 peptide antibodies by Western blot (A) and immunoprecipitation (B and C). (A) Proteins of whole rat liver nuclei were separated by SDS-PAGE and transferred to nitrocellulose filters which were incubated with anti-NAP57 antibodies in the absence (A, strip 1) or the presence of free competing NAP57 peptide (A, strip 2). (B) Amido black stain of immunoprecipitated proteins from native nuclear extracts by anti-NAP57 antibodies after separation of SDS-PAGE and transfer to nitrocellulose membrane. Precipitation was performed as in Fig. 1 either in the presence of free control Nopp140 peptide (B, lane 1) or of competing NAP57 peptide (B, lane 2). (C) Nitrocellulose membrane from B incubated with anti-Nopp140 antibodies. Antibodies on Western blots (A and C) were detected by ^{125}I protein A and autoradiography. The mobility of molecular mass marker proteins is indicated by bars on the left, that of Nopp140 and NAP57 on the right by arrowheads, and that of IgG heavy and light chains by H and L, respectively.

Nopp140 was not coprecipitated if the nuclear extracts were denatured with SDS before antibody incubation (not shown). Moreover, in the presence of free competing NAP57 peptide neither NAP57 nor Nopp140 was precipitated (Fig. 2, B and C, lane 2) indicating that coprecipitation of Nopp140 with NAP57 was specific.

Immunolocalization of NAP57

To determine the subcellular distribution of NAP57, the affinity purified anti-NAP57 peptide antibodies were employed in indirect immunofluorescence experiments on fixed and permeabilized BRL cells. Comparison of the same field of cells in phase contrast (Fig. 3 A) with that in fluorescence (Fig. 3 A') showed that anti-NAP57 antibodies strongly stained the nucleoli in a granular pattern and diffusely labeled the nucleoplasm. Moreover, extranucleolar dots (usually 1-3) were observed in most nuclei (Fig. 3 A', arrows), reminiscent of coiled body staining by anti-p80 coilin antibodies (Raska et al., 1990; Andrade et al., 1991; Raska et al., 1991). This nuclear pattern of NAP57 was indistinguishable from that previously reported with anti-Nopp140 antibodies (Meier and Blobel, 1990; Meier and Blobel, 1992). In addition to the nuclear staining however, there was a finely speckled labeling throughout the cytoplasm (Fig. 3, A' and

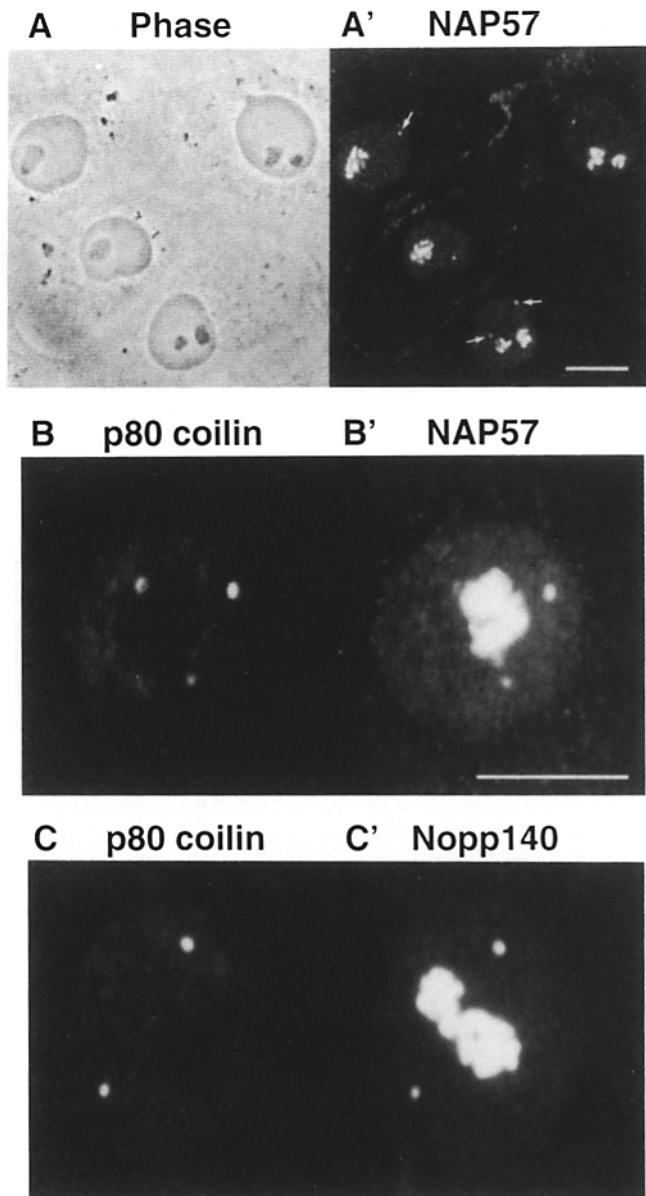


Figure 3. Immunolocalization of NAP57 on fixed and permeabilized BRL cells by confocal laser scanning microscopy. (A) Simultaneous visualization of a field of cells by phase contrast (A) and indirect immunofluorescence (A') generated by affinity purified anti-NAP57 peptide antibodies and Texas red-labeled secondary antibodies. Note the heavy labeling of the nucleoli, extranucleolar dots (arrows in some of the nuclei) and staining along the edge of the cells (compare A' to A). (B) Indirect double immunofluorescence staining of cells with human anti-p80 coilin (B) and rabbit anti-NAP57 (B') antibodies. A high magnification of the same nucleus was viewed simultaneously in the fluorescein (B) and Texas red (B') channel. Note labeling of the same nucleoplasmic dots around the nucleolus by the anti-NAP57 antibodies as by the anti-p80 coilin antibodies. (C) Indirect double immunofluorescence staining of cells with human anti-p80 coilin (C) and rabbit anti-Nopp140 (C') antibodies. Other specifications as in B. Bars: (A' and B) 10 μm , B and C same magnification.

B') and a more concentrated signal along the plasma membrane (Fig. 3 A). Western blots of postnuclear fractions from BRL cells and rat liver, presumably containing at least some of the plasma membrane, sometimes showed reactivity of the anti-NAP57 peptide antibodies also with bands that migrated slower than NAP57 (not shown). Therefore, we can presently not rule out the possibility that the cytoplasmic immunofluorescence was due to a protein other than NAP57. However, as on Western blots (Fig. 2 A, strip 2), free competing NAP57 peptide abolished all immunofluorescent signals, nuclear and cytoplasmic, of the anti-NAP57 peptide antibodies (not shown).

To determine whether the extranucleolar dots indeed corresponded to coiled bodies, indirect double immunofluorescence experiments were performed using the rabbit anti-NAP57 peptide antibodies, and the human autoimmune anti-p80 coilin antibodies. Comparison of the fluorescence pattern of both antibodies within the same nucleus clearly showed that the extranucleolar dots of NAP57 (Fig. 3 B') corresponded to the coiled bodies stained by the anti-p80 coilin antibodies (Fig. 3 B). In fact, when the two images were electronically merged the coiled bodies and the extranucleolar NAP57 dots matched perfectly (not shown). Similar extranucleolar dots had previously been observed with anti-Nopp140 peptide antibodies (Meier and Blobel, 1992). Indeed, analogous double immunofluorescence experiments localized Nopp140 to coiled bodies as well (Fig. 3, compare C with C').

To localize NAP57 at the ultrastructural level, immunoelectron microscopy experiments were performed on cryosections (Fig. 4 a) and thin sections of L. R. White embedded BRL cells (Fig. 4 b). The affinity purified anti-NAP57 peptide antibodies were detected with secondary antibodies coated with 10-nm gold particles. NAP57 was mostly concentrated in the dense fibrillar component of the nucleolus surrounding the fibrillar centers which were devoid of gold particles (Fig. 4 a). Occasional gold particles were also observed in the granular component. The nucleoplasm was labeled irregularly (Fig. 4, a and b). On some occasions, the

gold particles were seen in a curvilinear track extending from the nucleolus to the nuclear envelope (Fig. 4 b, arrowheads) reminiscent of tracks previously detected with anti-Nopp140 antibodies (Meier and Blobel, 1992). As in the case of Nopp140, the NAP57 tracks detected within the same plane as the thin sections were too few to allow statistical analysis.

cDNA Deduced Primary Structure of NAP57

The NAP57 amino acid sequences obtained from peptide sequencing were used to design degenerate oligonucleotides which served in a polymerase chain reaction with rat cDNA as template to produce a NAP57 specific DNA probe. Screening of rat cDNA libraries with this probe led to the identification of multiple, overlapping clones. One of these, pTM575, contained an insert of 1801 nucleotides (Fig. 5).

Nucleotide sequencing of pTM575 showed it to be unusually GC-rich (65%) and revealed a single open reading frame encoding a protein of 466 amino acids with a calculated molecular mass of 52,070 and an isoelectric point of 9.38 (Fig. 5). It is likely that the ATG in position 91-93 rather than 113-115 was the initiation codon because its surrounding sequence conformed to the translation initiation consensus sequence (Kozak, 1991), and because it was the first ATG in frame 30 nucleotides downstream of an in frame STOP codon. The cDNA deduced amino acid sequence agreed with the sequences determined for three NAP57 peptides (Fig. 5, *underlined residues*). The primary structure of NAP57 showed none of the common motifs found in other nucleolar proteins, i.e., RNA recognition motifs (nucleolin, fibrillarin, Ssbl, Nsr1, Nop3/Npl3), glycine and arginine rich domains (nucleolin, fibrillarin, Ssbl, Nsr1, Gar1, Nop3/Npl3), or acidic stretches containing serines (Nopp140, No38, nucleolin, UBF, Nsr1: Lischwe et al., 1985; Lapeyre et al., 1987; Schmidt-Zachmann et al., 1987; Jantzen et al., 1990; Lapeyre et al., 1990; Lee et al., 1991; Bossie et al., 1992; Meier and Blobel, 1992; Russell and Tollervey, 1992). The most remarkable features of the NAP57 amino acid se-

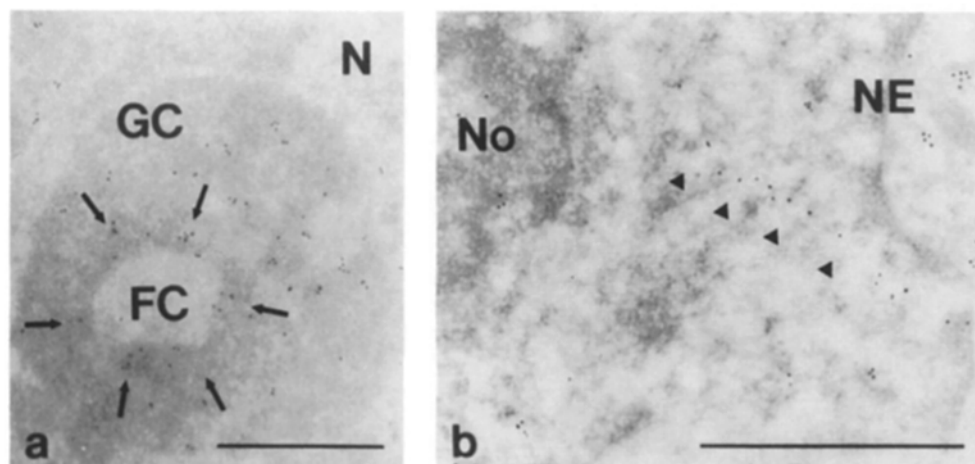


Figure 4. Immunoelectron microscopy with affinity purified anti-NAP57 peptide antibodies on BRL cells. (a) Cryosection incubated with anti-NAP57 antibodies and secondary antibodies coupled to 10-nm gold particles showing a nucleolus. Note the strong labeling of the dense fibrillar component (arrows) surrounding the fibrillar centers (FC) which were devoid of gold particles. The granular component (GC) of the nucleolus surrounded by nucleoplasm (N) exhibited only occasional staining. Note that

only one of two FCs and only parts of the total DFC are pointed out. (b) Immunogold labeling of NAP57 on a thin section of L. R. White resin embedded cells. Note the labeling in a linear array in the nucleoplasm (arrowheads) between the nucleolus (No) and the nuclear envelope (NE) and the granular staining in the cytoplasm. Bars, 1 μ m.

quence were two highly charged stretches near the amino and carboxy termini (Fig. 5, shaded gray) containing two minimal NLS consensus sequences (Fig. 5, open black boxes, Chelsky et al., 1989) and two strong consensus sites for casein kinase II phosphorylation (Fig. 5, S*, Marin et al., 1986; Kuenzel et al., 1987). It is noteworthy that both charged stretches were immediately followed by the sequence Pro-Leu-Pro. In addition, a hydrophobic amino acid repeat motif was detected between residues 286 and 370. Thus, with one exception, every seventh residue consisted of a hydrophobic amino acid (Fig. 5, circled residues) that was often flanked by an additional hydrophobic and two charged residues.

Northern blot analysis on poly(A)⁺ RNA isolated from BRL cells using the NAP57 cDNA as probe revealed a single

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1  GTCGCCGGCGCGCGTGGTGCACCGTGGCGTGCAGCGTTCGGCTCGCGCGCGCGGTGAG 10
                                     M A D A E A A M T F 10
61  CGCGGGTGGCGGGTGTCTGCCTGCACATGGCGGACGGGAAGCCGCATGACGTTTC 10
P [K K H K K K K E] R [K P L P E A D V A E] 30
121 CCGAAGAAGCAAGAGAGAAGAGAGCGGAAGCGCTCCGGAAGCTGACGTGGCTGAG 30
I Q H A E D F L I K P E S K A A Q L D T 50
181 ATCCAGCAGCGGGAAGATTTCTGATCAAAACCGGAGTCCAAAGCGCGCTCACTGGACCC 50
S Q W P L L L L K N F D R L N V R T T H Y 70
241 TCCAGTAGGCCCCCTGCTGCTCAAGAACTTTGACAGACTGAACCTGGCGAGCCACCTAC 70
T P I P C G S N P L K R E I G E Y V R T 90
301 ACGCCCATCCCTGGCGCTCAACCCGCTGAAGAGGAGATCGGGGAGTACCTCAGGACC 90
G F I N L D K P S N A W I 110
361 GGCPTCATCACTGGCAAGCACTCAACCCCTCTTCGCAAGGTCGTGGCTGGATC 110
R I L R V E K T G H S G T L D P K V T 130
421 CGCCGATCCTGGCGTGGGAAGACGGGGACAGCGGGACACTTGACCCCAAGGTCACC 130
G C L I V C I E R A T R L V K S Q Q S A 150
481 GGGTGCCTCATCGTCTGCTGAGCGCCGCGCGCTGGTCAAGTCAACAGCAGAGCGGG 150
G K E Y V G V V R L H N A I E G T A Q L 170
541 GGCAAGAGTATGTCGGGGTCTGCGGCTGCACAACTGCTATCGAGGGACCCCGCAGCTC 170
S R A L E T L T G A L F Q R P P L I A A 190
601 TCCAGGCCCCCTGGAGACCTGACAGGCGCCCTGTTCACGCTCCCGCTCATCGAGCA 190
V K R Q L R V R T I Y E S R V V E Y D P 210
661 GTGAAGCGGACAGTGGCGTGGCCCAATCTATGAGAGCGGTGTGTGAGATGACCT 210
E R R L G V F W V S C E A G T Y I R T L 230
721 GAGCGCAGGCTGGGTGTGTCTGGTGGCTGCGAGGCTGGCACCTACATCCGAGCGCTG 230
C V H L G L L L G V G G Q M Q E L R R V 250
781 TCGGTGCACTGGGCTGCTGCTGGCGTTCGGGGCGAGATGACAGGAGCTGGCGCGCTG 250
R S G V V G E R D H M V T M H D V L D A 270
841 CGCTCGGGGCTGTCGGGAGCGGGACACATGTTGACCATGACAGCAGCTCCTGGACGG 270
Q Y L Y D H H R D E S Y L R R V V F P L 290
901 CAGTATCTATACGACCCACCGGGAGCGAGCTACCTCCGCGCGTGGTTCCTCACTG 290
E K L T S H K R V M K D S A V N A I 310
961 GAGAGCTCTGACGCTGCACAAAGCCCTGCTGATGAGGACAGTCCGCTCAGCCGATC 310
C Y G K I M L P G L R Y E D G E V 330
1021 TGCTACGCGCGGAGATCATGCTCCCGGCTCTCGGCTACGAGGACCGCATCGAGGTC 330
N Q E V V I T T K G A V C V A I A L 350
1081 AACAGGAGGCTGCTGCTATCACCACCAAGGGCGAGCGGCTGCTGCTGGCCATCGCAGT 350
M T T A V S T C D H G V A K I R V 370
1141 ATGACGACGGCTGTGATCTCCAGTGCAGCCAGGGGTAGTAGGCAAGATCAAGCGCTC 370
I M E R D T Y P R K W G L G P K A S Q K 390
1201 ATCATGGAGCGGACACCTACCCCGCAAGTGGGGCTGGGGCGGAGGCAAGCGAGAG 390
K Q L I K Q G L L D K H G R P T D G T P 410
1261 AAGCAGCTGATCAAGCAGGGCTCTCGGACAAACATGGCCGACCCAGCAGGACCCCG 410
A S W T R D Y V D Y S D S S K K A T A A 430
1321 GCCTCTGGACGCGGACTACGTGACTACAGTACTCCAGCAAGAAGCGGACCGCGCC 430
E A T P G P G V T A D A A S I V K R K R 450
1381 GAAGCCACCGCGCCCTGGGTCACCGCGGACCGCCCGCAGCATCGTGAAGGAAACGC 450
D S D S D A D E R R P L P R P G 466
1441 GATAGCGACAGCGACGCGGACGAGCGACGCCACTACCACGCGCGGGTGAAGAAGGAGA 466
1501 AGAAGAAGAAAAGGAGAAAGCGGACCGCGGGAGGAGGAGGAGGAGGAGGAGGAGGAG 486
1561 ACGCCACAAGGAAGAAAAGAAAGAAAGGCAAGGGCGGAGAGGAGGAGGAGGAGGAGG 506
1621 GACTACCCCGGACCCCGGACGACCACTCCGCTCTGTTTAAAGGACGCGAGGCGTATG 526
1681 GAACGGGCTTCCACCACTGACCCCTCCACTCTGAGCCCCGACGACCGCCCGCGCG 546
1741 GATTCGCGGAGGCGATGTTAGCGACACCGCAGACGCGCCCTGGCGCTGCCCTCCATTT 566
1801 TT

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Figure 5. Nucleotide and predicted amino acid sequence of pTM575. The nucleotide derived amino acid sequence of NAP57 is shown in single letter code above with the residues underlined corresponding to the sequenced peptides. Two minimal NLS consensus sequences are pointed out by open black boxes while two highly charged stretches of amino acids are highlighted by gray shading. S* denotes two strong acceptor sites for casein kinase II phosphorylation. Circled amino acids form a hydrophobic repeat motif at every seventh position with one exception (circled by a broken line). Nucleotides are numbered on the left and amino acids on the right. These sequence data are available from EMBL/GenBank/DBJ under accession number Z34922.

hybridizing species of mRNA with an approximate size of 1,800 nucleotides corresponding well to that of the isolated cDNA of NAP57 (Fig. 6 A). In addition, in vitro transcription and translation of NAP57 mRNA in a reticulocyte lysate yielded a single protein migrating on SDS-PAGE like authentic NAP57 (Fig. 6 B). In summary, these data indicated that the correct cDNA containing the full-length open reading frame of NAP57 was cloned and sequenced.

NAP57 Is a Highly Conserved Protein

To determine whether NAP57 or homologous proteins had previously been identified, the NAP57 protein and nucleotide sequence was used to search GenBank. The search revealed related bacterial and yeast proteins. Regions of homology between these proteins and NAP57 are depicted schematically in Fig. 7 A. The essential *S. cerevisiae* CBF5p (Jiang et al., 1993b) was 71% identical and 85% homologous to the rat NAP57 while the corresponding numbers for the *E. coli* p35 (Sands et al., 1988) were 34 and 56%, and for the *B. subtilis* Bsp35 (Shazand et al., 1993) were 48 and 65%. In fact, the two bacterial proteins were only slightly

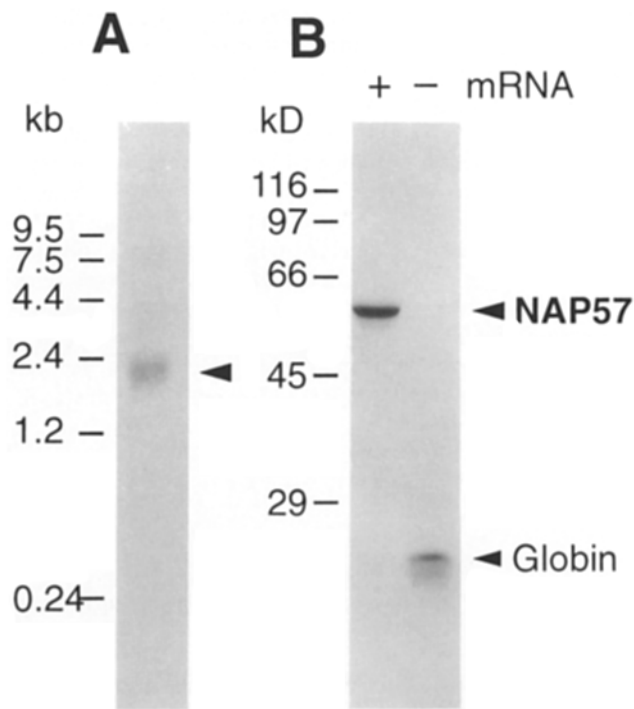


Figure 6. Characterization of the NAP57 cDNA by Northern blot (A) and in vitro transcription/translation analysis. (A) Northern blot of BRL cell poly(A)⁺ RNA hybridized with a ³²P-labeled NAP57 DNA probe corresponding to the first 1337 nucleotides of pTM575 and detected by autoradiography. Mobility of RNA size markers in kb is indicated by bars on the left and the only hybridizing band is pointed out by an arrowhead on the right. (B) Fluorograph of in vitro translated NAP57 analyzed by SDS-PAGE. pTM575 was in vitro transcribed and the RNA translated in rabbit reticulocyte lysate in the presence of [³⁵S]methionine (+ lane). In the right lane (- lane), translation products were analyzed in the absence of exogenous RNA. The NAP57 RNA translation product is indicated by an arrowhead as is globin, originating from the translation of residual globin mRNA in the reticulocyte lysate.

(~3%) more homologous and identical to each other than to NAP57. While the yeast CBF5p amino acid sequence contained a highly charged carboxy terminus beyond the region of homology with NAP57, NAP57 showed two short-charged stretches at both termini. Fig. 7 B shows the amino acid alignment of the homologous regions between the four proteins with the identically conserved residues between NAP57 and the other proteins highlighted by gray boxes. Interestingly, the hydrophobic amino acid repeat motif mentioned above was conserved between NAP57 and CBF5p but not between the pro- and eukaryotic homologs (Fig. 7 B, *black squares*). In addition, GenBank homology searches revealed that the partial cDNA of a human NAP57 homolog was identified as an expressed sequence tag among randomly sequenced human pancreatic islet cDNAs (Takeda et al., 1993). Thus the translation product of the human cDNA hbc1064 comprising 242 nucleotides showed 93% identity and 98% similarity to the corresponding NAP57 amino acid sequence (not shown).

Discussion

We have identified a novel mammalian nucleolar protein, NAP57, that is associated with the previously identified nucleolar protein Nopp140. NAP57 appears to be a highly conserved protein with putative homologs, albeit of unknown function, in yeast and even prokaryotes. This suggests that NAP57 and its putative homologs perform a highly conserved function in both pro- and eukaryotic cells. As the eukaryotic nucleolus is the site of ribosomal subunit assembly,

NAP57's nucleolar localization and its physical association with the nucleolo-cytoplasmic shuttler Nopp140 suggests that such a highly conserved function might be the chaperoning of newly synthesized ribosomal proteins or of ribosomal subunit assembly intermediates in both pro- and eukaryotic cells.

Since Nopp140 is one of the most highly phosphorylated proteins in the cell (Meier and Blobel, 1992), it is important to review the conditions under which NAP57 was complexed with Nopp140. The physical association of NAP57 with Nopp140 was detected in a nuclear extract of low salt (25 mM Tris-HCl) and alkaline pH (8.1). These alkaline/low salt conditions reportedly disintegrate nucleoli (Kay et al., 1972) and extract most of Nopp140 (Meier and Blobel, 1990). The NAP57/Nopp140 complex also survived the subsequent immunoprecipitation conditions of physiological salt (150 mM NaCl), alkaline pH (50 mM Tris-HCl, pH 8.1), and SDS in mixed micelles (0.2% SDS/1% Triton X-100). Furthermore, heating to 50°C of nuclear extracts in the presence of 0.4% SDS was not sufficient to dissociate the two proteins but required 90°C (not shown). This collective evidence demonstrates a strong interaction between these two proteins. In addition, NAP57 coprecipitated with Nopp140 in roughly equimolar amounts as judged by comparison of their Coomassie blue (not shown) and silver stain intensities on SDS-PAGE (Fig. 1, lane 5). Finally, the ratio of the two proteins did not vary when increasing amounts of Nopp140 were precipitated (not shown) indicating a stoichiometric association of NAP57 with Nopp140.

While the exclusive precipitation of only two proteins

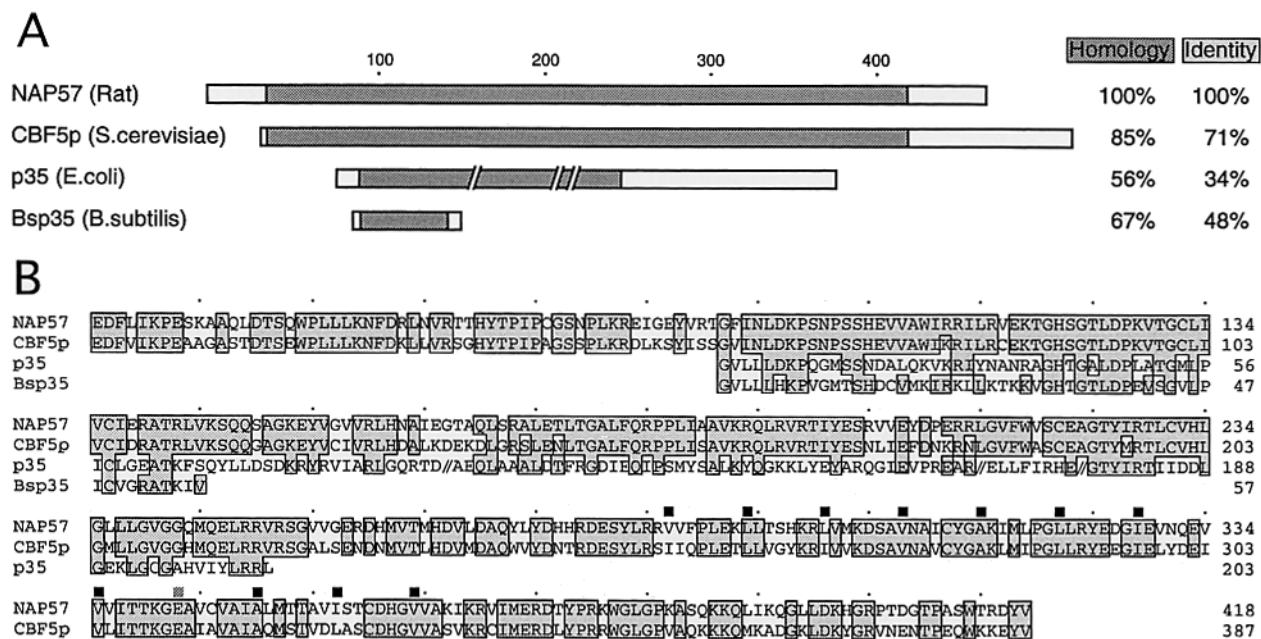


Figure 7. NAP57 is an evolutionary highly conserved protein. (A) Schematic alignment of the open reading frames corresponding to rat NAP57, yeast CBF5p, *E. coli* p35, and *B. subtilis* Bsp35 drawn to scale with regions of homology highlighted by dark gray. Percent homology (including conserved amino acid changes) and identity to NAP57 across the highlighted regions are listed on the right. Amino acid residue numbers of NAP57 are marked on top. The interruptions (//) in the p35 sequence stand for insertions of 17, 5, and 12 residues from left to right, respectively. (B) Alignment of the regions of homology in single letter code with residues identical to NAP57 boxed in light gray. The conserved hydrophobic residues forming a seven amino acid repeat motif with one exception (*gray square*) are indicated by black squares. Insertions (//) in p35 are as in A.

(Fig. 1, lane 5) from a complex mixture of polypeptides (Fig. 1, lane 2) provides good evidence for a specific protein-protein interaction, we cannot exclude the possibility that the observed NAP57/Noppl40 complex is an *in vitro* artifact resulting from the nuclear extraction and immunoprecipitation conditions. However, the observed immunocytochemical colocalization of Noppl40 and NAP57 argues strongly against such an artifact and for a physiologically meaningful complex. Like Noppl40, NAP57 is absent from the fibrillar centers (FC) of the nucleolus and is occasionally found in the granular component (GC) but localizes primarily to the dense fibrillar component (DFC). These three ultrastructurally distinct regions of the nucleolus are thought to represent distinct, if overlapping regions of vectorial ribosome formation. Thus the central FCs serve largely as the seat of ribosomal genes and together with the neighboring DFCs as the site of transcription while the DFCs and the further peripheral GCs are involved in co- and posttranscriptional ribosomal RNA processing and preribosome assembly (for review see, e.g., Somerville, 1986; Warner, 1990; Scheer et al., 1993). Therefore, the subnucleolar localization of NAP57 suggests that it functions in ribosomal subunit assembly and/or possibly ribosomal RNA processing. Moreover, its association with Noppl40 together with its nucleoplasmic localization, occasionally in the form of curvilinear tracks similar to those observed for Noppl40, may indicate that NAP57, like Noppl40, shuttles between the nucleolus and the cytoplasm. Thus like Noppl40, NAP57 may be involved in nucleolo-cytoplasmic transport. A dual role in ribosomal subunit assembly as well as in nucleolo-cytoplasmic transport would be consistent with a chaperoning function of NAP57 either in the import of ribosomal proteins into the nucleolus and/or in the export of nucleolar preribosomal subunits.

In this context it should be mentioned that the tools presently available did not allow us to address directly whether NAP57 indeed shuttles. For instance, labeling of anti-NAP57 antibodies with fluorescent markers led invariably to inactivation of the antibodies rendering a duplication of the experiments used to demonstrate Noppl40 shuttling (Meier and Blobel, 1992) impossible (not shown). In addition, the cytoplasmic reactivity of the anti-NAP57 antibodies (Fig. 3, *A* and *B*) would interfere with the interpretation of any shuttling experiments involving immunodetection. However, we are presently raising additional antibodies to bacterially expressed NAP57 and other synthetic peptides to resolve the interesting question of NAP57 shuttling.

Noppl40 and NAP57 also colocalized to coiled bodies which were discovered as nucleolar accessory bodies (Ramon y Cajal, 1903; Hardin et al., 1969; Monneron and Bernhard, 1969) and are thought to be related to the nucleolus (Raska et al., 1990). The definition of the coiled body as a biochemically distinct entity was made possible only recently by the identification of the coiled body marker p80 coilin, an autoimmune antigen exclusively located in the coiled bodies (Raska et al., 1990; Andrade et al., 1991; Raska et al., 1991). Noppl40 and NAP57 are the only two nucleolar proteins that have so far been found in the coiled bodies aside from fibrillarin (Raska et al., 1990, 1991; and topoisomerase I (Raska et al., 1991). Like fibrillarin, Noppl40 and NAP57 are located within the dense fibrillar component of the nucleolus suggesting thus a link between

this substructure of the nucleolus and the coiled body. Such a connection is in fact supported by recent reports of a direct physical association of coiled bodies with the dense fibrillar component of the nucleolus under certain conditions (Malatesta et al., 1994; Ochs et al., 1994). Noppl40 and possibly also NAP57 are the only shuttling proteins present in the coiled bodies which are devoid of the other nucleolar shuttling proteins, NO38 and nucleolin, or of pre-mRNA and associated shuttling proteins (Borer et al., 1989; Raska et al., 1991; Pinol-Roma and Dreyfuss, 1992). The coiled bodies appear to mainly consist of small nuclear RNPs involved in pre-mRNA splicing (Carmo-Fonseca et al., 1992; Spector et al., 1992) and of fibrillarin involved in ribosomal RNA processing (Kass et al., 1990; Raska et al., 1990, 1991). Thus they contain a high local concentration of RNPs which is not unlike the nucleolus harboring ribosomal assembly intermediates. Such an accumulation of highly charged RNPs may in general require the presence of chaperones like Noppl40 and NAP57 to allow them to concentrate in and/or leave such a RNP-containing structure.

The cDNA derived primary structure of NAP57 did not yield clues as to its function. It exhibited only two noteworthy features: First, it contained two putative NLSs in a charged context, one near the amino and the other near the carboxy terminus with the latter being close to two casein kinase II phosphorylation consensus sites. Second, the carboxy terminal half revealed a hydrophobic amino acid repeat motif (Figs. 5 and 7 *B*) that when folded into a possible α helix aligned 13 hydrophobic residues found in every seventh position with one exception. Searches in the data banks, however, revealed the existence of putative homologs in yeast, and, most strikingly, also in prokaryotes (*E. coli* and *B. subtilis*, see Fig. 7). The putative yeast homolog of NAP57 is the product of the essential *CBF5* gene (Jiang et al., 1993*b*). *CBF5p* is 71% identical to NAP57 across 384 amino acid residues. It was identified as a low affinity centromere-binding protein by chromatography on yeast centromere DNA (Jiang et al., 1993*b*). There is also genetic evidence for either direct or indirect interaction of *CBF5p* with *CBF3*, the multisubunit protein complex that binds with high affinity to the centromere (Lechner and Carbon, 1991). Thus, overexpression of *CBF5* can partially suppress a temperature sensitive mutation in *cbf2/ndc10* (Goh and Kilmartin, 1993; Jiang et al., 1993*a*) encoding the 110-kD subunit of *CBF3* (Jiang et al., 1993*b*). Nevertheless, the physiological relevance of *CBF5p* for centromere function is presently not clear. Moreover, *CBF5p* has recently been localized to the yeast nucleolus (Jiang, W., and John Carbon, personal communication) consistent with the localization in mammalian cells.

Most remarkable is the homology of NAP57 with proteins of prokaryotes namely p35 of *E. coli* and Bsp35 of *B. subtilis* (see Fig. 7). The amino-terminal halves of these two proteins are nearly 50% identical to that of NAP57, a degree of homology that equals or exceeds that between most ribosomal protein homologs of pro- and eukaryotes (Wittmann-Liebold et al., 1990; Arndt et al., 1991). The function of p35 and Bsp35 is not known. However, an important clue comes from the location of the genes encoding these proteins in the highly conserved *nusA-infB* operon of the two organisms (Sands et al., 1988; Shazand et al., 1993). This operon contains genes coding for some transcription and translation fac-

tors. Moreover, in *E. coli*, the *p35* gene is cotranscribed to some extent with the immediately 3'-adjacent *rpsO* gene coding for the ribosomal protein S15 (Sands et al., 1988). Taken together, these data are consistent with a putative chaperoning function of p35 (or Bsp35) for newly synthesized ribosomal proteins and/or for ribosomal subunit assembly in the bacterial cytosol in analogy to related chaperoning function of NAP57 in eukaryotes.

We are grateful to Helen Shio from the Rockefeller University EM facility for performing the immunoelectron microscopy, to Jun Sukegawa for his gift of unamplified cDNA library, to Robert Ochs for his gift of anti-p80 coilin antisera, to the members of the Rockefeller University biopolymer facility for peptide sequencing and synthesis and for oligonucleotide synthesis, and to the Albert Einstein College of Medicine image analysis facility for the use of the confocal microscope. We appreciate the personal communication on the immunolocalization of CBF5p by Weidong Jiang and John Carbon. We thank Susan Smith for advice and critical reading of the manuscript.

Received for publication 22 June 1994 and in revised form 1 September 1994.

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