Comparison of the Rat Nucleolar Protein Nopp140 with Its Yeast Homolog SRP40

DIFFERENTIAL PHOSPHORYLATION IN VERTEBRATES AND YEAST*

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Rat Nopp140, a nonribosomal protein of the nucleolus and coiled bodies, was characterized as one of the most highly phosphorylated proteins in the cell. Based on its subcellular location, its nuclear localization signal binding capacity, and its shuttling between the nucleolus and the cytoplasm, Nopp140 was proposed to function as a chaperone in ribosome biogenesis. This study shows that casein kinase II phosphorylates Nopp140 to its unusual high degree and identifies the yeast SRP40 gene product as immunologically and structurally related to rat Nopp140. SRP40 encodes an acidic (pI = 3.9), serine-rich (49%) protein of 41 kDa whose carboxyl terminus exhibits 59% sequence identity to that of Nopp140. SRP40 localizes to the yeast nucleolus and is required at a specific cellular concentration for optimal growth as indicated by the negative effect on cell growth of both overexpression and deletion of its gene. Like Nopp140, SRP40 is phosphorylated by casein kinase II, but to a much lesser extent. While the parallels between these two proteins suggest that SRP40 is the bona fide yeast Nopp140 homolog, their disparities reflect the differences in nucleolar dynamics and regulation of ribosome biogenesis between yeast and vertebrates.

The nucleolus is the site of ribosomal RNA transcription and processing and of assembly of preribosomal particles. The latter requires import from the cytoplasm of ribosomal proteins and subsequent export of the preassembled ribosomes. While functional bacterial ribosomes can be assembled under nonphysiological conditions in vitro (1), in vivo, cellular factors or chaperones are required for ribosome biogenesis. Several nonribosomal proteins within the nucleolus have been identified that are candidates for such chaperones (for a recent review see Ref. 2).

Nonribosomal nucleolar proteins were identified over 20 years ago (3–5) and, like ribosomal proteins, are often evolutionarily conserved. This was illustrated recently by our identification of a rat liver nucleolar protein, NAP57, that exhibits sequence similarity to yeast (71% sequence identity with yeast CBF5; Ref. 6) and prokaryotic proteins (7). Indeed, most vertebrate nucleolar proteins contain a variety of characteristic domains, such as RNA recognition motifs and glycine/arginine-rich repeats, both for association with RNA, and acidic stretches apparently for interaction with basic ribosomal proteins. The most unusual of the nucleolar proteins with an acidic stretch is Nopp140 (22). Its central domain harbors 10 repeats consisting of runs of 13–17 consecutive serines, aspartic, and glutamic acid residues separated by exclusively basic stretches of 23–46 residues rich in lysine, alanine, and proline. We originally identified Nopp140 as a nuclear localization signal binding protein in the nucleolus of rat liver (23) and subsequently also localized it to coiled bodies (7). It is one of the most highly phosphorylated proteins in the cell with up to 82 mol of phosphate/molecule (22). The phosphorylation apparently occurs in the acidic serine repeats and leads to the dramatic drop from the theoretical isoelectric point of 10.3 to 4.1. While located mostly in the nucleolus, Nopp140 constantly shuttles between the nucleolus and the cytoplasm on nucleoplasmic tracks (22). In addition, Nopp140 exists in a stoichiometric complex with the highly conserved protein NAP57 (see above; Ref. 7). We have proposed Nopp140, based on its nucleolar location, shuttling, nuclear localization signal binding, and acidic serine repeats, to function as a chaperone in ribosome assembly and/or nuclear transport of nucleolar components. Yet many questions regarding its function and mode of action remain. How does phosphorylation affect shuttling, and what is the kinase responsible for the enormous degree of phosphorylation? Is Nopp140 as highly conserved as its associated protein, NAP57, and can that be exploited to further define its function?

This study shows that casein kinase II (CKII)1 phosphorylates Nopp140 to its unusual high degree and that this phosphorylation also occurs in yeast cells. Antibody cross-reactivity reveals the yeast SRP40 gene product as immunologically related to rat Nopp140, and sequence comparison shows the two proteins to be structurally similar, with the carboxyl terminus exhibiting 59% sequence identity. While the SRP40 gene has been identified in several genetic screens (24–26), its product, SRP40, remained uncharacterized. SRP40 is an acidic (pI = 5.9) transferase; HA, hemagglutinin; SC, synthetic complete; MOPS, 4-morpholinopropanesulfonic acid; PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction.

1 The abbreviations used are: CKII, casein kinase II; BRL, buffalo rat liver; GST, glutathione S-transferase; HA, hemagglutinin; SC, synthetic complete; MOPS, 4-morpholinopropanesulfonic acid; PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction.

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Characterization of SRP40, a Novel Yeast Nucleolar Protein

Yeast Strains and Cell Growth

All strains used in this study were derived from the diploid W303 (MATa/MATa ade2–1 ade2–1 ura3–1 ura3–1 his3–11,15 trp1–1 trp1–1 leu2–3,112/leu2–3,112 can1–100 can1–100; kind gift from Amy Chang) and DFS (MATa/MATa ura3–52/ura3–52 his3–1200/his3–1200 trp1–1 trp1–1 leu2–3,112/leu2–3,112 lys2–801/lys2–801; kind gift from John Aitchison). TMX14 corresponds to W303 (URA3::URA3) and, analogously, TMX15 corresponds to DFS (Ura3::URA3). F1104 is the haploid Mata strain isogenic with W303, while TMY20 corresponds to F1104 with the SRP40 gene replaced by the URA3 gene (srp40::URA3). The following strains were generated by transformation with the appropriate plasmids (in parentheses) using the lithium acetate method (34) and selection on synthetic complete (SC) medium containing 2% glucose but lacking the corresponding marker (see respective plasmids): TMX12 = W303 (ptm25), TMX13 = W303 (pRS315G), TMX19 = W303 (pYE52), TMX23 = W303 (ptm36), TMX33 = W303 (ptm41), and TMY28 = F1104 (ptm41).

Expression of the genes encoded by the plasmids was induced from the GAL promoter by either growing the cells overnight in the presence of 2% galactose or by switching from 2% raffinose to 2% galactose containing SC medium lacking the corresponding markers for 90 min followed by suppression of the expression in rich medium (YPD; 1% yeast extract, 2% bactopeptone, 2% dextrose) as described under “Results.” No difference in localization of the induced gene products was observed between the two methods.

The strains deleted in one copy of the SRP40 gene, TMX14 and TMX15, were created by homologous recombination through transformation of W303 and DFS, respectively, with the linearized ptm33 construct and selection for growth on SC medium lacking the uracil marker. The haploid strain TMY20 (Mata srp40::URA3) was selected from the segregants that resulted from sporulation and subsequent tetrad dissection of TMX14 and that grew when replica-plated on SC medium lacking uracil (see Fig. 7, A–D). Proper genomic integration of the URA3 gene and consequent deletion of the SRP40 gene was confirmed in all strains by the production of the expected size products upon PCR amplification when using the corresponding genomic DNA as template combined with the primers, one outside the pTM33 construct and one each within the URA3 and the SRP40 gene (not shown).

Growth rates of F1104 and TMY20 were determined in liquid SC medium and, in the case of TMY28, in SC medium lacking leucine by diluting a freshly grown late stationary phase culture about 100-fold in fresh medium. Growth at 30°C was observed by measuring the optical density at 600 nm. Yeast cell culture, genomic DNA preparation, total yeast extracts for SDS-PAGE, and general manipulations were performed essentially as described (36).

Production and Purification of Recombinant Proteins

E. coli BL21(DE3) cells (28) transformed with pET8c/Nopp140, ptm25, or ptm50 were grown in L-broth containing 100 μg/ml ampicillin until A600 = 0.8 and expression of Nopp140 and the GST-SRP40 fusion proteins was induced by the addition of 1 mM isopropyl-1-thio-
β-D-galactopyranoside and continued for 2 h at 37°C. At this point, the cells were either lysed directly in SDS sample buffer for analysis of whole cell extracts or further processed for purification of the recombinant proteins.

Overexpressed Nopp140 was predominantly insoluble and segregated into inclusion bodies, which were isolated and washed as described (37). The inclusion bodies were solubilized in 6 M urea followed by denaturing dilution (10-fold final) into 10 mM potassium phosphate buffer (pH 8.1) at room temperature. After stirring for an additional 30 min, the solubilized Nopp140 solution was cleared at 27,000 × g for 30 min and loaded onto a hydroxyapatite (Bio-Gel HTP; Bio-Rad) column equilibrated with 10 mM potassium phosphate buffer (pH 8.1) at room temperature. The column was washed consecutively with equilibration buffer (pH 8.1), 1 M NaCl, and 200 mM NaCl, and the homogeneous recombinant Nopp140 eluted with 300 mM and 400 mM phosphate buffer. Recombinant Nopp140 was subsequently dialyzed against phosphate-buffered saline and concentrated in Centricon 50 concentrators (Amicon, Inc., Beverly, MA).

About 20% of overexpressed GST-SRP40 was soluble in the 10 mM Tris/HCl, pH 8.0, 1 mM EDTA supernatant, after the bacteria were lysed first by incubation with 10 μg/ml lysozyme for 30 min at room temperature, second by one freeze-thaw cycle, third by the addition of 20 mM magnesium chloride, 5 μg/ml DNase I (Sigma) (a protease inhibitor mixture previously used in the purification of Nopp140 (23)) and incubation at 37°C for 15 min, and fourth by tip sonication on ice after the

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3.9, serine-rich (49%) protein of 41 kDa that is localized to the nucleolus. It is required at a specific cellular concentration for optimal growth as indicated by the negative effect on growth of both overexpression and deletion of its gene. Bacterially expressed SRP40 is phosphorylated by CKII like Nopp140, but to a much lesser extent. These data are discussed in the context of the similarities and differences in the regulation of ribosome biogenesis and nucleolar dynamics between yeast and vertebrates.

EXPERIMENTAL PROCEDURES

DNA Constructs

All DNA manipulations were performed employing standard procedures (27) and as described previously (7, 22). The polymerase chain reaction (PCR) was routinely performed with the hot start technique to avoid false priming, and the newly generated linker sequences of the PCR products were confirmed by DNA sequencing. The SRP40 DNA inserted in the constructs below was isolated by PCR amplification using yeast genomic DNA as template and primers that created the indicated restriction sites.

pTM33 (Nopp140 Bacterial Expression Vector)—The open reading frame of Nopp140 was amplified by PCR with primers, generating an Ncol site at the initiating methionine and a BamHI site 25 nucleotides downstream of the stop codon, using the original λ DNA (pTM17; Ref. 22) as template. The PCR product was cloned into the corresponding sites of the prokaryotic expression vector pET8c (28), and the resulting construct was transferred into BL21 (DE3) cells for expression.

pTM25 (Nopp140 Yeast GAL10, LEU2, CEN6, ARS4 Expression Vector)—The open reading frame of Nopp140 was amplified as described for the pET8c/Nopp140 construct with the exception that the 5′-primer added a BamHI instead of an Ncol site. The product was cloned into the BamHI site of pRS315G (a kind gift from Susan Smith; Ref. 29) such that the complete coding sequence of Nopp140 plus an extra amino-terminal serine and glycine were fused in frame to the second amino acid of the CYC1 gene under control of the GAL10 promoter.

pTM32 (GST-SRP40 Bacterial Expression Vector)—The coding region of the SRP40 gene with a Ncol site at the initiating methionine and a Xhol site in place of the stop codon was cloned into those sites of pGEX-KG (a kind gift from Gang Liu; Ref. 30), creating a continuous open reading frame between glutathione S-transferase (GST) and SRP40 with an additional eight codons after which a stop codon was provided by the vector in all three reading frames.

pTM50 (GST-SRP40C-term Bacterial Expression Vector)—The DNA encoding the conserved last 51 amino acids of SRP40 with a 5′-Ncol site (encoding an in frame methionine) and a 3′-Xhol site in place of the stop codon was cloned into the pGEX-KG vector as described for pTM52.

pTM36 (SRP40-HA Yeast GAL1, URA3, 2μ Expression Vector)—The coding region of SRP40 with a 5′ EcoRI site and a 3′ KpnI site was cloned into those sites of pJ D35 (Jürgen Dohmann, Heinrich Heine University, Düsseldorf, Germany; based on pLSG5D, Ref. 31) in frame with a cDNA encoding the hemagglutinin (HA) epitope tag (2) generating pTM35. The SRP40-HA construct was excised from pTM35 with EcoRI and XbaI and cloned into those sites of pYE52 (Invitrogen, San Diego, CA) placing the SRP40-HA construct under the inducible control of the GAL1 promoter.

pTM41 (HA-SRP40 Yeast GAL10, LEU2, CEN6, ARS4 Expression Vector)—The open reading frame of SRP40 was subcloned into pCIP1,5 generating an in frame amino-terminal HA epitope tag. The HA-SRP40 construct was further subcloned into the SpeI and NotI sites of pRS315G (29) in frame with the first five amino acids of the CYC1 gene under the inducible control of the GAL10 promoter.

pTM33 (Construct for SRP40 Gene Deletion)—The SRP40 gene including 1020-nucleotide 5′- and 960-nucleotide 3′-untranslated region with a 5′ SalI and a 3′ XbaI site was cloned into the Xhol and XbaI sites of pBluescript II SK+ (Stratagene, La Jolla, CA) generating pTM32. The URA3 gene, isolated by PCR amplification with pRS316 (33) as template, was inserted in reverse orientation into the Xhol and EcoRI sites of pTM32 while dropping out the entire SRP40 coding region as shown schematically in Fig. 7A. The construct carrying the SRP40 3′- and 5′-flanking region with the URA3 gene inserted was excised from pTM33 using Xbal and KpnI and used directly to transform diploid yeast strains.


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addition of 150 mM sodium chloride and 1% Triton X-100. The bacterial lysate was clarified by centrifugation at 47,000 × g for 10 min at 4°C and GST-SRP40 adsorbed onto glutathione-Sepharose 4B (Pharmacia Biotech Inc.) was packed in a column and equilibrated with phosphate-buffered saline containing 1% Triton X-100. The column was washed consecutively with equilibration buffer; 10 mM Tris (pH 8.0), 1 mM sodium chloride, and 50 mM Tris (pH 8.0); and purified recombinant GST-SRP40 was eluted with 10 mM glutathione in 50 mM Tris (pH 8.0). GST-SRP40 was concentrated and free glutathione was removed by two cycles of concentration and dilution into 10 mM Tris, pH 8.0, in Centri-con-50 concentrators (Amicon).

**Immunofluorescence**

Indirect immunofluorescence on buffalo rat liver (BRL) cells was done essentially as described (23) using the antirecombinant Nopp140 antiserum at a dilution of 1:1000, affinity-purified anti-Nopp140 peptide IgGs (22) at 0.2 μg/ml, anti-pp135 antisera (kindly provided by Alfred Anderer; Ref. 38) at 1:500, and 12CA5 anti-HA ascites fluid (kindly provided by Jonathan Bacskay and directed against the HA peptide YYDPCDYDA; Ref. 32) at 1:5000.

**Phosphorylation**

Recombinant Nopp140 and GST-SRP40, 0.5 μg each, were incubated for 1 h at 37°C in the presence or absence of 16 ng of purified sea star CKII (Upstate Biotechnology Inc., Lake Placid, NY) in 50 mM MOPS buffer (pH 7.0) containing 50 mM sodium chloride, 5 mM magnesium chloride, 5 mM EGTA, and 1 mM each ATP and GTP (10 μl total volume). The reaction was stopped by the addition of 10 μl of double-concentrated sample buffer and analyzed by SDS-PAGE and Coomassie Blue or silver stain as described (7). When [γ-32P]-ATP (10 μCi/sample, Amersham Corp.) was used in phosphorylation assays, only half the amounts of substrates and CKII were added, and the dried SDS-PAGE gels were exposed for autoradiography to Kodak XAR film for an average of 1 min.

Protein analyses, such as isoelectric point calculations and sequence alignments, were performed using the GeneWorks software package (IntelliGenetics, Inc., Mountain View, CA). The pK values for phosphoserine were approximated from those of phosphocarboxy esters (41), which resulted in calculated isoelectric points close to those experimentally determined, i.e., pI for Xenopus Nopp140 (42) = 4.0 (theoretical) and 4.2 (experimental; Ref. 43). GenBank™ searches were performed by the BLAST algorithm (44).

**RESULTS**

Nopp140 is an Extreme Substrate for Casein Kinase II—One of the hallmarks of Nopp140 is its high degree of phosphorylation and its mobility shift on SDS-PAGE from M, 140 to M, 100 upon phosphatase treatment (22). Nopp140 contains 49 phosphorylation consensus sites for CKII, and upon their phosphorylation an additional 33 (22). Full phosphorylation of Nopp140, therefore, causes the enormous drop of its theoretical isoelectric point from extremely basic (10.3) to very acidic (4.1). To determine if Nopp140 was a substrate for CKII and if phosphorylation by CKII could account for the anomalous mobility of Nopp140 on SDS-PAGE, Nopp140 was expressed in bacteria and incubated in the absence (−) and presence (+) of purified sea star CKII (Fig. 1). When analyzed by SDS-PAGE, recombinant Nopp140 migrated with a M, of 100 (Fig. 1, A and B, lanes 1). This mobility was slower than predicted from the calculated molecular mass of 73.6 kDa but was identical to the mobility of phosphatase-treated Nopp140 (not shown; Ref. 22). Upon incubation with CKII, Nopp140 was phosphorylated and showed the characteristic mobility shift from M, 100 to M, 140 (Fig. 1, A and B, lanes 2). As observed previously in vivo and in reticulocyte lysates in vitro, phosphorylation of recombinant Nopp140 by purified CKII also proceeded in an all-or-none fashion with only minor intermediate forms of phosphorylated Nopp140 present. Phosphorylation occurred equally well with ATP or GTP as phosphate source (not shown), a hallmark of phosphorylation by CKII. Taken together, these data indicate that phosphorylation alone accounts for the prominent mobility shift of Nopp140. Furthermore, the identical electrophoretic behavior of Nopp140 upon phosphorylation in vivo (22) and upon incubation by purified CKII in vitro, strongly incriminates CKII as the Nopp140 kinase in vivo.

The ability of CKII to phosphorylate Nopp140 to a high degree (Fig. 1; Ref. 22) supported our previous hypothesis that Nopp140 is identical to the mouse nucleolar protein pp135 (38), which was experimentally demonstrated to incorporate 75 phosphate groups per molecule upon incubation with CKII (45). To test this hypothesis directly, anti-pp135 antisera (38) was obtained and used to probe Western blots of recombinant rat Nopp140. These antibodies recognized the bacterially expressed Nopp140 (not shown), thus confirming the identity of the two proteins.

In vitro phosphorylation experiments also revealed that the phosphorylated form stained more strongly with silver than the unphosphorylated one even though there was less of the

![Figure 1. CKII phosphorylates recombinant Nopp140 in vitro.](https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3891887/bin/fig1.jpg)
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Rat Nopp140 expressed in yeast and became phosphorylated when it was induced by growth in the presence of galactose (Fig. 2). The identity of these bands as Nopp140 was confirmed by their reactivity with anti-Nopp140 peptide antibodies and subsequent repression by growth in 2% galactose for 1.5 h (lane 2). The kinase(s) necessary for phosphorylation of Nopp140 were also detected, which were chased, however, into the fully phosphorylated form after Nopp140 expression was suppressed in glucose containing medium (Fig. 2, lanes 3 and 4).

To localize the heterologously expressed Nopp140, indirect immunofluorescence was performed on fixed and permeabilized spheroplasts using the anti-Nopp140 peptide antibodies (Fig. 2B; Ref. 22). Rat Nopp140 localized predominantly to an area corresponding to one-third of the yeast nucleus (Fig. 2B, b), closely apposing the DNA stain (Fig. 2B, b′) best visualized by the superimposition of the two images in false color (Fig. 2B, b′). This pattern was very characteristic of yeast nucleolar staining (see Fig. 6B). No labeling was observed prior to induction with galactose (not shown). Taken together these data demonstrated that yeast contained the kinase(s) necessary to phosphorylate and the cellular machinery to properly localize rat Nopp140 to the nucleolus.

Immunocross-reacting Species in Yeast Nucleoli—Antibodies raised previously against Nopp140 excised from SDS-PAGE gels (23) and against synthetic peptides of Nopp140 (22) did not cross-react with yeast proteins. To identify Nopp140 homologs in other species, a new antiserum was raised against the recombinant rat Nopp140 (see Figs. 1A, lane 1, and 8B, lane 2), which was available in large amounts. On Western blots this antiserum reacted with the recombinant Nopp140 (not shown) and with a single protein band of 140 kDa of rat liver nuclei (Fig. 3A, lane 1) and of whole BRL cell extracts (Fig. 3A, lane 2), demonstrating its specificity for Nopp140. In indirect immunofluorescence experiments on fixed and permeabilized BRL cells, this antiserum exhibited Nopp140-characteristic staining of the nucleolus and the coiled bodies (Fig. 3B; Refs. 7 and 23). When used to probe yeast cells, the anti-rat Nopp140 antiserum reacted with a crescent-shaped structure in yeast nuclei (Fig. 3, C and D). This structure was reminiscent of the yeast nucleolus in closely apposing the DNA stain (Fig. 3C), particularly evident when the two images were superimposed in false colors (Fig. 3C). Indeed, double immunofluorescence with antibodies against the bona fide yeast nucleolar antigen, Nop1 (40), revealed an identical pattern (Fig. 3, D and D′). When the two images were superimposed in false colors, red and green, the resulting yellow-orange color showed a perfect overlap of the two antigens (Fig. 3D′). Therefore, the anti-rat Nopp140 antibodies clearly cross-reacted with a yeast nucleolar antigen, possibly a Nopp140 homolog.

SRP40 Is Structurally and Immunologically Related to Nopp140—Nopp140 consists of three domains, the unique amino and carboxyl termini separated by the signature central domain of acidic serine clusters that alternate with exclusively basic stretches (Fig. 4A; Ref. 22). This overall structure is conserved among the rat (22), human (53–55), and Xenopus (42) Nopp140 homologs, as shown schematically in Fig. 4A. While the central domain is structurally conserved, the amino and carboxyl termini are conserved on an amino acid level, with the last 51 residues being most highly conserved and exhibiting 81% sequence identity between rat and frog (Fig. 4A). GenBank™ searches after deposition of the entire yeast genomic sequence with this conserved carboxyl terminus identified a single homologous yeast gene, SRP40 (24), which corresponds to the open reading frame YKR12 (56), and also expressed sequence tags from nematode (Caenorhabditis elegans) and plant (Arabidopsis thaliana). The alignment of the carboxyl termini of all related proteins, including the translations of the expressed sequence tags, is depicted in Fig. 4B. Analysis of this

Fig. 2. Rat Nopp140 becomes fully phosphorylated (A) and is localized to the nucleolus (B) when expressed in yeast. Yeast cells (TMX12) carrying the rat Nopp140 cDNA under the inducible control of the GAL10 promoter were analyzed by Western blotting (A) and indirect immunofluorescence (B) using anti-Nopp140 peptide antibodies. A, cells were grown in selective medium containing 2% raffinose (lane 1) followed by the induction of Nopp140 expression by switching to 2% galactose for 1.5 h (lane 2) and subsequent repression by growth in YPD for 3 h (lane 3) and 6 h (lane 4). The arrowheads mark the mobility of unphosphorylated and phosphorylated Nopp140. B, after growth for 1.5 h in the presence of 2% galactose, the cells were prepared for indirect immunofluorescence as described under “Experimental Procedures” and probed simultaneously for Nopp140 (Fig. 2A, lane 2), pp135, which was characterized as a 44-kDa protein with the mobility of unphosphorylated proteins, and nucleolin, which was characterized as a 135-kDa protein with the mobility of phosphorylated proteins. The identity of these bands as Nopp140 was confirmed by their mobility shift upon CKII phosphorylation (Fig. 1, lane 2), intermediate forms of phosphorylated Nopp140 could also be detected, which were chased, however, into the fully phosphorylated form after Nopp140 expression was suppressed in glucose containing medium (Fig. 2A, lane 3 and 4).
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SRP40 Is a Nucleolar Protein—Because acidic serine clusters are also present in other yeast nucleolar proteins, such as NSR1 (14), it could not be established whether the yeast nucleolus (Fig. 3) were able to cross-react with the yeast nucleolus (Fig. 3) to be immunologically related, SRP40 (24). The legend for the graphic depiction is as in A. The boxed and shaded residues highlight the conserved carboxyl terminus, and the underlined residues indicate minimal nuclear localization signals.

Fig. 3. Antibodies against the recombinant Nopp140 are specific for Nopp140 in rat cells (A and B) and cross-react with the yeast nucleolus (C and D). A. Western blots of rat liver nuclei (lane 1) and whole BRL cell lysates (lane 2) probed with the anti-Nopp140 antiserum. B, indirect immunofluorescence of the anti-Nopp140 antiserum on fixed and permeabilized BRL cells and the corresponding phase contrast picture (B1). Note the nucleolar and called body (extranucleolar dots) labeling. C, indirect immunofluorescence of the anti-Nopp140 antiserum on yeast spheroplasts probed simultaneously for DNA (C), C', electronically merged image of the anti-Nopp140 (green) and DNA fluorescence (red) in false colors. D, indirect double immunofluorescence with anti-Nopp140 antiserum (D) and monoclonal antibodies against the nucleolar yeast Nop1 (D'). D', electronically merged image of the anti-Nopp140 (green) and anti-Nop1 (red) fluorescence in false colors. Note the perfect overlap indicated by the yellow-orange color. Bars, 5 μm.

conserved Nopp140 tail for protein motifs revealed a consensus site for cAMP-dependent protein kinase that was conserved across all species (Fig. 4B, asterisk), suggesting a common sensory function of the carboxyl terminus for cAMP-mediated signals. SRP40 encodes a serine-rich protein of 41 kDa, SRP40, consisting of 48% serine residues that are mostly clustered in two long acidic stretches (Fig. 4C) containing 52 CKII consen-
sus sites. In addition to these acidic serine stretches, which show structural resemblance to the acidic serine clusters of Nopp140, the carboxyl-terminal 51 amino acids of SRP40 exhibit 59% sequence identity to Nopp140 (Fig. 4C, boxed). Fur-
thermore, SRP40 contains two minimal nuclear localization signal sequences, one SV40 large T antigen type sequence (57) and one bipartite sequence (Fig. 4C, underlined; Ref. 58).

To determine whether the anti-rat Nopp140 antibodies that cross-reacted with the yeast nucleolus (Fig. 3) were able to recognize SRP40, the SRP40 gene was isolated from yeast genomic DNA using PCR and employed to express GST-SRP40 fusion proteins in Escherichia coli. Surprisingly, when full-length SRP40 was fused to GST (GST-SRP40), the 69-kDa fusion protein migrated with a Mr of close to 110 (Fig. 5A, lane 2). This aberrant migration was caused by the SRP40 moiety of the fusion protein, because GST alone (not shown) or GST fused to the conserved carboxyl-terminal 51 amino acids of SRP40 (GST-SRP40C-term) migrated according to their predicted molecular weight (Fig. 5A, lane 3). Therefore, the expected mobility of SRP40 alone would correspond to approximately 80 kDa or twice its actual molecular weight, analogous to the situation with rat Nopp140 (22). This abnormal migration is most likely caused by the long acidic serine stretches in SRP40, resulting in the very acidic theoretical isoelectric point of 3.9, close to that of fully phosphorylated Nopp140.

When the fusion proteins were probed on Western blots with the anti-rat Nopp140 antibodies, only the full-length fusion protein (Fig. 5B, lane 2), but not the conserved carboxyl-terminal 51-amino acid fusion protein (Fig. 5B, lane 3), was recognized by the polyclonal antiserum. This showed the rat Nopp140 and the yeast SRP40 to be immunologically related, apparently, through their acidic serine stretches and not their conserved carboxyl termini.

SRP40 Is a Nucleolar Protein—Because acidic serine clusters are also present in other yeast nucleolar proteins, such as NSR1 (14), it could not be established whether the yeast nucleolar signal observed with the anti-rat Nopp140 antibodies (Fig. 3) was due solely to cross-reactivity with SRP40. To de-

Fig. 4. Homologs of Nopp140. A, schematic alignment of rat Nopp140 (22) and its human (53, 54) and Xenopus (42) homologs drawn to scale. Black boxes indicate acidic serine stretches containing exclusively serines and glutamic and aspartic acid residues; gray boxes show exclusively basic stretches rich in lysine, alanine, and proline residues; striped boxes point out domains of sequence similarity with the numbers reflecting the percentage identity to rat Nopp140. The dots above the alignment indicate the numbers of residues with an increment of 100. B, amino acid sequence alignment of Nopp140 carboxyl termini from different species. Identical residues are boxed, but note, in addition, the many conservative changes. The asterisk indicates a conserved serine residue that constitutes a consensus site for cAMP-dependent protein kinase phosphorylation. The plant and nematode sequences were translated and assembled from expressed sequence tags with the accession numbers Z26471 (A. thaliana) and D36503, D27732, D32928, and D33633 (C. elegans). Note that all the sequences, despite considerable differences in overall length (see numbers, where available, on the left), end within three residues. C, schematic and amino acid sequence representation of the yeast SRP40 (24). The legend for the graphic depiction is as in A. The boxed and shaded residues highlight the conserved carboxyl terminus, and the underlined residues indicate minimal nuclear localization signals.
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Antirecombinant rat Nopp140 antibodies recognize bacterially expressed GST-SRP40 fusion protein on Western blots. Whole bacterial lysates, before (lanes 1) and after (lanes 2 and 3) the induction by isopropyl-1-thio-β-D-galactopyranoside of SRP40 expression as GST-fusion protein, either full-length (GST-SRP40, lanes 1 and 2) or the conserved carboxyl terminus alone (GST-SRP40C-term, lanes 3), were analyzed by Amido Black stain (A) and antibody reactivity (B) after SDS-PAGE and transfer to nitrocellulose. The antirecombinant Nopp140 antiserum also cross-reacted with some bacterial proteins (asterisks), which was not surprising, since the antibodies were raised against bacterially expressed Nopp140.

To verify that the fluorescence pattern observed with the anti-HA antibodies corresponded to the full-length SRP40 and not to a breakdown product, total cell extracts were prepared and analyzed by SDS-PAGE, transferred to nitrocellulose, and visualized with Amido Black staining and by ECL after probing with anti-HA antibodies (Fig. 6C). As done for indirect immunofluorescence, cells transformed with the 2μ expression vector alone (Fig. 6C, lanes 2) or containing the HA-tagged SRP40 (Fig. 6C, lanes 1), were grown overnight in selective medium containing 2% galactose. The HA-tagged SRP40 was detected on Western blots as a single band migrating at about twice its actual molecular weight, like the bacterially expressed protein (see Fig. 5).

DISCUSSION
This study reveals the yeast SRP40 gene product, SRP40, as the structural and immunologically related homolog of the rat nucleolar phosphoprotein Nopp140. Nopp140 together with its partner NAP57 has been proposed to function as a chaperone in the nuclear transport of nucleolar components and/or ribosome assembly (7, 22). Such a chaperone function is also indicated for SRP40 in yeast by the requirement for a specific level of SRP40 expression for optimal cell growth, as evidenced by the slow growth phenotype after both overexpression and deletion of SRP40 (Fig. 7). This could be envisioned as follows. In the

1. The yeast nucleolus is an organelle involved in the assembly of ribosomes.
2. SRP40, a novel yeast nucleolar protein, is essential for ribosome assembly.
3. Overexpression of SRP40 leads to a severe growth defect, while deletion of SRP40 results in a growth phenotype similar to that of the wild type.

These findings support the role of SRP40 as a key player in the nucleolar function of yeast.
absence of SRP40, ribosome biogenesis continues, albeit at a slowed pace, due to partially overlapping functions of other nucleolar proteins with acidic stretches, such as NSR1 (14), NPI46/FPR3 (59, 60), and UBF1 (61). Overexpression of SRP40, however, titrates out certain ribosomal proteins, all of which are required in equimolar amounts for ribosome assembly and consequent cell growth.

SRP40 was previously identified in two separate genetic screens. It was first described as a weak multicopy suppressor of a temperature-sensitive mutation in one of the common subunits of RNA polymerase I and III, AC40 (24), and second as a multicopy suppressor of a thermosensitive mutation in transcription factor IIIC (25). Since SRP40 localizes to the same subcellular compartment as RNA polymerase I, a physical interaction could occur between these two proteins. A direct interaction between SRP40 and RNA polymerase III or transcription factor IIIC, however, is more difficult to explain. One possible explanation for both phenotypes is that the dramatically reduced growth rate caused by overexpression (Fig. 7F; Ref. 26) allows the mutant RNA polymerases I and III and transcription factor IIIC to keep up with their functions well enough for slow cell growth to occur, reflecting an indirect and pleiotropic effect.

Based on the following observations, yeast SRP40 is the bona fide homolog of rat Nopp140. First, analysis of the primary sequence shows the two proteins contain at least two distinct domains that are conserved across evolution: the highly conserved carboxyl terminus, also present in nematode- and plant-expressed sequence tags (see Fig. 4B), and the acidic serine stretches, also found in mammalian nucleolin (13), budding yeast NSR1 (14), fission yeast GAR2 (62), and plant Rab17 (63, 64). Second, Nopp140 and SRP40 are immunologically related as demonstrated by the cross-reactivity of the antibodies raised against the rat protein with the yeast protein. Third, SRP40, like Nopp140, is predominantly located in the nucleolus as judged by immunolocalization of the epitope-tagged protein. Fourth, the proposed function of Nopp140 as a chaperone in ribosome biogenesis is compatible with the phenotype of both SRP40 gene deletion and overexpression. In fact, when rat Nopp140 was overexpressed in yeast it caused growth impairment like overexpression of SRP40, even though less pronounced (not shown).

While Nopp140 and SRP40 are similar in many ways, they also differ. In particular, Nopp140 is more highly phosphorylated by CKII than SRP40 leading to the dramatic drop of its theoretical isoelectric point of over 6 units from 10.3 to 4.1. These numbers closely match those of the larger Xenopus Nopp140 homolog (42), namely 10.4 and 4.0, the latter being in good agreement with the experimentally determined isoelectric point of 4.2 (43), thereby validating the theoretical values. Yeast SRP40, however, possesses an acidic isoelectric point of 3.9 even in the absence of phosphorylation. This makes it already one of the more acidic proteins in the cell and may explain why CKII barely introduces any further negative charges despite its 52 phosphorylation consensus sites. We previously showed that phosphorylation of rat Nopp140 was required for binding of basic nuclear localization signal peptides, demonstrating a potential regulatory role for phosphorylation in vivo (22). In contrast, the yeast Nopp140 homolog appears to be a constitutively acidic protein lacking the additional level of regulation found in its vertebrate counterparts.

Further information on the function of the two proteins can be gained from another difference. While yeast SRP40 lacks any consensus sites for Cdc2 kinase phosphorylation, Nopp140...
contains 10, all of which are situated in the basic regions that separate the 10 acidic serine stretches (22). Insertion of negative charges by phosphorylation into these exclusively basic regions of Nopp140 could have dramatic structural consequences. Indeed, the human Nopp140 homolog has been demonstrated to become hyperphosphorylated during mitosis (53) concomitantly with the segregation of the nucleolus and the dispersion of Nopp140 (23, 53). Thus, Nopp140 is apparently phosphorylated by Cdc2 kinase during mitosis like two other vertebrate nucleolar proteins, nucleolin and NO38 (65, 66). In yeast, however, the nucleolus remains intact during mitosis (67, 68) and does not, therefore, require the phosphorylation and consequent dispersion of SRP40.

In summary, analysis of both the similarities and differences between Nopp140 and SRP40 points toward a common function in vertebrates and yeast. Thus, Nopp140 and SRP40 may serve as the glue or skeleton that holds the nucleolus together via ionic interactions between their negative charges and the basic ribosomal proteins. The observation of oligomeric forms (see Fig. 1) and tracks (22) of Nopp140 are particularly intriguing in this context. Having the yeast homolog of Nopp140 in hand now adds the possibility of a genetic approach to further define its function.

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REFERENCES
Characterization of SRP40, a Novel Yeast Nucleolar Protein