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Identification of a Yeast Karyopherin Heterodimer That Targets Import Substrate to Mammalian Nuclear Pore Complexes*

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Targeting of import substrate to nuclear pore complexes of permeabilized vertebrate cells was previously shown to require a protein complex composed of two subunits, termed karyopherin. Yeast contain a homologue of karyopherin α named Srp1p, which was initially identified as a genetic suppressor of mutations in a subunit of RNA polymerase I. To determine whether yeast contain a karyopherin complex that includes Srp1p as the karyopherin α homologue, we genetically replaced Srp1p with a Srp1-Protein A chimera. Cytosol from this strain contained a complex, composed of the chimera and a protein of 95 kDa, that was purified using affinity chromatography on IgG Sepharose. Microsequence analysis showed that the 95-kDa protein was identical with a yeast protein encoded by gene L8300.15 on chromosome XII. Sequence comparison revealed that the L8300.15 gene product is the closest structural homologue of vertebrate karyopherin β. The yeast α and β karyopherin subunits were expressed in *Escherichia coli* and were purified. When combined, they formed a heterodimeric complex and were active in targeting import substrate to nuclear envelopes of mammalian cells. We propose that all karyopherins function as α/β heterodimers.

Several factors that mediate import of proteins into nuclei of permeabilized mammalian cells have been isolated and characterized. A protein complex composed of two subunits termed karyopherin α and β was shown to target substrates that contain a nuclear localization sequence (NLS)[1] to nuclear pore complexes (NPCs) (1–5). A more detailed analysis showed that karyopherin α mediates recognition of the NLS substrate (2, 4, 6), whereas karyopherin β functions to dock the karyopherin α/NLS-substrate complex to a subgroup of nucleoporins that contain peptide repeats (3, 7, 8). Subsequent transport into the nucleus is mediated by the small GTPase Ran (9, 10) and a Ran interacting protein (11). The mechanics of transport and the involvement of other proteins such as Ran-GTPase activating protein(s) (12), Ran-GDP/GTP exchange protein(s) (13), and the heat-shock cognate protein hsc70 (14, 15) remain to be elucidated (16).

In the yeast *Saccharomyces cerevisiae*, protein import into nuclei was examined *in vitro* using spheroplasts that were permeabilized by freeze-thaw (17). In contrast to the mammalian import reaction, docking of import substrate to the yeast nuclear envelope did not require addition of cytosolic proteins (17). To explain this difference, it was proposed that previously identified NLS receptors of yeast (none of them karyopherin homologues) remain tightly bound to the NPCs of freeze-thawed cells and do not need to be added back (17). The recent identification of karyopherin in vertebrates and the fact that karyopherin α is homologous to yeast Srp1p (4, 18–20) suggested that targeting of NLS substrates to NPCs in yeast is mediated by a homologue of vertebrate karyopherin and that Srp1p is the α subunit.

Here we report that yeast Srp1p is indeed the functional homologue of vertebrate karyopherin α and that it can be isolated from yeast cytosol in a heterodimeric complex with a protein of 95 kDa that is a functional homologue of vertebrate karyopherin β. Yeast karyopherin α and β were expressed separately in *Escherichia coli*, and each was purified to homogeneity. Recombinant α and β assembled into a heterodimer *in vitro* and were required in combination for NLS substrate recognition and docking to nuclear envelopes in digitonin-permeabilized mammalian cells. The yeast proteins are therefore termed Kap60p for karyopherin α subunit of 60 kDa and Kap95p for karyopherin β subunit of 95 kDa. The alternate term Kap60p for Srp1p was deemed necessary to avoid further confusion with the previously issued acronym SRP (signal recognition particle) (21).

EXPERIMENTAL PROCEDURES

Strains—*S. cerevisiae* strains used were DF5 (*MATa/MATα trp1–1/trp1–1 ura3-52/ura3-52 his3-Δ200/his3-Δ200 leu2-3, 112/leu2-3, 112 lys2-801/lys2-801 gal/gal*) (22) and the DF5-derived strains CEY1 (*Mata/Matα KAP60/kap60Δ::URA3*), CEY1A (*Mata KAP60*), and CEY1B (*Matα kap60Δ::URA3*, pRS315-Kap60-ProtA). Standard molecular biological and yeast genetic techniques were used (23). To construct CEY1, nucleotides −450 to 1950 that contain the *SRP1/KAP60* open reading frame (ORF) (19) were amplified from *S. cerevisiae* genomic DNA by PCR using the primers 5′-ATT GAT CCC TCG AGG TTA ACT TAA TCG ACC G-3′ and 5′-CTA GGA AGA TCT TTC AGC TGT GGA-3′. The PCR product was digested with *Xho*I and *Bgl*II and inserted into *Xho*I-*Bam*HI-digested pBluescript (Stratagene). The 1.1-kilobase *Hin*dIII-*Eco*RV fragment (nucleotides 149-1255) within the *SRP1/KAP60* ORF was replaced by the 1.1-kilobase *Hin*dIII-*Sma*I fragment that contains the *URA3* gene from vector pJJ244 (24). The *kap60Δ::URA3* deletion allele was transformed as a *Pvu*II fragment into the diploid strain DF5. The replacement of one chromosomal *KAP60* copy by the deletion allele in the Ura$^+$ transformant CEY1 was confirmed by Southern blot hybridization. To express Kap60-ProtA in yeast, nucleotides −450 to 1626 that contain the *SRP1/KAP60* ORF (19) were amplified by PCR from yeast genomic DNA using primers that incorporate a *Xho*I site at the 5′ end and an in-frame *Bam*HI site replacing the stop codon of *SRP1/KAP60*. The PCR fragment was

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[1] The abbreviations used are: NLS, nuclear localization sequence; NPC, nuclear pore complex; GST, glutathione *S*-transferase; IgG Sepharose, immunoglobulin G-Sepharose; PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; PMSF, phenylmethylsulfonyl fluoride.

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inserted into *Xho*I-*Bam*HI-digested *CEN6-ARS-LEU2* vector pRS315 (25) to generate pRS315-Kap60. The segment of the staphylococcal Protein A gene (nucleotides 272-1104) that encodes the five IgG binding domains was amplified from pET8c-pS/protA (26) by PCR using primers that incorporate an in-frame *Bam*HI site at the 5′ end and a stop codon followed by a *Bam*HI site at the 3′ end. The PCR product was digested with *Bam*HI and ligated in pRS315-Kap60 to generate pRS315-Kap60-ProtA, which was used to transform CEY1. Leu$^+$ transformants were selected and sporulated. After tetrad dissection Ura$^+$ Leu$^+$ spores were selected, one of them was named CEY1B. To verify the replacement of the wild-type copy of *SRP1/KAP60* by the plasmid-born *SRP1/KAP60*-ProtA, cell lysates of CEY1B were analyzed by SDS-PAGE and ECL Western blotting (Amersham).

Purification of the Kap60-ProtA/Kap95p Complex—Yeast strain CEY1B was grown in 5 liters of YPD medium at 30 °C for 16 h to early stationary phase. Cells (80 g) were harvested and converted into spheroplasts as described (27), using only Zymolyase 20T. Spheroplasts were lysed in 300 ml of 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM PMSF with a Dounce homogenizer. Cell debris was removed by centrifugation at 12,000 × *g* for 15 min at 4 °C, and the supernatant was clarified by centrifugation at 200,000 × *g* for 15 min at 4 °C. The high speed supernatant (25 mg of protein/ml) was mixed with 3 ml of packed IgG-Sepharose beads (Pharmacia Biotech Inc.) that were equilibrated in loading buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.05% Tween 20, 1 mM PMSF), and the mixture was incubated for 2 h at 4 °C in batch. Beads were transferred to a column and washed with 30 bed volumes of loading buffer and 2 bed volumes of 5 mM NH_4OAc, pH 5.0. Bound proteins were eluted with 10 bed volumes of 0.1 M glycine-HCl, pH 3.0, and neutralized with 1 M Hepes-KOH, pH 7.4. The eluted 90-kDa protein was processed for microsequence analysis as described (28).

Purification of Recombinant Kap60 and Kap90 Proteins from E. coli—*KAP60* and *KAP95* genes were amplified separately from yeast genomic DNA by PCR using synthetic oligonucleotides (5′-CCG GGA TCC ATG GAT AAT GGT ACA GAT TCT TCC ACG AGC A-3′ and 5′-CCG GGA TCC TTA ATT GAA TTG TTG GTT GAC ATT AGA ACC A-3′ for *KAP60*, and 5′-CCG GGA TCC ATG TCC ACC GCT GAA TTT GCT CAA and 5′-CCG GGA TCC TTA TAA GGA TAA TTG ACG CTT CTG TTG-3′ for *KAP95*) that incorporate a *Bam*HI endonuclease restriction site in-frame with the initiation codon and another after the stop codon of each gene. The purified *Bam*HI fragments were ligated into vector pGEX-2TK (Pharmacia Biotech Inc.) to create in-frame fusions with the glutathione *S*-transferase gene (*GST*). Plasmids that contained *GST-KAP60* and *GST-KAP90* gene fusions were introduced into *E. coli* strain BLR (Novagen). Fusion proteins were purified as follows. Reagents used were obtained from Sigma, unless otherwise indicated. Cells were grown in 1 liter of 2 × YTA (Difco Laboratories) at 37 °C to a cell density of 1–2 A_{600} units. Cells were shifted to 28 °C, and isopropyl-1-thio-β-D-galactopyranoside was added to a final concentration of 0.5 mM to induce synthesis of fusion proteins. After 1 h, cells were harvested at 4 °C by centrifugation, and cell pellets were resuspended with 18 ml of chilled lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl). PMSF and lysozyme were added to final concentrations of 0.5 mM and 1 mg/ml, respectively, and the cell suspension was incubated for 15 min at room temperature. Sodium deoxycholate and DNase I were added to final concentrations of 1 mg/ml and 10 μg/ml, respectively, and lysates were incubated again at room temperature for 15 min. Cell debris was removed by centrifugation at 20,000 × *g* for 10 min at 4 °C, and the supernatant was filtered through a 0.45-μm syringe filter (Schleicher and Schuell). The filtrate was mixed with 0.5 ml of packed glutathione-agarose beads that were equilibrated in transport buffer (TB: 20 mM Hepes-KOH, pH 7.4, 110 mM KOAc, 2 mM MgOAc, 1 mM EGTA, 2 mM dithiothreitol), and the mixture was incubated at 4 °C for 1–6 h. Beads were collected by centrifugation at 2,000 × *g* for 2 min at 4 °C and were washed 4 times with 15 ml of TB by repeated resuspension and centrifugation. To elute fusion protein, beads were resuspended in 1 ml of TB with 10 mM reduced glutathione and were incubated for 10 min at 4 °C. Pooled eluates (3 ml) contained fusion protein at an average concentration of 0.5 mg/ml. To cleave Kap proteins from GST, thrombin was added to eluates that had been concentrated (1.5 NIH units of thrombin per 100 μg of GST-Kap60p and 3 NIH units per 100 μg of GST-Kap95p), and the mixture was incubated for 10–30 min at room temperature. Samples were chilled, and PMSF was added to a final concentration of 0.5 mM. Proteins in the samples were resolved in a Superdex 200 FPLC sizing column (Pharmacia Biotech Inc.) to yield near-pure Kap proteins in fractions depleted of GST, thrombin, and uncleaved fusion protein.

Nuclear Docking Assay—Assays were performed as described previously (4, 29) with a few technical variations that are outlined below.

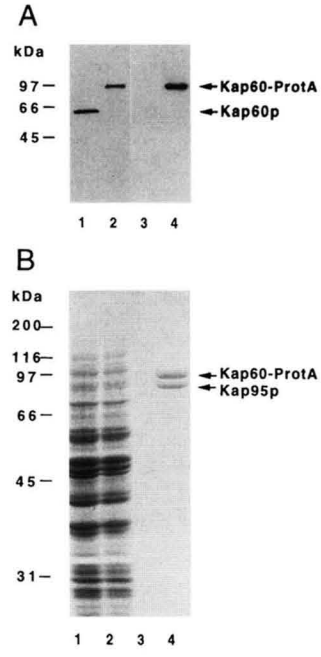

FIG. 1. **Kap60-ProtA can be isolated from yeast cytosol in a complex with Kap95p.** *A*, expression of Kap60-ProtA in yeast. Whole cell lysates (50 μg of protein per lane) from yeast strains CEY1A (*KAP60*) (*lanes 1* and *3*) and CEY1B (*kap60Δ::URA3*, pRS315-Kap60-ProtA) (*lanes 2* and *4*) were subjected to 10% SDS-PAGE and analyzed by immunoblotting using anti-Srp1p (Kap60p) antibodies (19) and ProtA-horseradish peroxidase conjugates (*lanes 1* and *2*) or anti-rabbit IgG-horseradish peroxidase conjugates (*lanes 3* and *4*); horseradish peroxidase conjugates were obtained from Amersham. *B*, co-purification of Kap60-ProtA and Kap95 proteins by IgG-Sepharose affinity chromatography. Fractions were analyzed by 10% SDS-PAGE and Coomassie Blue staining: *lane 1*, high-speed supernatant of a cell extract of CEY1B (50 μg of protein); *lane 2*, flow-through (50 μg of protein); *lane 3*, combined wash fractions; *lane 4*, concentrated acid eluate (3 μg of protein).

Buffalo rat liver cells were permeabilized in 50 μg/ml digitonin in TB, during a 5-min incubation at room temperature. Cells were then placed on ice and washed twice with chilled TB. Reactions were incubated for 15 min on ice and contained additions as indicated in the figure legends. Completed reactions were washed twice with chilled TB, and cells were fixed with 3% formaldehyde in TB. Cells were visualized and photographed under a Zeiss Axiophot microscope (Carl Zeiss, Inc.).

RESULTS

To facilitate isolation of yeast Srp1p/Kap60p and to determine whether it, like vertebrate karyopherin α, is associated with a β subunit, we genetically replaced cellular Kap60p with a Kap60p-Protein A chimera that could be easily isolated using IgG-Sepharose chromatography. Immunoblot analysis of wild-type and mutant cell lysates using anti-Srp1p(Kap60p) antibodies (19) showed that Kap60p was absent in mutant cells (Fig. 1*A*, compare *lanes 1* and *2*) but was replaced by the Kap60p-Protein A chimera (*lane 2*). When the same blot was probed with anti-rabbit IgG-horseradish peroxidase, the chimera (*lane 4*) but not wild-type Kap60p (*lane 3*) was detected.

The tagging of Kap60p with Protein A allowed its purification from a high-speed supernatant of a cell lysate using IgG Sepharose chromatography (Fig. 1*B*). Acid elution of tightly bound proteins and subsequent analysis of the eluate by SDS-PAGE showed two major bands in approximately stoichiometric amounts (*lane 4*). The slower moving polypeptide was the Kap60p-Protein A chimera, as determined by immunoblot analysis (data not shown). To determine the chemical nature of the faster moving protein, we subjected it to microsequence analysis and obtained a peptide (KQFYGQDWVIDYKRTRS-GQLFSQATKD) that was identical with the C terminus of a

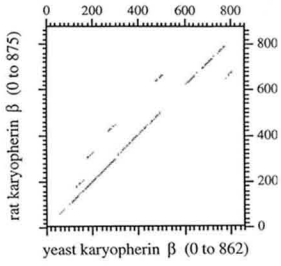

FIG. 2. **Dot-matrix comparison of yeast and rat karyopherin β sequences.** Stringency: 10; window: 30 (Lasergene, DNAstar). The amino acid sequences of yeast karyopherin β (Kap95p) and rat karyopherin β are available in GenBank™, accession numbers U19028 and L38644, respectively.

95-kDa protein of unknown function, encoded by the essential[2] gene L8300.15 on yeast chromosome XII (GenBank™ accession number U19028). A protein homology search in GenBank identified rat karyopherin β as the closest structural homologue of the L8300.15 gene product, with 33% identity and 63% similarity (Fig. 2). Because of the high degree of similarity to vertebrate karyopherin β and because of its apparent stoichiometric association with yeast karyopherin α (Kap60p), we termed this protein Kap95p. Taken together the data suggest that yeast, like vertebrate cells, possess a cytosolic karyopherin complex.

To determine whether yeast karyopherin functions in targeting import substrate to NPCs, we expressed the two subunits separately in *E. coli* and purified each to homogeneity. Molecular sieving of isolated Kap60p and Kap95p showed that each migrated as expected for a monomeric globular protein (Fig. 3, *A* and *B*). When the two subunits were incubated together at 0 °C and the mixture was subjected to molecular sieving, approximately 50% of the subunits assembled into a heterodimeric complex (Fig. 3*C*). To test whether recombinant yeast karyopherin subunits are competent to target an import substrate to NPCs, we employed a well characterized assay that uses digitonin-permeabilized mammalian cells (29). Indeed, as previously shown for vertebrate karyopherin subunits (1, 2, 4, 8), both yeast subunits were required to obtain docking of import substrate at the nuclear envelope (Fig. 4, *panel 3*). Neither subunit alone was sufficient to obtain docking (*panels 1* and *2*). Thus, we conclude that Kap60p and Kap95p are the functional homologues of karyopherin in yeast.

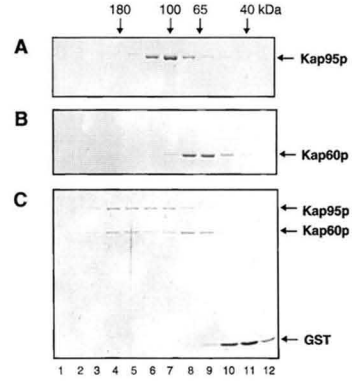

FIG. 3. **Recombinant Kap60 and Kap95 proteins assemble into a 180-kDa heterodimer.** *A*, 100 μg of isolated GST-Kap95p were digested with thrombin and applied to a Superdex-200 FPLC sizing column, as described under "Experimental Procedures." Proteins in eluted fractions were resolved by electrophoresis in a 9% polyacrylamide gel and were stained with Coomassie Blue. *B*, 100 μg of isolated GST-Kap60p were digested, fractionated, and analyzed as described in *A*. *C*, the digested products described in *A* and *B* were mixed and incubated for 30 min on ice, prior to fractionation and analysis as described in *A*.

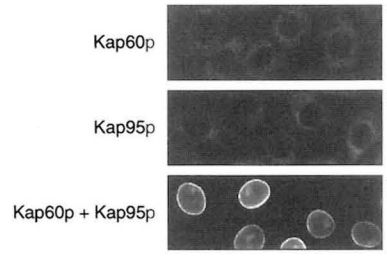

FIG. 4. **The Kap60/Kap95 heterodimer promotes docking of import substrates to nuclear envelopes.** Nuclear docking assays were performed using digitonin-permeabilized Buffalo rat liver cells as described under "Experimental Procedures." All reaction mixtures contained 20 μg/ml fluorescent import substrate (TRITC-labeled human serum albumin with conjugated peptides that contain the NLS of SV40 large T-antigen (29), 1 mg/ml bovine serum albumin, 1 mM ATP, 0.1 mM GTP, 5 mM phosphocreatine, 20 units/ml creatine phosphokinase, and protein fractions as indicated below). Reactions contained 15 μg/ml (250 nM) purified Kap60p, 15 μg/ml (150 nM) purified Kap95p, or both, as indicated. Cells were visualized and photographed under a Zeiss Axiophot microscope. All panels correspond to reactions that were incubated, photographed, and printed under identical conditions.

DISCUSSION

The data presented here demonstrate that yeast Srp1p is the functional homologue of vertebrate karyopherin α and that it is present in yeast cytosol in a heterodimeric complex with a novel 95-kDa protein (Fig. 1). We show the latter to be the functional homologue of vertebrate karyopherin β (Figs. 2 and 4). We demonstrate that recombinant yeast karyopherin α (Kap60p) and karyopherin β (Kap95p) assemble into a heterodimeric complex *in vitro* (Fig. 3) and are required in combination to target import substrates to nuclear envelopes of permeabilized mammalian cells (Fig. 4). Based on these data, we propose that yeast karyopherin mediates the docking of import substrate to nuclear envelopes of yeast. Proof awaits the development of a karyopherin-dependent import assay in yeast (see introduction). Vertebrates contain several homologues of karyopherin α (3–6, 18). Other yeast homologues of karyopherin α have not been identified, but there appear to be several homologues of karyopherin β.[2]

Two lines of evidence suggest that yeast karyopherin functions as an α/β heterodimer. First, neither Kap60p nor Kap95p displayed docking activity when added alone (Fig. 4, *panels 1* and *2*); docking was only detected when Kap proteins were mixed prior to a reaction (Fig. 4, *panel 3*), under conditions that allow the formation of a 180-kDa heterodimer (Fig. 3*C*) (see below). Second, Kap60 and Kap95 proteins were isolated from yeast cytosol as a heterodimeric complex (Fig. 1). We concluded that yeast karyopherin is a heterodimer based on the expected molecular size of a 1:1 complex (65 + 100 = 165 kDa) (see Fig. 3) and on the equimolar amount of Coomassie Blue stain adsorbed by each subunit in the complex (Figs. 1*B* and 3*C*). Our conclusion provides proof of our previous proposal that karyopherins function as α/β heterodimers (3, 4, 8) and is fully consistent with the results of others in that two distinct proteins are required to target NLS substrates to NPCs (1, 2, 5).

We believe that Kap60p functions like vertebrate karyopherin α to recognize the NLS substrate (4), and Kap95p functions like vertebrate karyopherin β (3) to dock the Kap60p/NLS-substrate complex to nucleoporins that contain peptide repeats (3, 7, 8). We therefore suspect that the reported genetic and physical interaction between Srp1p and the nucleoporin Nup1p (30) is indirectly mediated by Kap95p; indeed, the uncharacterized 95-kDa protein that co-purifies with Nup1 and Srp1 proteins (30) is probably Kap95p. Docking of yeast karyo-

[2] M. Rout, G. Blobel, and J. Aitchison, unpublished results.

pherin to multiple sites on the NPC would explain previous immunofluorescence data that show co-localization of Srp1p and nucleoporins in a punctate pattern on the nuclear surface (19). We recently showed that vertebrate karyopherin α enters the nucleus, whereas karyopherin β remains at the NPC (8). If Kap60p functions in a similar manner in yeast, it would explain the finding that Srp1p is also localized in the nucleoplasm (19).

Why was Srp1p/Kap60p detected as a genetic suppressor of mutations in a subunit of RNA polymerase I (19)? Although at present there is no obvious answer, it is likely that Kap60p performs a critical step in the assembly of RNA polymerase I by mediating nuclear import of polymerase subunits.

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