Yeast *NPI46* Encodes a Novel Prolyl *cis-trans* Isomerase That Is Located in the Nucleolus

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Abstract. We have identified a gene (*NPI46*) encoding a new prolyl *cis-trans* isomerase within the nucleolus of the yeast *Saccharomyces cerevisiae*. The protein encoded by *NPI46* was originally found by us in a search for proteins that recognize nuclear localization sequences (NLSs) in vitro. Thus, NPI46 binds to affinity columns that contain a wild-type histone H2B NLS but not a mutant H2B NLS that is incompetent for nuclear localization in vivo. NPI46 has two domains, a highly charged NH_2 terminus similar to two other mammalian nucleolar proteins, nucleolin and Noppl40, and a COOH terminus with 45% homology to a family of mammalian and yeast proline isomerases. NPI46 is capable of catalyzing the prolyl *cis-trans* isomerization of two small synthetic peptides, succinyl-Ala-Leu-Pro-Phe-*p*-nitroanilide and succinyl-Ala-Ala-Pro-Phe-*p*-nitroanilide, as measured by a chymotrypsin-coupled spectrophotometric assay. By indirect immunofluorescence we have shown that NPI46 is a nucleolar protein. *NPI46* is not essential for cell viability.

EARLY experiments on the folding of ribonuclease A in vitro suggested that all the information necessary for proper protein folding resides within the nascent polypeptide chain (Anfinsen, 1973). Therefore, it was thought that protein folding, unlike most cellular processes, is spontaneous and does not need to be catalyzed by other proteins. However, based on the peptide-binding affinity of two members of the hsp70 family, BiP and hsc70, Rothman and colleagues have proposed a different model whereby catalysts can determine the nature of protein folding in vivo (Flynn et al., 1989). Thus, families of proteins known as chaperones and two enzymes, protein disulfide isomerase (PDI) and peptidyl-prolyl *cis-trans* isomerase or proline isomerase (PPI), are thought to play a role in a protein reaching its final conformational state (Stamnes et al., 1992; Gething and Sambrook, 1992).

Proline isomerases belong to an abundant class of enzymes that catalyze the *cis-trans* isomerization of X-Pro peptide bonds and can accelerate the refolding of proline-containing polypeptides in vitro and in vivo (Gething and Sambrook, 1992; Heitman et al., 1992). This activity has led investigators to propose that these enzymes may be important in the folding of cellular proteins in vivo. As mentioned above, even though proteins can reach their final conformational state in vitro without the aid of catalysts, in vivo the isomerization of the peptidyl-prolyl bond may be a rate limiting step in determining protein folding (Schmid et al., 1986; Brandts et al., 1975). Indeed, a few examples exist that support the notion that proline isomerases play a role in protein folding in vivo (Heitman et al., 1992). As pointed out in a review by Stamnes et al. (1992), even a modest increase in the isomerization of the peptidyl-prolyl bond could be of considerable biological importance because the rate of folding of a particular protein within the cell would increase (Stamnes et al., 1992).

Proline isomerases are widely distributed, and they have been identified in bacteria (Liu and Walsh, 1990; Hayano et al., 1991; Wülfing et al., 1994; Roof et al., 1994), as well as in a variety of tissues in different organisms (see review by Gething and Sambrook, 1992). These enzymes were first discovered by their ability to bind to drugs that cause inhibition of T lymphocyte activation (Heitman et al., 1992). On this basis, two classes of enzymes have been identified that have prolyl *cis-trans* isomerase activity. However, they have no structural or sequence homology. One class binds the immunosuppressant drug cyclosporin A (CsA)[1] and are known as cyclophilins, while the second class binds the immunosuppressant drugs rapamycin or FK506 and are known as FKBP or rapamycin-binding proteins (Heitman et al., 1992; Stamnes et al., 1992).

Investigators have demonstrated that inhibition of T cell activation by immunosuppressant drugs is not a result of inhibition of the isomerase activity of cyclophilins or FKBPs. Instead, the drug-proline isomerase complex acts dominantly in a different pathway to inhibit a cellular target and some-

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1. *Abbreviations used in this paper*: ADH, alcohol dehydrogenase; CsA, cyclosporin A; DAPI, 4,6-diamidino-2-phenylindole; FKBP, FK506-binding protein; GST, glutathione-S-transferase; HA, hemagglutinin; NLS, nuclear localization sequence.

how causes a toxic effect on the cell (Heitman et al., 1991; Tropschug et al., 1989; Koser et al., 1991). For example, the CsA and FK506 toxicity is caused by the inhibition of a serine-threonine phosphatase, known as calcineurin (Heitman et al., 1991). In one instance, inhibition of this phosphatase disrupts the regulation of the nuclear import of the cytoplasmic subunit of the transcription factor, NF-AT, required for expression of genes involved in T cell activation (Flanagan et al., 1991). Complexes formed between proline isomerases and rapamycin inhibit a different cellular target molecule than the complex formed between proline isomerases and cyclosporin A or FK506 (Heitman et al., 1992).

Although some of the targets of the proline isomerase–CsA and FK506 drug complexes have been identified and their role in inhibiting the signal transduction pathway involved in T cell activation has been shown, little is known about the function of these enzymes in normal cellular processes. If cellular location is any indication of the function of proline isomerases, they undoubtedly will act on different substrates because they are found in the cytoplasm, rough endoplasmic reticulum, mitochondria, and the nucleus (Gething and Sambrook, 1992; Heitman et al., 1992).

In the yeast, *Saccharomyces cerevisiae*, four cyclophilins and two FKBPs have been identified and cloned (for review see Heitman et al., 1992). In this manuscript, we describe a novel FKBP-type proline isomerase that contains a highly charged NH_2-terminal domain not present in other known proline isomerases, and which is located within the nucleolus. The protein was identified by us as a nuclear localization signal (NLS)-binding protein, and it is capable of specifically recognizing a wild-type but not a mutant NLS by affinity chromatography. The NH_2 terminus of NPI46 is highly acidic, containing multiple potential casein kinase II sites, and is homologous to the NH_2 terminus of Nopp140 and nucleolin, proteins suggested to be involved in the nuclear import of proteins required for ribosome biogenesis (Meier and Blobel, 1992; Lapeyre et al., 1987). Our data show that the activity of the nucleolar proline isomerase is low when compared to cytoplasmic proline isomerase FKBP12 (lacking an acidic NH_2 terminus), suggesting possible regulation of the enzyme activity by its NH_2 terminus.

Materials and Methods

Plasmids

pXS4: A 4.6-kb XbaI fragment was excised from a positive clone of a YCp50 yeast genomic library that contains the entire *NPI46* gene, and was subcloned into the XbaI site in pBluescript SK(−). The 5′ end of the gene is toward the BamHI site in the vector.

pXS5: A 1.5-kb SpeI fragment from pXS4 including the entire coding region of *NPI46* was cloned into the SpeI site in pBluescript SK(−). A ClaI to EcoRI DNA fragment corresponding to amino acid residues 43–275 of the NPI46 protein was replaced by the yeast selectable marker *LEU2*.

pXS6: A 168-bp fragment was synthesized by the polymerase chain reaction. This contains nucleotides $^{-}26$ to $^{+}142$ of the *NPI46* gene. A BamHI site was engineered at the $^{-}20$ position in the primer for the polymerase chain reaction, and the fragment was cloned into pBluescript SK(−) between the BamHI and ClaI sites. A 1.1-kb ClaI-SspI fragment containing the *NPI46* gene from position 126 to the end was cloned between ClaI and HincII sites of the above plasmid. This recreated the entire *NPI46* gene with a BamHI site at its 5′ end.

pXS7: The ends of a 1.3-kb BamHI-XhoI fragment from pXS6 including the entire *NPI46* coding region were filled in with Klenow and ligated into a previously constructed yeast centromeric plasmid pZX1 (Xue et al., 1993) that had been cut with HindIII and the ends filled in with Klenow. The resulting plasmid contains the yeast selectable marker *URA3* with the *NPI46* gene under control of the inducible GAL10 promoter.

pXS8: The same BamHI-XhoI fragment as above was cloned into the SmaI site in pAD5 (Field et al., 1988) that contains the yeast selectable marker *LEU2* and the yeast alcohol dehydrogenase (ADH) promoter, followed by an epitope from the influenza hemagglutinin (HA) protein. The *NPI46* gene was fused in frame behind the HA epitope and was placed under control of the constitutive yeast ADH promoter.

pXS9: A BamHI fragment containing the ADH promoter and the HA epitope, as well as a BamHI-SacI fragment containing the *NPI46* gene, were cut out from pXS8 and cloned into pZX1 between the BamHI and SacI sites. The resulting plasmid expresses an HA epitope–NPI46 fusion protein under the control of the yeast ADH promoter. The HA epitope is a 9–amino acid sequence (YPYDVPDYA) from the hemagglutinin of the influenza virus (HA1) that is recognized by the monoclonal anti-HA antibody 12CA5 and a polyclonal anti-HA antiserum.

pXS10: The ADH promoter and the HA epitope were cut out from pXS8 with BamHI and cloned into yeast centromeric plasmid pRS316 (Sikorski and Hieter, 1989) at the BamHI site.

pXS11: The XhoI ends of a BamHI-XhoI fragment from pXS6 containing the entire *NPI46* gene were filled in with Klenow and cloned into pGEX-FLA (a gift from Dr. Jack D. Keene, Duke University, Durham, NC; see Hoffman et al., 1991) between the BamHI and Klenow-filled EcoRI ends. This created an in frame fusion of glutathione-S-transferase (GST) and *NPI46* with a thrombin cleavage site in between, and put the fusion protein under the control of the isoprylthiogalactoside-inducible *tac* promoter.

pXS12: Both ends of a 4.9-kb BamHI-EcoRI fragment of pGEX-FLA were filled with Klenow and religated. This plasmid expresses the glutathione-S-transferase under the control of the *tac* promoter.

Strains

Escherichia coli strain XL-1 was used for all cloning. *E. coli* strain BL-21 was used for expressing proteins in bacteria. All DNA manipulations and bacterial transformations were done according to published procedures (Sambrook et al., 1989). The yeast strains used were W303-1A, *Matα, ade2-1, can1-100, ura3-1, leu2-3,112, trp1-1, his3-11,15*; WLY353, same as W303-1A except *nsr1Δ::HIS3*; W303, isogenic a/α diploid strain; and XSY1, *Mata, ade2-1, can1-100, ura3-1, leu2-3,112, trp1-1, his3-11,15, npi46Δ::LEU2*.

Yeast transformations were done using the lithium acetate method of Ito et al. (1983). Standard media preparation and yeast cell culture were carried out according to Sherman et al. (1986).

Chemical and Enzymes

Succinyl-Ala-Ala-Pro-Phe-*p*-nitroanilide, α-chymotrypsin, and bovine antithrombin were from Sigma Immunochemicals (St. Louis, MO). Succinyl-Ala-Leu-Pro-Phe-*p*-nitroanilide was from BACHEM Bioscience Inc. (Philadelphia, PA). Bovine thrombin was from ICN Biomedicals, Inc. (Costa Mesa, CA) and FK506 was provided by Fujisawa Pharmaceuticals (Deerfield, IL) and Dr. Stephen Gotschlich at The Rockefeller University (New York). Rapamycin was provided by the Drug Synthesis and Chemistry Branch, Developmental Therapeutics Program, Division of Cancer Treatment, National Cancer Institute (Bethesda, MD). Monoclonal anti-NSR1 antibody was a gift from Dr. John Woolford at Carnegie Mellon University (Pittsburgh, PA). Monoclonal and polyclonal anti-HA antibodies were from BAbco (Berkeley, CA). FITC-conjugated goat anti-mouse IgG and lissamine rhodamine-conjugated donkey anti-rabbit IgG were from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA).

Preparation of a Yeast Nuclear Extract and Cytosol

A yeast nuclear extract and cytosol were generated by the following procedure. Yeast cells were grown in YPD medium to an OD_{600} of 0.8. Cells were harvested by centrifugation at 5,000 rpm for 5 min. The cell pellet was resuspended in a minimum volume of 20 mM Tris, 2 mM EDTA, 0.5 mM PMSF, 1 μg/ml each of leupeptin and pepstatin. Cells were broken by vortexing with glass beads. Unbroken cells were removed by centrifugation at 2,000 rpm for 2 min. A crude nuclear pellet was generated by centrifugation at 14,000 rpm for 15 min in an Eppendorf centrifuge. The supernatant was further centrifuged at 100,000 rpm for 60 min in an ultracentrifuge (TL-100; Beckman Instruments, Inc., Fullerton, CA) to generate a clear cytosol. The crude nuclear pellet was extracted with 2 M NaCl, 10 mM Tris, pH 8.0, and centrifuged at 100,000 rpm for 60 min. The supernatant was dialyzed overnight against either 20 mM phosphate, pH 8.0, 150 mM NaCl (for antibody generation), or 20 mM Tris, pH 8.0 (for testing antisera), to generate a nuclear extract.

Affinity Chromatography and Antibody Preparation

Wild-type and mutant histone H2B NLS affinity columns were prepared according to the procedure described in Lee et al. (1991). The sequence of the wild-type NLS peptide is NH_2-Ser-Thr-Asp-Gly-Lys-Lys^{31}-Arg-Ser-Lys-Ala-Arg-Lys-Glu-Tyr-Cys-COOH, and the sequence of the mutant NLS peptide is the same except that Lys^{31} was replaced by Met (University of California at Los Angeles peptide facility).

To obtain potential NLS-binding proteins, a yeast nuclear extract and cytosol made from strain WLY353, as described above, were passed over the wild-type NLS affinity column. The column was washed with 20 mM phosphate, pH 8.0, 0.15 M NaCl, and eluted with 20 mM phosphate, pH 8.0, 2 M NaCl. Generation of antibodies against eluant from the NLS affinity column (potential NLS-binding proteins) using rabbits was done essentially as described in Lee et al. (1991).

To characterize the antisera generated against the eluant from the NLS affinity column, a yeast nuclear extract made from yeast WLY353 was applied to a wild-type H2B NLS affinity column (5 ml bed volume). The column was washed with 25 ml of 20 mM Tris, pH 8.0, eluted with 15 ml each of 0.2, 0.5, and 1 M NaCl in 20 mM Tris, pH 8.0. Fractions were collected, and each was precipitated with 20% trichloroacetic acid overnight at 4°C. The precipitated proteins were separated on a 10.5% SDS polyacrylamide gel and were transferred onto two sheets of nitrocellulose paper. One sheet was analyzed by immunoblotting with the antisera against potential NLS-binding proteins and HRP-conjugated goat anti-rabbit IgG using 4-chloro-1-naphthol as color reagent. The other sheet was stained with India ink.

To test the NLS-binding ability of NPI46, a nuclear extract was made from yeast XSY1 carrying plasmid pXS9 (expressing HA-NPI46 fusion protein). The extract was diluted with 20 mM Tris, pH 8.0, to a final NaCl concentration of 0.25 M, without dialyzing, and applied to a wild-type or a mutant H2B NLS affinity column (both 5 ml). The columns were washed with 25 ml of 0.25 M NaCl in 20 mM Tris, pH 8.0, eluted with 15 ml each of 0.3, 0.5, and 1 M NaCl in 20 mM Tris, pH 8.0. The fractions were analyzed by immunoblotting with the monoclonal anti-HA antibody 12CA5 as above.

Isolation of NPI46 Gene

Affinity-purified antibody was used to screen a λZAP yeast genomic expression library (Stratagene, La Jolla, CA; see Megraw and Chae, 1993), as described in Snyder et al. (1987). Positive clones were used to generate probes to screen a YCp50 yeast genomic library (a gift from Mark Rose, Princeton University, Princeton, NJ) using the colony hybridization method (Sambrook et al., 1989). Unidirectional nested deletions of pXS4 were performed in both orientations using exonuclease III according to the procedure of Sambrook et al. (1989). The deletion series were transformed into *E. Coli* XL-1. Dideoxy sequencing was carried out using a Sequenase Kit (U.S. Biochemical Corp., Cleveland, OH) according to the procedure provided by the manufacturer. Sequence editing and analysis were performed using the GenBank databank version 81 "*FASTA*" program (Pearson and Lipman, 1988).

Disruption of the NPI46 Gene and Tetrad Analysis

One copy of the *NPI46* gene on the chromosome was disrupted with a partial deletion (*npi46Δ::LEU2*) by the one-step gene disruption method (Rothstein, 1983). A linear 2.8-kb fragment from pXS5 containing the entire *NPI46* gene, with the ClaI to EcoRI region being replaced by the yeast selectable marker *LEU2*, was isolated and transformed into yeast strain W303. The $LEU2^+$ transformants were further confirmed by Southern blot analysis. Heterozygous diploids were sporulated, and the tetrads were dissected and analyzed according to the procedure of Sherman et al. (1986).

Purification of the GST-NPI46 Fusion Protein

E. Coli strain BL-21 carrying pXS11 or pXS12 were grown overnight in Luria broth containing ampicillin. After a 1:10 dilution, the cells were grown for an additional hour in the same medium. IPTG was then added to 1 mM to induce the expression of the proteins. Cells were harvested after a 2-h induction period and were resuspended in a minimum volume of a buffer containing 20 mM phosphate, pH 8.0, 0.15 M NaCl, 1% Triton X-100, and 0.5 mM PMSF. Lysozyme was added to the suspension to a concentration of 1 mg/ml, and the suspension was incubated on ice for 20 min. The suspension was then sonicated four times for 30 s and was centrifuged in an Eppendorf centrifuge at 0°C and 14,000 rpm for 30 min. The supernatant was applied to a glutathione–Sepharose 4B column (Pharmacia LKB Biotechnology, Piscataway, NJ), and GST-NPI46 fusion protein or GST was purified according to Smith and Johnson (1988).

Results

Isolation of a Yeast Gene Encoding an NLS-binding Protein with an Apparent Molecular Mass of 70 kD

Our laboratory had previously identified a protein in yeast, NSR1, that specifically recognized NLSs in vitro (Lee and Mélèse, 1989; Lee et al., 1991). However, it is clear from our analysis that although NSR1 is the major NLS-binding protein by this assay, other NLS-binding proteins are also present in yeast (Lee et al., 1991; Xue, Z., and T. Mélèse, unpublished results). The fact that *NSR1* is not essential for yeast viability, although disruption of this gene results in a slow-growth phenotype, made us wonder if any of these other NLS-binding proteins had overlapping function with NSR1.

To isolate additional NLS-binding proteins, we prepared nuclear and cytosolic extracts from a yeast strain containing a disruption in *NSR1* (*nsr1⁻Δ::HIS3*; *WLY353*). Potential NLS-binding proteins were purified on an affinity column containing the wild-type histone H2B NLS (Lee et al., 1991). The eluant from the affinity column was used to generate antibodies (see Materials and Methods). The antiserum was tested against whole cell extracts from yeast by immunoblot analysis, and several protein bands were recognized by the antisera. We chose to further characterize a major 70-kD protein present in the immunoblots. The 70-kD protein band on a strip of nitrocellulose paper, corresponding to the 70-kD protein in the immunoblot, was used to affinity purify antiserum against this protein from the crude antiserum (Pringle et al., 1989).

To ensure that the purified anti–70-kD antiserum we obtained recognized the same 70-kD NLS-binding protein that we originally observed in the eluant of a wild-type NLS affinity column, whole-cell extracts and yeast nuclear extracts made from strain WLY353, as well as fractions resulting from the passage of nuclear extracts over a wild-type NLS affinity column, were analyzed using the purified antiserum (Fig. 1). A 70-kD protein was recognized in whole-cell extracts, as well as in the nuclear extract (Fig. 1, *Immunoblot*, lanes *1* and *2*). When the nuclear extract was fractionated by passage over the NLS affinity column, most proteins were present in the flow-through, buffer wash, and 0.2-M salt fractions (Fig. 1, *India ink*, lanes *3–7*). However, the 70-kD protein band was absent from the flow-through, buffer wash, and 0.2-M salt–eluted fractions, but present in the 0.5-M salt–eluted fraction (Fig. 1, *Immunoblot*, lanes *3–7*). We conclude from these results that the affinity-purified antiserum recognizes a potential NLS-binding protein with an apparent molecular mass of 70 kD.

The purified antiserum was then used to screen a λZAP yeast genomic expression library. Several identical positive clones were isolated. The entire gene along with flanking regions was isolated from a yeast plasmid library using a probe made from the positive clone obtained from the ex-

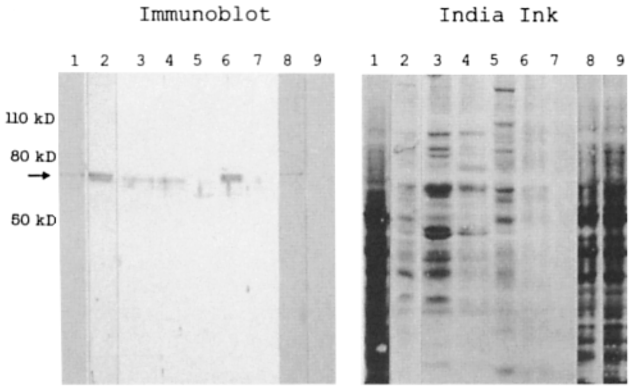

Figure 1. Detection of an NLS-binding protein with an apparent molecular mass of 70 kD by an affinity-purified polyclonal antiserum. Yeast whole cell and nuclear extracts were made as described in the Materials and Methods. A nuclear extract made from strain WLY353 was dialyzed against 20 mM Tris, pH 8.0, overnight, and fractionated on a wild-type H2B NLS affinity column (see Materials and Methods). (Lane *1*) WLY353 cells; (lane *2*) nuclear extract from WLY353; (lane *3*) flow-through fraction from the NLS affinity column; (lane *4*) 20 mM Tris buffer wash fraction; (lane *5*) 0.2 M NaCl eluant; (lane *6*) 0.5 M NaCl eluant; (lane *7*) 1.0 M NaCl eluant; (lane *8*) XSY1 carrying pXS7 grown on galactose medium (the expression of NPI46 is induced); (lane *9*) XSY1 carrying pXS7 grown in glucose medium (the expression of NPI46 is repressed). The approximate positions of the molecular mass markers are indicated on the left. To allow detection of proteins in all fractions by India ink staining, the 0.2-, 0.5-, and 1.0-M NaCl eluant lanes were loaded with twice as much sample as the flow through and buffer wash lanes.

pression library. Sequencing of the clone revealed the presence of a long open reading frame, coding for a protein with 411 amino acid residues, and a molecular mass of 46,541 D. The predicted amino acid sequence of the COOH-terminal 106 residues share a high degree of homology with a class of proline isomerases that bind to FK506 and rapamycin (Heitman et al., 1992). We have designated this gene, Nucleolar Proline Isomerase or *NPI46*.

One copy of the wild-type *NPI46* gene in the diploid strain W303 was replaced with a deletion allele (see Materials and Methods) using the one-step gene disruption method (Rothstein, 1983). Two independent heterozygous diploids were sporulated, and a total of 52 tetrads were dissected. Four viable spores were recovered from each tetrad, two of them being LEU^+. All four spores grew normally to form colonies of the same size as the normal haploid W303. The *npi46*$^-$ strain has no observable phenotype. We also compared the sensitivity of the *npi46*$^-$ and *NPI46*$^+$ strains toward FK506 and rapamycin, and we found that the sensitivity level of both strains toward either drug is identical (data not shown).

Consistent with *NPI46* being the gene encoding the 70-kD protein, the protein was absent in LEU^+ colonies (containing a disrupted copy of *NPI46*; this strain was used for the experiments discussed below) and present in leu^- colonies (containing the normal *NPI46* gene; data not shown), as analyzed in immunoblots using the affinity-purified anti-70-kD antibody. To further ensure that the *NPI46* gene encoded p70, we checked that p70 expression coincided with transcription from the *NPI46* gene. To do this, the *NPI46* gene was placed under the inducible yeast GAL10 promoter in plasmid pXS7, and subsequently transformed into the yeast strain XSY1 (*npi46Δ::LEU2*). Cells were grown on media containing either glucose or galactose as a carbon source, and whole-cell extracts were then made and subsequently analyzed by immunoblotting. The 70-kD protein band is present in extracts from cells grown on galactose medium (*NPI46* is induced), but absent in cells grown in glucose medium (*NPI46* is repressed) (Fig. 1, *Immunoblot*, lanes *8* and *9*).

All the data above demonstrate that *NPI46* is indeed the gene encoding for p70, and that it is not essential for cell viability. The difference between the apparent molecular mass of NPI46 protein measured on an SDS gel (~70 kD) and the actual molecular mass calculated from the predicted amino acid sequence of the gene (46.5 kD) likely results from the highly charged nature of the NH_2 terminus of the protein.

NPI46 Binds Specifically to an Affinity Column Conjugated with a Peptide Containing the Wild-type Histone H2B NLS

To test whether the *NPI46* gene encodes a protein that specifically recognizes a wild-type but not a mutant NLS, we tested whether the protein product bound specifically to a wild-type NLS affinity column. A plasmid (pXS9) was constructed that expresses an in-frame fusion of NPI46, and the HA epitope that is under the control of the constitutive ADH promoter. The *npi46*$^-$ yeast strain XSY1 was transformed with this plasmid. Cells containing the plasmid were grown overnight on synthetic medium lacking uracil, diluted into YPD medium, and allowed to grow to an OD_{600} of 0.8.

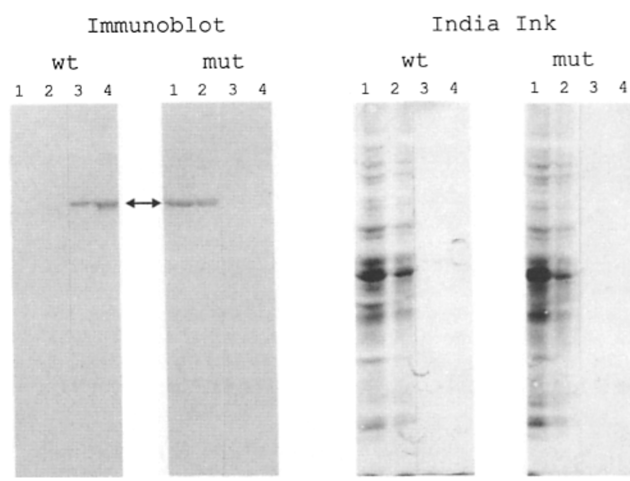

Figure 2. NPI46 binds to a wild-type NLS affinity column. A nuclear extract from yeast XSY1 carrying plasmid pXS9 was passed over an affinity column conjugated with peptide containing either the wild-type or a mutant histone H2B NLS, as described in the Materials and Methods. Proteins in fractions collected from the column were detected by immunoblotting or India ink staining, as described in Materials and Methods. The immunoblot panels (*wt* and *mut*) show the result of immunoblotting with anti-HA monoclonal antibody 12CA5; the India ink panels (*wt* and *mut*) show the result of India ink staining. *wt*, Samples collected from the wild-type H2B NLS affinity column; *mut*, samples collected from the mutant H2B NLS affinity column. (Lane *1*) flow-through; (lane *2*) 0.25-M salt wash; (lane *3*) 0.3-M salt elution; and (lane *4*) 0.5-M salt elution. The arrow indicates the positon of NPI46.

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   1 ACTAGTATTAGCTCGCTGTTCAATTTTCAGCCCTCTACGGACTGTCGTCATTCCTAAAGG
  61 ACGGCAAAATTTAAAAGTGAAGGTAAAATTTTTAAATTCGAAAAAAGAGCCTACTAACAC
 121 GTTTCTATATAATACATAATTGTGTGAAAGTTCATACATAATTGAAAGCAAGCATCCAAC
 181 CAGCCCAATATGTCTGATTTGTTACCACTAGCTACCTACAGTTTGAATGTTGAACCTTAT
               M  S  D  L  L  P  L  A  T  Y  S  L  N  V  E  P  Y
 241 ACCCCGGTTCCAGCAATCGACGTCACGATGCCCATCACCGTTCGTATTACTATGGCTGCT
      T  P  V  P  A  I  D  V  T  M  P  I  T  V  R  I  T  M  A  A
 301 TTGAACCCGGAAGCCATCGATGAAGAGAACAAACCATCGACTTTAAGAATTATCAAAAGA
      L  N  P  E  A  I  D  E  E  N  K  P  S  T  L  R  I  I  K  R
 361 AACCCGGACTTTGAAGATGATGATTTTTTAGGTGGTGATTTTGATGAAGACGAAATAGAC
      N  P  D  F  E  D  D  D  F  L  G  G  D  F  D  E  D  E  I  D
 421 GAAGAATCCTCTGAGGAAGAAGAAGAGGAAAAAACCCAAAAGAAAAAGAAGAGTAAAGGC
      E  E  S  S  E  E  E  E  E  E  K  T  Q  K  K  K  K  S  K  G
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      K  K  A  E  S  E  S  E  D  D  E  E  D  D  D  E  D  D  E  F
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      Q  E  S  V  L  L  T  L  S  P  E  A  Q  Y  Q  Q  S  L  D  L
 601 ACCATTACTCCAGAAGAAGAAGTCCAATTCATTGTCACTGGTTCTTACGCTATCTCCTTG
      T  I  T  P  E  E  E  V  Q  F  I  V  T  G  S  Y  A  I  S  L
 661 AGCGGTAACTATGTTAAGCATCCATTTGATACTCCAATGGGAGTCGAAGGTGAAGACGAA
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 781 ATTATTGGCGACGACATGGACGACTTGGATGACGAAGAGGAAGAAGAAGTTCGTATTGAA
      I  I  G  D  D  M  D  D  L  D  D  E  E  E  E  E  V  R  I  E
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      E  E  E  Q  K  E  E  V  K  P  E  P  K  K  S  K  K  E  K  K
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1081 AAACCAAAGAGTAAGGTTTTGGAAGGCGGCATAGTAATCGAAGACCGTACTATCGGTGAT
      K  P  K  S  K  V  L  E  G  G  I  V  I  E  D  R  T  I  G  D
1141 GGCCCACAGGCTAAGAGAGGTGCCAGAGTAGGCATGAGGTACATTGGTAAGTTAAAGAAC
      G  P  Q  A  K  R  G  A  R  V  G  M  R  Y  I  G  K  L  K  N
1201 GGTAAAGTTTTCGACAAGAACACCAGCGGTAAACCATTTGCATTCAAACTTGGCCGTGGT
      G  K  V  F  D  K  N  T  S  G  K  P  F  A  F  K  L  G  R  G
1261 GAAGTTATCAAAGGCTGGGACATTGGTGTTGCCGGTATGTCTGTTGGTGGCGAACGTAGA
      E  V  I  K  G  W  D  I  G  V  A  G  M  S  V  G  G  E  R  R
1321 ATCATCATTCCAGCACCATATGCCTACGGGAAGCAAGCTCTGCCAGGTATTCCTGCCAAT
      I  I  I  P  A  P  Y  A  Y  G  K  Q  A  L  P  G  I  P  A  N
1381 TCCGAACTGACATTCGACGTTAAATTGGTTTCTATGAAAAACTAGTACACGCGCTCGTAC
      S  E  L  T  F  D  V  K  L  V  S  M  K  N
1441 GGATAGATGTTTATATATATTATTCTTTTTACATAATATTGTAACCTTCTGTATGTAAAT
1501 CCGAAAACATTCTATTTCTTTTTAGTATCTATTGCTCTTTATAAGCTTTTCATTTTACTT
1561 TTCTCCGCGGATGATACAAATAAAGAAGCTGTGTTGTATAGAAAGAAATATATGGCGGCA
1621 AAAAAAGCGTTATCTTCTTTCTGTTCCTATTTCACTGAGTGCTACTGGATGGTCGTCAAG
1681 AAAGGTGAAGAAATCTTCCAAAGCAATAAGAATTC
```

Figure 3. The nucleotide and deduced amino acid sequence of *NPI46*. The three acidic stretches are underlined, and the region homologous to the FKBPs is double underlined. These sequence data are available from EMBL/GenBank/DDBJ under accession number X79379.

At this time, the cells were harvested, and crude nuclear and cytosolic fractions were made as described in Materials and Methods. The two fractions were analyzed by immunoblotting with both affinity-purified anti-p70 antisera and monoclonal anti-HA antibody 12CA5. NPI46 was present only in the nuclear fraction (data not shown).

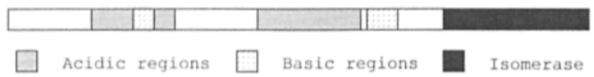
NPI46 protein was solubilized from the nuclear fraction by salt extraction, and the extract was then diluted and applied to a wild-type or mutant histone H2B NLS affinity column, as described in Materials and Methods. Fractions collected were analyzed by immunoblotting with the anti-HA antibody 12CA5. As shown in Fig. 2 (*wt*), after passage over a wild-type H2B NLS affinity column, the majority of NPI46 remained bound to the column (as noted by its absence in the flow-through and wash fractions; lanes *1* and *2*). NPI46 was partially eluted when the salt concentration in the buffer was 0.3 M (lane *3*) and fully eluted at a salt concentration of 0.5 M (lane *4*). When the extract was passed over a mutant H2B NLS column all the NPI46 protein was found in the flow-through and wash fraction (Fig. 2; *mut*, *1* and *2*). India ink staining of the proteins in the different column fractions indicated that most proteins in the extract were not retained by either the wild-type or the mutant histone H2B NLS affinity column (all the lanes in this gel were loaded with the same amount of sample, thus the proteins in the salt eluted fractions are not visible by India ink staining). Thus, NPI46 has a higher affinity in vitro for the wild-type as opposed to the mutant H2B NLS.

The NPI46 Protein Contains a Highly Acidic NH_2 Terminus and a COOH Terminus That is Homologous to Proline Isomerases

The nucleotide and deduced amino acid sequences of *NPI46* are shown in Fig. 3. Fig. 4 *A* shows the overall structure of the NPI46 protein. The NH_2-terminal domain of the protein is highly charged, containing three separate regions of acidic residues and two separate regions that are rich in basic residues. Each acidic region contains serine or threonine residues that are potential phosphorylation sites for casein kinase II. The first and second acidic regions of NPI46 are separated by the first of the two basic regions. The second basic region is located behind the third acidic region. There is a potential bipartite NLS in the second basic region, containing residues KKRK and KKAKK, separated by seven other amino acid residues (Robbins et al., 1991).

The NH_2-terminal domain of NPI46 is similar to mammalian nucleolin and Noppl40, and to a lesser degree, the yeast NSR1. The charged domains share >20% sequence identity with nucleolin and Noppl40. Aside from the se-

A



B

```
          300    G        G G      G     HYTG L  G
NPI46     KSKVLEGGIVIEDRTIGDGPQ-AKRGARVGMRYIGKLKNGKV
ScFPR1    MSEVIEGNVKIDRISPGDGATKPKTGDLVTIHYTGTLENGQK
mFKBP12         MGVQVETISPGDGRTFPKRGQTCVVHYTGMLEDGKK

          FDSS     PF F LG   VI GW  G   M  G    L I
NPI46     FDKN-TSGKPFAFKLGRGEVIKGWDIGVAGMSVGGERRIIIP
ScFPR1    FDSSVDRGSPFQCNIGVGQVIKGWDVGIPKLSVGEKARLTIP
mFKBP12   FDSSRDRNKPFKFTLGKQEVIRGWEEGVAQMSVGQRAKLIIS

             AYG  G    IPP  TL FEVEL    411
NPI46     APYAYGKQALPG-IPANSELTFDVKLVSMKN
ScFPR1    GPYAYGPRGFPGLIPPNSTLVFDVELLKVN
mFKBP12   SDYAYGATGHPGIIPPHATLVFDVELLKLE
```

Figure 4. The domain structure of NPI46 and sequence homology between NPI46 and FKBP12. (*A*) Structural features of NPI46. The acidic and basic regions and the proline isomerase domain are labeled in the figure. (*B*) Comparison of the sequences of yeast and mouse FKBP12 with NPI46. Residues in bold letters are those involved in FK506 binding, as revealed by x-ray crystallography (Van Duyne et al., 1991). Conserved residues among known FKBPs are indicated in italics above the NPI46 sequence (from listings in GenBank, version 81).

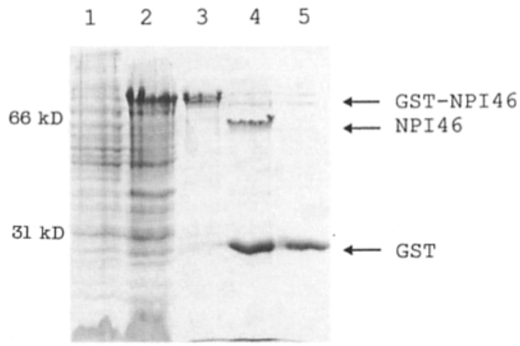


Figure 5. Purification of a GST-NPI46 fusion protein. A GST-NPI46 fusion protein and GST alone were purified as described in Materials and Methods. The purified proteins and proteins from IPTG-induced and uninduced *E. coli* cultures were first separated on a 12% SDS gel and then stained with Coomassie brilliant blue. (Lane *1*) Protein profile of an uninduced culture; (lane *2*) protein profile of an induced culture expressing the GST-NPI46 fusion protein; (lane *3*) purified GST-NPI46 fusion protein; (lane *4*) purified fusion protein after digestion with thrombin to remove the GST moiety; (lane *5*) purified GST. Positions of the molecular mass markers are indicated on the left side.

quence homology, the nature of the arrangement of acidic and basic stretches, as well as the presence of multiple potential casein kinase II phosphorylation sites, are similar in all four proteins. Nucleolin, Nopp140, and NSR1 are all nucleolar proteins that like NPI46, have been shown to bind NLSs in vitro (Lee et al., 1991; Xue et al., 1993; Meier and Blobel, 1992). The extremely charged nature of the NH_2 terminus most likely accounts for the discrepancy between the apparent molecular weight of NPI46, estimated from mobility on SDS gels, and its predicted molecular weight based on its DNA sequence. We also found this to be the case for NSR1 and nucleolin (Lee et al., 1991; Xue et al., 1993).

The COOH-terminal domain of the protein, containing 106 residues, is homologous to a class of prolyl *cis-trans* isomerases that bind the immunosuppressant drug FK506. These enzymes are referred to as FK506-binding proteins or FKBPs. Fig. 4 *B* shows the comparison of the NPI46 protein sequence with two other FKBPs. More than 45% of the residues in this domain are identical to yeast and mammalian FKBP12, including most of the residues that are conserved among the known FKBPs as listed in GenBank, version 81. Those residues that are involved in FK506 binding, as revealed by x-ray crystallography, are also conserved in NPI46 (Van Duyne et al., 1991).

NPI46 Protein has Proline Isomerase Activity

Because of the strong sequence homology with proline isomerases, we decided to test if NPI46 had proline isomerase activity. A plasmid that expressed a hybrid protein containing GST at the NH_2 terminus followed by NPI46 at the COOH terminus (pXS11) was constructed under the control of the IPTG inducible *tac* promoter. pXS11 was transformed into *E. coli* strain BL21, and the expression of the fusion protein was induced by IPTG. The fusion protein was purified using the procedure described in Material and Methods. As a control, a plasmid that contained only GST rather than the GST-NPI46 hybrid protein (pXS12) was constructed. GST was expressed and purified identically to the fusion protein, using pXS12. Fig. 5 displays the separation of the purified proteins on a 12% SDS gel. Both the purified GST-NPI46 fusion protein and GST protein were tested for isomerase activity by using a standard chymotrypsin-coupled spectrophotometric assay (Heitman et al., 1993), as described in the legend of Fig. 6.

Fig. 6 *A* shows the results of the activity assays using 100 μg of the purified GST-NPI46 fusion protein and 50 μM succinyl-Ala-Leu-Pro-Phe-*p*-nitroanilide as substrate. In this assay, we observe proline isomerase activity for purified GST-NPI46, but not for the purified GST alone, even though double the amount of the protein was used. When a different synthetic peptide, succinyl-Ala-Ala-Pro-Phe-*p*-nitroanilide, was used as a substrate, the isomerase activity of GST-NPI46 was lower, only 15% of that measured with the first peptide (data not shown). We estimate that the activity of the NPI46 fusion protein is only ~5% of that previously reported for FKBP12. To ensure that the low isomerase activity of the GST-NPI46 protein was not caused by the presence of the GST moiety, the purified fusion protein was digested with thrombin to remove the GST moiety. Antithrombin was added to inactivate excess thrombin, and the isomerase activity of the sample was measured. The same level of activity was observed with both GST-NPI46 and NPI46. Thus, the results indicate that GST does not interfere with the activity of NPI46 (data not shown).

The isomerase activity of FKBPs is inhibited by either FK506 or rapamycin. Thus, we tested the ability of these two drugs to inhibit the activity of NPI46. Fig. 6 *B* shows the results. Both rapamycin and FK506 inhibit the isomerase activity of NPI46 almost completely at a concentration of 1 μM. The concentration of inhibitors required for 100% inhibition of activity is lower than the concentration of NPI46 protein used in the assay. This may either result from an inaccuracy in the measurement of the protein concentration of the Lowry assay, or because some of the purified protein has

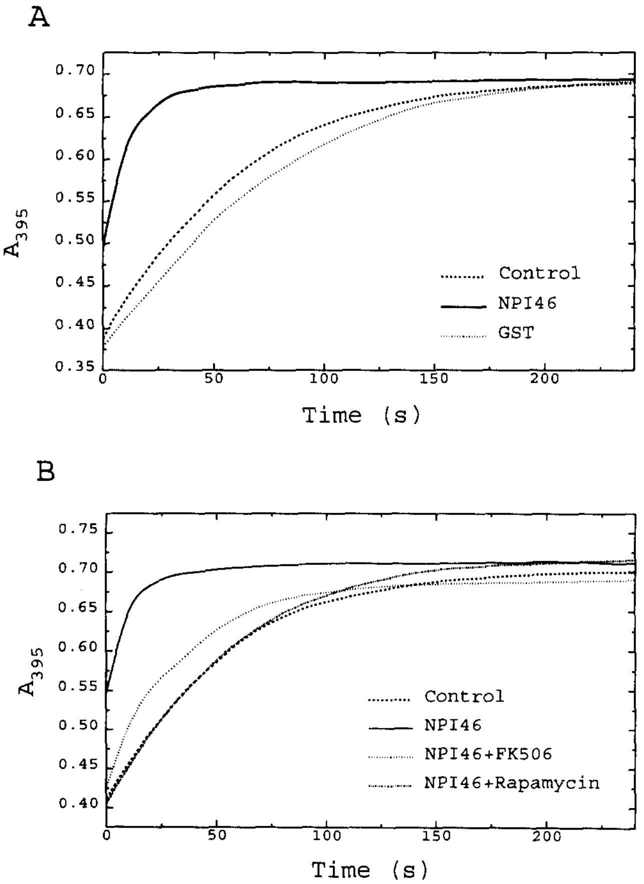


Figure 6. Proline isomerase activity of NPI46. (*A*) The isomerase activity of the purified GST-NPI46 fusion protein and the purified GST were measured according to the procedure of Heitman et al. (1993). Samples were assayed in a mixture containing; 50 mM Hepes, pH 8.0, 100 mM NaCl, 500 μg of chymotrypsin, and either 100 μg of purified GST-NPI46 fusion protein (labeled as NPI46 in the figure) or 200 μg of purified GST (labeled as GST) in a 1-ml vol. The assay mixtures were chilled to 4°C, and 10 μl of 5 mM succinyl-Ala-Leu-Pro-Phe-*p*-nitroanilide, dissolved in trifluoroethanol containing 470 mM LiCl, was added to initiate the reaction. The *cis* to *trans* isomerization was monitored by a change in the absorbance at 395 nm. A control sample (labeled as control) that contained all the ingredients except for GST-NPI46 or GST was also assayed. (*B*) Inhibition of the proline isomerase activity of NPI46 by rapamycin and FK506. Rapamycin or FK506 was added to the assay mixture containing 100 μg of purified GST-NPI46 fusion protein to a final concentration of 1 μM, in the absence of chymotrypsin. The sample was then incubated on ice for 5 min, after which time chymotrypsin was added, and the assay was performed identically as in *A*. Control curve: buffer only; NPI46 curve: 100 μg GST-NPI46 fusion protein with no inhibitor; NPI46+FK506 curve: 1 μM FK506 and 100 μg GST-NPI46 fusion protein; NPI46+rapamycin curve: 1 μM rapamycin and 100 μg GST-NPI46 fusion protein.

its isomerase moiety in an inactive conformation, even though the GST moiety of the protein is functional.

NPI46 is a Nucleolar Protein

As indicated earlier, NPI46 protein is present in the nuclear, but not cytosolic fraction of yeast. Analysis of the subcellular location of NPI46 was carried out by expression of NPI46 on a plasmid in a yeast strain (XSY1) disrupted in the chromosomal copy of *NPI46*. The strain either harbored plasmid pXS9 (NPI46 fused with an HA epitope) or pXS10 (HA epitope alone). By indirect immunofluorescence, NPI46 showed distinct nucleolar staining (Fig. 7). In yeast, the nucleolus forms a crescent that lines the nuclear envelope and occupies a sizeable volume of the nucleus. As discussed in a previous paper from our laboratory, in most cases, the orientation of the cells will be such that the nucleolar antigen (stained by FITC) will not overlap the DNA (stained by DAPI [4,6-diamidino-2-phenylindole]), and because the nucleolar region is not stained well by DAPI, the two staining patterns will be nearly independent (see Fig. 7). The control cells show little or no FITC staining. The observed pattern of nucleolar staining by NPI46 is similar to that visualized for NSR1 (Lee et al., 1991) and nucleolin (when expressed in yeast; see Xue et al., 1993). To further confirm that NPI46 is a nucleolar protein, we also performed double immunofluorescence labeling using a monoclonal antibody against the previously identified nucleolar protein NSR1 and a polyclonal anti-HA antiserum. The staining pattern of the two antibodies completely overlapped with each other (Fig. 7, NPI46/NSR1). We conclude that NPI46 is a novel nucleolar proline isomerase.

Discussion

We have identified a novel prolyl *cis-trans* isomerase in the yeast, *Saccharomyces cerevisiae*, as the result of a search for proteins that specifically recognize NLSs. NPI46 has a highly charged NH_2 terminus, where presumably the recognition of the basic NLS occurs, and a 106–amino acid stretch at the COOH terminus that contains the proline isomerase activity. Analysis by indirect immunofluorescence demonstrated that NPI46 is a nucleolar protein. Since the nucleolus is the site of ribosome biogenesis, and proline isomerases are known to facilitate the folding of proteins, we propose that NPI46 is involved in the assembly or folding of ribosomal proteins.

Although proline isomerases are quite abundant, and a number of them have been identified, their physiological substrates are unknown. Interestingly, only a few proline isomerases have been identified that contain two domains: an isomerase domain and another variable domain. Two examples are FKBP52/60 or hsp56 from calf thymus, which associates with the 90-kD heat shock protein and is a component of steroid receptor complexes (Sanchez et al., 1990; Yem et al., 1992; Tai et al., 1986; Peattie et al., 1992), and FKBP25 from human thymus, which contains putative NLSs and is located in the nucleus by subcellular fractionation (Galat et al., 1992; Jin and Burakoff, 1993). NPI46 is the first yeast isomerase to have such an additional domain, and the first proline isomerase to be found in the nucleolus of any organism.

Yeast disrupted in the chromosomal copy of the *NPI46* gene do not have an apparent phenotype, indicating that the function of NPI46 is not essential for cell growth. Thus far, none of the identified genes encoding yeast proline isomerases are essential (Heitman et al., 1992). This is either caused by the fact that the *cis-trans* isomerization about the X-Pro sequences in most proteins occurs at a slow rate, even when this reaction is not catalyzed by a proline isomerase,

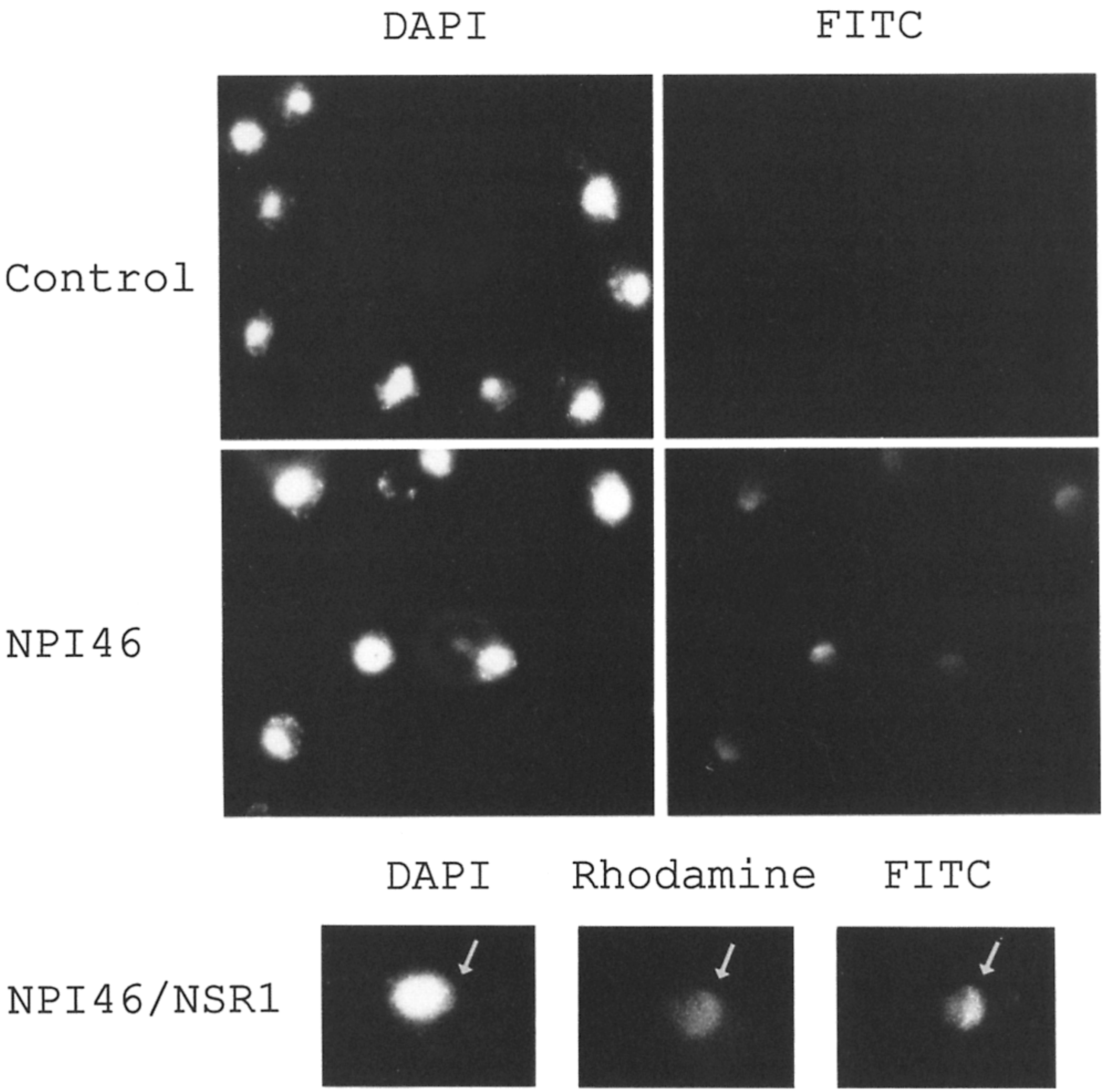


Figure 7. Indirect immunofluorescence on *npi46*⁻ strain XSY1 carrying the *NPI46* gene fused to the HA epitope on a plasmid under the control of the yeast constitutive ADH promoter, or the promoter followed by HA epitope alone. Indirect immunofluorescence was carried out as described by Pringle et al. (1989). The control panel is XSY1 carrying pXS10 (HA epitope alone); NPI46 panel is XSY1 carrying pXS9 (containing the *NPI46* gene fused to the HA epitope). *DAPI*, DAPI staining of DNA; *FITC*, FITC staining using monoclonal anti-HA antibody 12CA5 and FITC-conjugated goat anti-mouse IgG. The NPI46/NSR1 panel shows double immunofluorescent labeling of XSY1 carrying pXS9. *Rhodamine*, Rhodamine staining using the polyclonal anti-HA antibody and lissamine rhodamine-conjugated donkey anti-rabbit IgG; *FITC*, FITC staining using a monoclonal anti-NSR1 antibody and FITC-conjugated goat anti-mouse IgG. Arrows point to the nucleolus region.

or alternatively, other as yet unidentified isomerases provide a functionally redundant role.

The nucleolar proline isomerase, NPI46, belongs to a small group of known isomerases that contain domains separate from their isomerase domain. It is tempting and reasonable to speculate that the function of these additional domains is either in the regulation of isomerase activity or in the cellular localization of the enzyme. Proline isomerases that have no additional domains, like FKBP12, may act on any proteins present in the cytoplasm that contain an X-Pro bond. The addition of other domains to these enzymes may now allow them to interact with a higher specificity to a particular class of proteins. Interestingly, two of the enzymes with additional domains, FKBP52 and NPI46, have much lower activity than FKBP12 when assayed in vitro with a small peptide as the substrate (Peattie et al., 1992). A possibility is that the isomerase domain is somehow masked in FKBP52 and NPI46, and they only become fully active after binding to their natural substrates. FKBP25 has an activity profile closer to FKBP12 and thus its activity may not be regulated, although it does possess a small extra domain that is used to localize the isomerase within the nucleus (Jin and Burakoff, 1993).

The large size of the NH_2-terminal domain of NPI46 (∼75% of the protein) makes it feasible that this single domain could carry out both a regulatory and a localization function. The NH_2-terminal domain of NPI46 is similar to that found in Noppl40, nucleolin, and to some extent, NSRl in both overall structure and the ability of this region to bind NLSs in vitro. We and other laboratories have found that unlike NLSs, nucleolar targeting does not occur via a specific consensus sequence, but rather occurs through specific binding interactions with other proteins and/or nucleic acids. In the case of NSRl, the NH_2 terminus is one of the domains sufficient for the nucleolar accumulation of a hybrid protein (Yan and Mélèse, 1993). There are conflicting results concerning the ability of the NH_2 terminus of nucleolin to allow the accumulation of a hybrid protein in the nucleolus (Schmidt-Zachmann and Nigg, 1993; Creancier et al., 1993). Therefore, the NH_2 terminus of NPI46 is a good candidate for mediating the accumulation of the protein within the nucleolus.

The mammalian nucleolar proteins nucleolin, Noppl40, and the yeast nucleolar proteins NSRl and NPI46 have all been shown to bind NLSs and to contain NH_2 termini that are highly charged. The diversity of the cellular functions associated with the structural domains present in these proteins makes it hard to argue that they all belong to a family of general nuclear transport receptors. However, since all of them are present in the nucleolus, they could be involved specifically in the import of ribosomal proteins or export of preribosome particles. Another possibility, which we consider more likely, is that all of these proteins are involved in the assembly of ribosomal proteins.

Among the nucleolar NLS-binding proteins, nucleolin and NSRl contain well-conserved RNA-recognition motifs: nucleolin has been shown to bind ribosomal RNA in vitro, and NSRl affects rRNA processing (Herrera and Olson, 1986; Lee et al., 1991). Perhaps nucleolin and NSRl bind to pre-rRNA and other nucleolar proteins at the same time, facilitating the assembly of preribosome particles. NPI46, being a nucleolar proline isomerase, likely catalyzes the rearrangement of semifolded or assembling ribosomal proteins, as opposed to catalyzing the initial folding of a newly translated nascent polypeptide chain. The common acidic NH_2 terminus of NPI46, NSRl and nucleolin is likely to be the domain involved in protein–protein interactions within the nucleolus. Given the in vitro NLS-binding ability of these proteins, it is plausible that the protein–protein interaction occurs through recognition of NLSs in vivo because most, if not all, of the proteins in the nucleolus have an NLS. An alternative possibility is that the NLS-binding ability observed mimics the binding of the natural substrate, which may be a highly basic nucleolar protein. Identification of the natural substrate of NPI46 will help us understand how the NH_2 termini of this and other similar nucleolar proteins facilitate their function within the nucleolus.

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References

Anfinsen, C. B. 1973. Principles that govern the folding of protein chains. *Science (Wash. DC).* 181:223–230.

Brandts, J., H. Halvorsen, and M. Brennan. 1975. Consideration of the possibility that the slow step in protein denaturation reactions is due to *cis-trans* isomerism of proline residues. *Biochemistry.* 14:4953–4963.

Créancier, L., H. Prats, C. Zanibellato, F. Amalric, and B. Bugler. 1993. Determination of the functional domains involved in nucleolar targeting of nucleolin. *Mol. Biol. Cell.* 4:1239–1250.

Field, J., J.-I. Nikawa, D. Broek, B. MacDonald, L. Rodgers, I. A. Wilson, R. A. Lerner, and M. Wigler. 1988. Purification of a RAS-responsive adenylyl cyclase complex from *Saccharomyces cerevisiae* by use of an epitope addition method. *Mol. Cell. Biol.* 8:2159–2165.

Flanagan, W. M., B. Corthesy, R. J. Bram, and G. R. Crabtree. 1991. Nuclear association of a T-cell transcription factor blocked by FK-506 and cyclosporin A. *Nature (Lond.).* 352:803–807.

Flynn, G. C., T. G. Chappell, and J. E. Rothman. 1989. Peptide binding and release by proteins implicated as catalysts of protein assembly. *Science (Wash. DC).* 245:385–390.

Galat, A., W. S. Lane, R. F. Standaert, and S. L. Schreiber. 1992. A rapamycin-selective 25-kDa immunophilin. *Biochemistry.* 31:2427–2434.

Gething, M.-J., and J. Sambrook. 1992. Protein folding in the cell. *Nature (Lond.).* 355:33–45.

Hayano, T., N. Takahashi, S. Kato, N. Maki, and M. Suzuki. 1991. Two distinct forms of petidyl-prolyl *cis-trans* isomerase are expressed separately in periplasmic and cytoplasmic compartments of *Escherichia coli* cells. *Biochemistry.* 30:3041–3048.

Heitman, J., N. R. Movva, and M. N. Hall. 1991. Targets for cell cycle arrest by the immunosuppressant rapamycin in yeast. *Science (Wash. DC).* 253:905–909.

Heitman, J., N. R. Movva, and M. N. Hall. 1992. Proline isomerases at the crossroads of protein folding, signal transduction, and immunosuppression. *The New Biologist.* 4:448–460.

Heitman, J., A. Koller, M. E. Cardenas, and M. N. Hall. 1993. Identification of immunosuppressive drug targets in yeast. *Methods Enzymol. Compan.* 5:176–187.

Herrera, A. H., M. O. J. Olson. 1986. Association of protein C23 with rapidly labeled nucleolar RNA. *Biochemistry.* 25:6258–6264.

Hoffman, D. W., C. C. Query, B. L. Golden, S. W. White, and J. D. Keene. 1991. RNA-binding domain of the A protein component of the U1 small nuclear ribonucleoprotein analyzed by NMR spectroscopy is structurally similar to ribosomal proteins. *Proc. Natl. Acad. Sci. USA.* 88:2495–2499.

Ito, H., Y. Jukuda, K. Murata, and A. Kimura. 1983. Transformation of intact yeast cells treated with alkali cations. *J. Bacteriol.* 153:163–168.

Jin, Y. J., and S. J. Burakoff. 1993. The 25-kDa FK506-binding protein is localized in the nucleus and associates with casein kinase II and nucleolin. *Proc. Natl. Acad. Sci. USA.* 90:7769–7773.

Koser, P. L., D. J. Bergsma, R. Cafferkey, W.-K. Eng, M. M. McLaughlin, A. Ferrara, C. Silverman, K. Kasyan, M. J. Bossard, and R. K. Johnson. 1991. The CYP2 gene of *Saccharomyces cerevisiae* encodes a cyclosporin A-sensitive peptidyl-prolyl *cis-trans* isomerase with an N-terminal signal sequence. *Gene (Amst.).* 108:73–80.

Lapeyre, B., H. Bourbon, F. Amalric. 1987. Nucleolin, the major nucleolar protein of growing eukaryotic cells: an unusual protein structure revealed by the nucleotide sequence. *Proc. Natl. Acad. Sci. USA.* 84:1472–1476.

Lee, W.-C., and T. Mélèse. 1989. Identification and characterization of a nuclear localization sequence-binding protein in yeast. *Proc. Natl. Acad. Sci. USA.* 86:8808–8812.

Lee, W.-C., Z. Xue, and T. Mélèse. 1991. The *NSR1* gene encodes a protein that specifically binds nuclear localization sequences and has two RNA recognition motifs. *J. Cell Biol.* 113:1–12.

Liu, J., and C. T. Walsh. 1990. Peptidyl-prolyl *cis-trans*-isomerase from *Escherichia coli*: a periplasmic homolog of cyclophilin that is not inhibited by cyclosporin A. *Proc. Natl. Acad. Sci. USA.* 87:4028–4032.

Megraw, T. L., and C.-B. Chae. 1993. Functional complementarity between the HMG-1 like yeast mitochondrial histone HM and the bacterial histone-like protein HU. *J. Biol. Chem.* 268:12758–12764.

Meier, U. T., and G. Blobel. 1992. Nopp140 shuttles on tracks between nucleolus and cytoplasm. *Cell.* 70:127–138.

Pearson, W. R., and D. J. Lipman. 1988. Improved tools for biological sequence comparison. *Proc. Natl. Acad. Sci. USA.* 85:2444–2448.

Peattie, D. A., M. W. Harding, M. A. Fleming, M. T. DeCenzo, J. A. Lipke, D. J. Livingston, and M. Benasutti. 1992. Expression and characterization of human FKBP52, an immunophilin that associates with the 90-kDa heat shock protein and is a component of steroid receptor complexes. *Proc. Natl. Acad. Sci. USA.* 89:10974–10978.

Pringle, J. R., R. A. Preston, A. E. M. Adams, T. Stearns, D. G. Drubin, B. K. Haarer, and E. W. Jones. 1989. Fluorescence microscopy methods for yeast. *Methods Cell Biol.* 31:357–435.

Robbins, J., S. M. Dilworth, R. A. Laskey, and C. Dingwall. 1991. Two interdependent basic domains in nucleoplasmin nuclear targeting sequence: identification of a class of bipartite nuclear targeting sequence. *Cell.* 64:615–623.

Roof, W. D., S. M. Horne, K. D. Young, and R. Young. 1994. slyD, a host gene required for ϕX174 lysis, is related to the FK506-binding protein family of peptidyl-prolyl *cis-trans*-isomerases. *J. Biol. Chem.* 269:2902–2910.

Rothstein, R. J. 1983. One-step gene disruption in yeast. *Methods Enzymol.* 101:203–211.

Sambrook, J., E. F. Fritsch, and T. Maniatis. 1989. Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

Sanchez, E. R., L. E. Faber, W. J. Henzel and W. B. Pratt. 1990. The 56-59-kilodalton protein identified in untransformed steroid receptor complexes is a unique protein that exists in cytosol in a complex with both the 70- and 90-kilodalton heat shock proteins. *Biochemistry.* 29:5145–5152.

Schmid, F., R. Grafl, A. Wrba, and J. Beintema. 1986. Role of proline peptide bond isomerization in unfolding and refolding of ribonuclease. *Proc. Natl. Acad. Sci. USA.* 83:872–876.

Schmidt-Zachmann, M. S., E. A. Nigg. 1993. Protein localization to the nucleolus: a search for targeting domains in nucleolin. *J. Cell Sci.* 105:799–806.

Sherman, F., J. B. Hicks, and G. R. Fink. 1986. Methods of Yeast Genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

Sikorski, R., and P. Hieter. 1989. A system of shuttle vectors and yeast host strains designed for efficient manipulation of DNA in *Saccharomyces cerevisiae. Genetics.* 122:19–27.

Smith, D. B., and K. S. Johnson. 1988. Single-step purification of polypeptides expressed in *Escherichia coli* as fusions with glutathione-S-transferase. *Gene (Amst.).* 67:31–40.

Snyder, M., S. Elledge, D. Sweetser, R. A. Young, and R. W. Davis. 1987. λgt11: gene isolation with antibody probes and other applications. *Methods Enzymol.* 154:107–128.

Stamnes, M. A., S. L. Rutherford, and C. S. Zuker. 1992. Cyclophilins: a new family of proteins involved in intracellular folding. *Trends Cell Biol.* 2:272–276.

Tai, P. K. K., Y. Maeda, K. Nakao, N. G. Wakim, J. L. Duhring, and L. E. Faber. 1986. A 59-kilodalton protein associated with progestin, estrogen, androgen, and glucocorticoid receptors. *Biochemistry.* 25:5269–5275.

Tropschug, M., I. B. Barthelmess, and W. Neupert. 1989. Sensitivity to cyclosporin A is mediated by cyclophilin in *Neurospora crassa* and *Saccharomyces cerevisiae. Nature (Lond.).* 342:953–955.

Van Duyne, G. D., R. F. Standaert, P. A. Karplus, S. L. Schreiber, and J. Clardy. 1991. Atomic structure of FKBP-FK506, an immunophilin-immunosuppressant complex. *Science (Wash. DC).* 252:839–842.

Wülfing, C., J. Lombardero, and A. Plückthun. 1994. An *Escherichia coli* protein consisting of a domain homologous to FK506-binding proteins (FKBP) and a new metal binding motif. *J. Biol. Chem.* 269:2895–2901.

Xue, Z., X. Shan, B. Lapeyre, and T. Mélèse. 1993. The amino terminus of mammalian nucleolin specifically recognizes SV40 T-antigen type nuclear localization sequences. *Eur. J. Cell Biol.* 62:13–21.

Yan, C., and T. Mélèse. 1993. Multiple regions of NSR1 are sufficient for accumulation of a fusion protein within the nucleolus. *J. Cell Biol.* 123:1081–1091.

Yem, A. W., A. G. Tomasselli, R. L. Heinrikson, H. Zurcher-Neely, V. A. Ruff, R. A. Johnson and M. R. Deibel. 1992. The hsp56 component of steroid receptor complexes binds to immobilized FK506 and shows homology to FKBP-12 and FKBP-13. *J. Biol. Chem.* 267:2868–2871.