Cloning and characterization of human karyopherin *β*3

(nucleoporin/Ran-GTP/ribosomal proteins)

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ABSTRACT   Nuclear import of classical nuclear localization sequence-bearing proteins is mediated by karyopherin *α*/*β*1 heterodimers. A second nuclear import pathway, mediated by karyopherin *β*2 (transportin), recently was described for mRNA-binding proteins. Here we report the cloning and characterization of human karyopherin *β*3, which may be involved in a third pathway for nuclear import. Karyopherin *β*3 was localized mainly to the cytosol and the nucleus, particularly the nuclear rim. It bound to several of the repeat-containing nucleoporins (Nup358, Nup214, Nup153, Nup98, and p62) in overlay and solution-binding assays and was competed away by karyopherin *β*1. For Nup98, we localized this binding to the peptide repeat-containing region. Karyopherin *β*3 contains two putative Ran-binding homology regions and bound to Ran-GTP in a solution-binding assay with much higher affinity than to Ran-GDP. Furthermore, it interacted with two ribosomal proteins in an overlay assay. We suggest that karyopherin *β*3 is a nuclear transport factor that may mediate the import of some ribosomal proteins into the nucleus.

The nuclear import of different subsets of proteins appears to proceed via several distinct import pathways. Proteins bearing a traditional nuclear localization sequence are bound by a heterodimer consisting of karyopherin *α* and karyopherin *β*1, which then docks at the nuclear pore complex (1–11). Karyopherin *α* binds to the nuclear localization sequence of the import substrate, whereas docking at the nuclear pore complex is mediated by interaction of karyopherin *β*1 with nucleoporins. Several nucleoporins contain characteristic peptide repeat regions (12). Karyopherin *β*1 docks at the nuclear pore complex by interacting with the repeat regions of these nucleoporins (3, 13–15). Docking is followed by an energy-dependent translocation step that requires the GTPase Ran and p10 (16–19). Ran-GTP binds to karyopherin *β*1 leading to the dissociation of the karyopherin *α*/*β* heterodimer and translocation of karyopherin *α* and the import substrate into the nucleus (14, 20–22).

A separate nuclear import pathway recently was described for certain mRNA-binding proteins in yeast (23) and mammalian cells (24, 25). In this pathway, the substrate-binding and docking/import functions are performed by a single molecule of the karyopherin *β* family. The yeast homologue, Kap104p (karyopherin *β*2), binds to the mRNA-binding proteins Nab2p and Nab4p and to repeat-containing nucleoporins, and is required for the nuclear localization of Nab2p. Two other proteins with a high degree of similarity to karyopherin *β*1, Kap121p (also called Pse1p or karyopherin *β*3) and Kap123p (karyopherin *β*4), have been identified in yeast and are

involved in the import of ribosomal and other proteins (M. Rout, G.B., and J. Aitchison, unpublished data). Pse1p originally was described as an enhancer of protein secretion (26).

Here we describe the cloning and characterization of the human homologue of Kap121p (Pse1p). We show by immunofluorescence that it localizes to the cytosol, nuclear rim, and nucleolus of HeLa cells. It binds to repeat-containing nucleoporins in overlay assays and solution-binding assays and can be competed away by excess karyopherin *β*1. Using Nup98 fragments, we show that this binding is specific to the repeat-containing region of the nucleoporin. It also contains putative Ran-binding sites and binds to Ran-GTP in a solution-binding assay. These findings strongly suggest that this protein is a nuclear transport factor of the karyopherin *β* family. We therefore have named it karyopherin *β*3. Overlay assays show binding of karyopherin *β*3 to two ribosomal proteins, RL23 and RL13. We suggest a role for karyopherin *β*3 in the nuclear transport of ribosomal proteins.

MATERIALS AND METHODS

Cloning of Karyopherin *β*3 cDNA. A human bone marrow 5′ Stretch Plus cDNA library in λgt10 (CLONTECH) was used. The phage were plated according to manufacturer's instructions in 150-mm plates at a density of 30,000 plaques per plate with C600 *Hfl* bacteria as a host. A single-stranded antisense oligonucleotide, 5′-GGGCAAGCTGGCGTTG-CATATTGTTGAGGCTAGTGTCTCCACACAACTTTA-GACTTAGCTG-3′, corresponding to bases 174–234 of the expressed sequence tag (EST) database sequence H13499 was labeled with ^{32}P using polynucleotide kinase and [γ-^{32}P]ATP. This sequence was chosen from among several EST sequences with similarity to yeast Kap121p (Pse1p or karyopherin *β*3), because it matched sequences closer to the N terminus of yeast Kap121p, thus making it more likely to obtain a full-length clone. Duplicate replicas of the plates on nitrocellulose membrane were screened by plaque hybridization according to standard protocols (27). Four independent overlapping clones were obtained and sequenced, one of which (clone 3) matched the full length of Kap121p and predicted a protein of 123,656 daltons.

Expression of Recombinant Proteins and Antibody Production. The cDNA clone 25814 from the EST database was purchased from the IMAGE consortium, and its insert was amplified with two PCR primers: 5′-CGATTCTAGGATCC-GTGTGACAACAGCCCAGAAGT-3′ and 5′-TGAACAG-TCGTCGACGTGAATGAGAGACTACTGCC-3′ introducing a *Bam*HI and a *Sal*I site at the 5′ and 3′ ends, respectively.

The insert that encodes the C-terminal 183 amino acids of human karyopherin β3 then was subcloned in-frame into the *Bam*HI and *Sal*I sites of the pQE-31 vector (Qiagen) downstream of a sequence encoding six histidine residues. Recombinant protein was expressed in *Escherichia coli* M15 cells and purified by nickel affinity chromatography according to manufacturer's instructions. Polyclonal rabbit antiserum to the recombinant protein was produced at a commercial facility (HRP, Denver, PA). For affinity purification of the antibody, C-terminal karyopherin β3 was attached to Affi-Gel-15 (Bio-Rad) according to manufacturer's instructions at 4 mg/ml of packed beads. Antiserum was incubated with the beads overnight at 4°C with turning end over end. The beads were washed six times with 10 vol of PBS, and the antibody eluted with 0.1 M glycine at pH 2.5 followed by immediate neutralization with 1/10 vol of 1 M Tris at pH 8.

The full-length karyopherin β3 coding sequence was amplified by PCR from the λ phage clone 3 with a 5′ *Sma*I-containing primer 5′-CGATTCTACCCGGGTCATGGCGG-CGGCCGCGGCGGGAGC-3′ and a 3′ *Xho*I-containing primer 5′-TGAACAGTCCTCGAGGTTGTGTGTGGCTCTAC-AGC-3′ and was subcloned into the corresponding sites of the pGEX4T-3 vector (Pharmacia). This produced a glutathione *S*-transferase (GST)-karyopherin β3 fusion protein, which was purified from *E. coli* BL21 bacteria by binding to glutathione-Sepharose 4B beads. The recombinant protein was recovered from the beads either by elution with 10 mM reduced glutathione in 50 mM Tris·HCl at pH 8 or by cleavage with 6 NIH units of thrombin (Sigma) according to manufacturer's instructions. The former yields a GST-karyopherin β3 fusion protein while the latter yields karyopherin β3 only.

Immunoblotting. HeLa cytosol was purchased from Cellex (Minneapolis, MN). Proteins were transferred after SDS/PAGE onto nitrocellulose membrane in a Mini-Protean apparatus (Bio-Rad) at 300 mA for 3 h at room temperature with an ice pack. Transfer buffer consisted of 25 mM Tris base, 192 mM glycine, 0.025% SDS, and 20% methanol. After transfer, the membrane was stained in 0.1% amido black in 45% methanol, and 10% acetic acid and destained in water. The membrane was dried and blocked overnight in 20 mM Tris, 137 mM NaCl, 0.1% Tween 20 (TBS-T) with 5% nonfat dry milk (Carnation) at 4°C with rocking. All washes were performed in TBS-T and consisted of two quick rinses followed by three washes at room temperature for 3 min each with rocking. The membrane was washed with TBS-T and incubated with primary antibody in TBS-T with 5% milk for 1 h at room temperature with rocking. Anti-karyopherin β3 affinity purified antibody (384AP) was used at 1:250 dilution; anti-Ran antibody (16) was used at 1:500 dilution. The membrane then was washed and incubated for 30 min at room temperature with rocking in 1:5,000 secondary anti-rabbit Ig horseradish peroxidase-conjugated antibody (Amersham) in TBS-T with 5% milk. This was followed by a more extensive wash (with five 3-min washes) and detection with a Super Signal detection kit (Pierce) and exposure to Biomax film (Kodak).

Immunofluorescence Microscopy. HeLa cells were grown overnight on Teflon-coated 10-well glass slides (Polysciences). The slides were washed three times in transport buffer (20 mM Hepes/110 mM potassium acetate/2 mM magnesium acetate/1 mM EGTA, pH 7.3) with 2 mM DTT. They then were incubated with transport buffer/DTT with or without 35 μg/ml digitonin (Gallard-Schlesinger) for 10 min at room temperature. After washing three times with transport buffer/DTT, the slides were fixed for 30 min in 2% formaldehyde in transport buffer/DTT, washed three times in TBS-T, and permeabilized for 3 min in acetone at −20°C. The slides were washed three times with TBS-T and blocked in TBS-T with 5% milk for 30 min at room temperature followed by three washes in TBS-T and a 1-h incubation at room temperature with 1:200 384AP antibody in TBS-T with 5% milk. After five more

washes with TBS-T, Cy3-conjugated donkey anti-rabbit secondary antibody (Jackson ImmunoResearch) was added at 1:200 in TBS-T with 5% milk, and the slides were incubated for 30 min at room temperature. The slides finally were washed six times in TBS-T, and coverslips were mounted with 80% glycerol/50 mM Tris, pH 8/0.1% *p*-phenylenediamine. Images were captured from a Zeiss Axiophot fluorescence microscope with a digital Sony DKC-5000 camera and saved on a Power Macintosh 7200 computer using ADOBE PHOTOSHOP 3.0.5 software.

Overlay Blot Assay. Nuclear envelope proteins were prepared as described (28). Nup98 and its deletion constructs were a gift from A. Radu (The Rockefeller University) (13). Ribosomal proteins were prepared as described below. Overlay assay was performed essentially as described (23). Nitrocellulose blots prepared as described above were blocked in transport buffer with 0.1% Tween 20 and 5% milk (TB-T/milk) at room temperature for 1 h. All incubations were done on a rocking platform. All washes consisted of two quick rinses and a 1-min wash with rocking in TB-T/milk. One hundred micrograms per ml karyopherin β3 or 25 μg/ml karyopherin β1 in TB-T/milk was added, and the blots were incubated overnight at 4°C. The blots were washed and incubated with 1:250 dilution of 384AP antibody or 1:1,000 dilution of anti-karyopherin β1 antibody (3) in TB-T/milk for 1 h at room temperature. Another wash was followed by a 1-h incubation at room temperature with 1:500 secondary anti-rabbit Ig horseradish peroxidase-conjugated antibody (Amersham) in TB-T/milk. After a final wash and two quick rinses with TB-T without milk, the signal was detected as described for immunoblotting.

Solution-Binding Assay. Recombinant GST-karyopherin β3 fusion protein was bound to glutathione-Sepharose 4B beads at 1 μg/10 μl of beads in TB-T by turning end over end at room temperature for 30 min. Two micrograms of Nup98 (13) with or without 5 μg of karyopherin β1 was added in 30 μl TB-T to the beads and incubated with turning end on end for 45 min at room temperature. The beads were spun down and washed twice with TB-T, suspended in SDS sample buffer, and analyzed by SDS/PAGE. For Ran-binding assays, loading of Ran with GDP or GTP and the binding assays were performed essentially as described (14, 29). One microgram of recombinant Ran was loaded with either GDP or GTP in a 10-μl reaction containing 100 mM Hepes at pH 7.3, 100 mM potassium acetate, 10 mM GDP or GTP, and 5 mM EDTA. The mixtures were incubated on ice for 30 min, brought to 20 mM MgCl₂, and incubated for another 10 min on ice. Excess nucleotides were removed by passage through G-50 Micro Columns (Pharmacia) pre-equilibrated with TB-T. The mixtures incubated with GST-karyopherin β3 beads as described above and the bound and half of the unbound fractions were analyzed by SDS/PAGE followed by immunoblotting with anti-Ran antibody as described above.

Preparation of Ribosomal Proteins. Rat livers were processed as described (30), but after filtering the homogenate through cheesecloth, it was centrifuged at 800 × g, and the supernatant was collected and stored frozen. After thawing, this crude cytosol was centrifuged at 12,000 × g for 20 min at 4°C. The supernatant was layered over a step gradient of 1.6 M sucrose and 2.0 M sucrose in TB-T and centrifuged overnight at 200,000 × g. The ribosomal pellets were resuspended in TB-T/DTT, frozen in liquid nitrogen in aliquots, and stored at −80°C. Ribosomal protein separation was performed by reverse-phase HPLC essentially as described (31). One milligram of ribosomes was precipitated with trichloroacetic acid and resuspended in 100 μl of 4% SDS in 200 mM Tris at pH 7.4 by sonication. Four hundred microliters of 20 mM DTT in 200 mM Tris at pH 7.4 was added, and the mixture was heated at 60°C for 10 min. The proteins were brought up to 4 ml in 200 mM Tris at pH 7.4 and loaded on a C-4 100 × 2.1 mm

Cell Biology: Yaseen and Blobel

Proc. Natl. Acad. Sci. USA 94 (1997)   4453

Aquapore reverse-phase HPLC column (Perkin–Elmer). Ribosomal proteins were eluted with a 6–35% acetonitrile gradient in 60% formic acid. Forty-five 0.8-ml fractions were collected, dried down in a Speed Vac Concentrator (Savant), and resuspended in SDS sample buffer. The proteins were separated by SDS/PAGE and transferred either to nitrocellulose for overlay assays or to poly(vinylidene difluoride) membrane for internal peptide sequencing at the Protein/DNA Technology Center at The Rockefeller University.

RESULTS

Cloning of Karyopherin β3 cDNA. The EST database was searched using the Basic Local Alignment Search Tool (BLAST) algorithm with the tblastn program for sequences similar to yeast Kap121p (Pse1p). Several sequences were found with similarity to Kap121p, some of which were to a lesser extent similar to Kap123p. No sequences were found with significant similarity to Kap 123p besides those with a stronger similarity to Kap121p.

A radiolabeled anti-sense oligonucleotide based on one of the EST sequences (see *Materials and Methods*) was used to probe a human bone marrow λgt-10 library. Four overlapping clones were obtained and sequenced, one of which encoded a 1,097 amino acid protein with a predicted molecular mass of 123,656 daltons (Fig. 1). Based on its length, full-length homology to Kap121p (Fig. 1*A*), and comigration of its recombinant product with native karyopherin β3 (Fig. 2*B*), this clone probably encodes full-length karyopherin β3.

Comparison of the predicted amino acid sequence of human karyopherin β3 to yeast Kap121p (Fig. 1*A*) by the Clustal method reveals 65.2% similarity and 28.3% identity along the entire length of the protein. Furthermore, human karyopherin β3 is 58.9% homologous and 23% identical to yeast Kap123p (not shown). There is a lesser degree of similarity between human karyopherin β1 and human karyopherin β3 with 44.4% similarity and 17.6% identity (Fig. 1*B*). However, a high degree of homology exists between two putative Ran-GTP binding sites in karyopherin β1 (21) and their counterparts in karyopherin β3 (Fig. 1*C*). Human karyopherin β3 is also similar to human karyopherin β2 (transportin) with 44.1% similarity and 16.5% identity (not shown).

Expression of Recombinant Karyopherin β3 in *E. coli*. The coding sequence for the C-terminal 183 amino acids of karyopherin β3 was amplified by PCR and subcloned into the *Bam*HI and *Sal*I sites of the pQE-31 bacterial expression vector downstream of an in-frame histidine tag sequence. The fusion protein (Fig. 2*A*) was purified by nickel affinity chromatography and used to immunize a rabbit. The resulting antibody was affinity purified by binding to the same C-
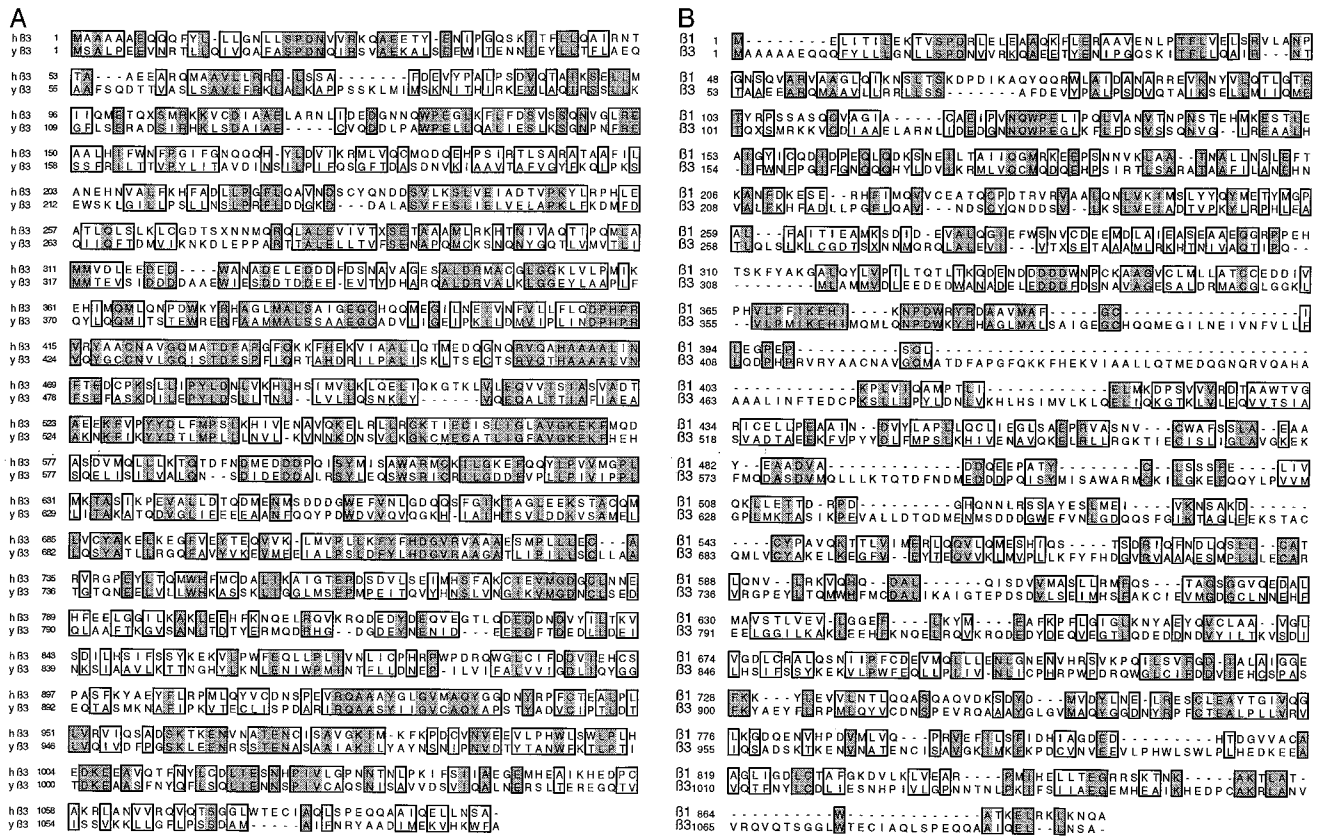
A

B

C

RanBP1	41E-EDEEE-------------64PEWKER
Karyopherin β1	333DENDDDDD----------377PDWRYR
Karyopherin β3	325ADELEDDD----------370PDWKYR

FIG. 1.   Human karyopherin β3 is homologous to yeast Kap121p and human karyopherin β1. (*A*) The amino acid sequence of human karyopherin β3 (hβ3) was aligned with that of Kap121p (yβ3) by the Clustal method using the Multiple Sequence Alignment module of the Lasergene software (DNASTAR). The aligned sequences were imported into SeqVu 1.1 (Garvan Institute) to highlight homologies (boxed sequences) and identities (shaded sequences); the setting used for homologies was GES scale at 85%. (*B*) The amino acid sequences of human karyopherin β1 and β3 were aligned as in *A*. (*C*) Comparison of Ran binding regions in RanBP1 (32), human karyopherin β1 (21) and β3; the numbers indicate amino acid positions.
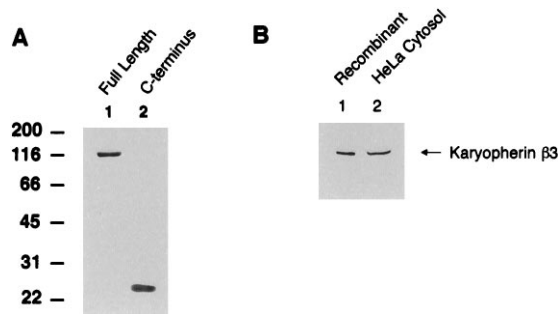
FIG. 2. Recombinant karyopherin β3. (*A*) Full-length (lane 1) and C-terminal 183 amino acids (lane 2) of karyopherin β3 were subjected to SDS/PAGE on a 5–20% acrylamide gradient gel and stained with Coomassie blue. Places of molecular mass markers are indicated to the left. (*B*) Fifty nanograms of recombinant karyopherin β3 (lane 1) and 40 μg of HeLa cytosol S100 fraction (lane 2) were electrophoresed, transferred to nitrocellulose, and immunoblotted with anti-karyopherin β3 antibody 384AP, and the signal was detected by chemiluminescence.

terminal karyopherin β3 fragment immobilized on Affi-Gel-15. This affinity-purified antibody (384AP) was used in all blotting and immunofluorescence experiments described below.

The complete coding sequence of karyopherin β3 was amplified by PCR and subcloned into the *Sma*I and *Xho*I sites of pGEX4T3 3′ to the GST gene to produce a GST-karyopherin β3 fusion protein. A thrombin site between the GST moiety and karyopherin β3 was used to cleave the karyopherin β3 (Fig. 2*A*) off the glutathione beads as described in *Materials and Methods*.

Immunoblotting of HeLa cell cytosol with 384AP antibody showed a single band that comigrates with recombinant full-length karyopherin β3 (Fig. 2*B*).

Immunofluorescence Localization of Karyopherin β3. HeLa cells grown on a 10-well Teflon-coated glass slide were stained with 384AP antibody (Fig. 3). Either intact or digitonin-permeabilized HeLa cells were fixed in 2% formaldehyde followed by acetone permeabilization. Primary staining with 384AP antibody followed by Cy3-conjugated secondary antibody revealed diffuse cytoplasmic staining with perinuclear accentuation in intact HeLa cells. Digitonin treatment resulted in the disappearance of most of the cytoplasmic staining, revealing a distinct nuclear rim signal with variable nucleolar staining.

Binding of Karyopherin β3 to Repeat-Containing Nucleoporins. Karyopherin β1 is known to dock at the nuclear rim by binding to several of the repeat nucleoporins (3). Because karyopherin β3 is homologous to karyopherin β1 (Fig. 1*B*) and localizes to the nuclear rim (Fig. 3), we sought to determine whether it also binds to repeat nucleoporins in an overlay assay. Nuclear envelope proteins were electrophoresed, transferred to nitrocellulose, and probed with recombinant full-length karyopherin β3 or β1 followed by detection with the appropriate primary antibody and horseradish peroxidase-
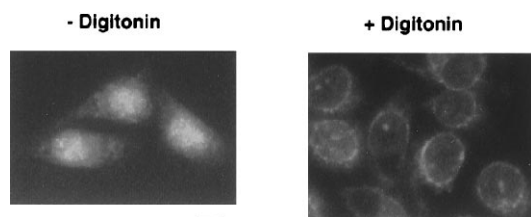

FIG. 3. Immunofluorescence staining for karyopherin β3. HeLa cells treated with or without digitonin as indicated were incubated with anti-karyopherin β3 primary antibody and Cy3-conjugated secondary antibody. (Bar = 10 μm.)

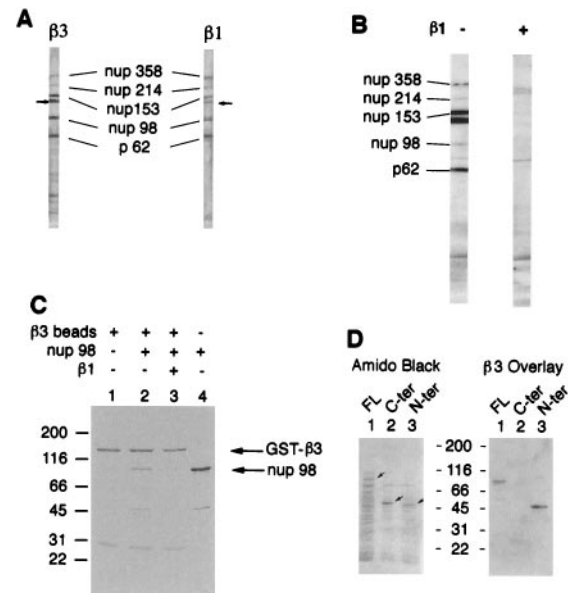FIG. 4. Karyopherin β3 and karyopherin β1 compete for binding to repeat nucleoporins in overlay assay and in solution. (*A*) Nuclear envelope proteins were electrophoresed and transferred to nitrocellulose. Karyopherin β1 or β3 was added followed by the appropriate primary antibody and a secondary horseradish peroxidase-conjugated antibody. The signal was detected by chemiluminescence. The small arrows indicate a previously observed (3) unidentified band that interacts with both karyopherin β1 and β3. (*B*) Nuclear envelope protein blots were subjected to overlay assay with karyopherin β3 as in *A* but in the presence or absence of 10-fold molar excess of karyopherin β1 as indicated. The control lane (without karyopherin β1) contained glutathione elution buffer (see *Materials and Methods*) to control for the presence of this buffer in the karyopherin β1 preparation. The weak Nup98 signal in this lane may be due to the glutathione tripeptide competing with Nup98 for binding to karyopherin β3. (*C*) GST-karyopherin β3 fusion protein was bound to glutathione beads (lane 1), and Nup98 was added in the absence (lane 2) or presence (lane 3) of 5-fold molar excess of karyopherin β3. Lane 4 shows the Nup98 preparation in the absence of beads. (*D*) Bacterial lysates containing recombinant near-full length Nup98 (13) (lane 1), its C terminus (lane 2), or its repeat-containing N terminus (lane 3) were electrophoresed and transferred to nitrocellulose. The blot was stained with amido black (*Left*) and subjected to overlay assay with karyopherin β3 as in *A* (*Right*). Small arrows indicate the positions of the Nup98 bands in the amido black-stained gel.

conjugated secondary antibody (Fig. 4*A*). As previously shown (3), recombinant karyopherin β1 showed more or less equal reactivity with Nup358, Nup214, Nup153, Nup98, and p62 (Fig. 4*A*). Karyopherin β3 bound to bands comigrating with those interacting with karyopherin β1, but with somewhat weaker reactivity to Nup214 and Nup358. Binding of karyopherin β3 in this assay was competed away by a 10-fold molar excess of karyopherin β1 (Fig. 4*B*) indicating that the two karyopherins bind to the same nucleoporins at identical or overlapping sites. To confirm these findings with nondenatured proteins, a solution-binding assay was performed (Fig. 4*C*). Full-length Nup98 bound to GST-karyopherin β3 fusion protein immobilized on glutathione beads, and the binding was also strongly inhibited by adding excess karyopherin β1 to the reaction.

To further characterize the karyopherin β3-binding site on nucleoporins, recombinant near-full-length Nup98, its repeat-containing N-terminal portion, and its nonrepeat C-terminal portion were used (13). Bacterial lysates containing each of these proteins were electrophoresed, transferred to nitrocellulose, and subjected to overlay assay with recombinant karyopherin β3 as described above (Fig. 4*D*). Binding was observed to Nup98 and to its repeat-containing N-terminal portion, but
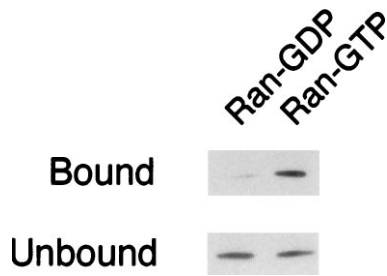
FIG. 5.   Karyopherin β3 binds to Ran-GTP. GST-karyopherin β3 fusion protein was immobilized on beads as in Fig. 4*C* and either Ran-GDP or Ran-GTP was added. The bound and half of the unbound fractions were electrophoresed and subjected to immunoblotting with anti-Ran antibody.

not to the C-terminal nonrepeat region, indicating that karyopherin β3 binds to the repeat region of nucleoporins.

Binding of Karyopherin β3 to Ran-GTP. Karyopherin β3 contains two putative Ran-binding consensus sequences (Fig. 1*C*). To determine whether Ran binds to recombinant karyopherin β3, a solution binding assay was used. Recombinant GST-karyopherin β3 fusion protein was immobilized on glutathione beads, and recombinant Ran that had been previously loaded with either GDP or GTP was added. The bound and half of the unbound fractions were electrophoresed, transferred to nitrocellulose, and immunoblotting was performed with anti-Ran antibody (Fig. 5). Ran-GTP showed significant binding to karyopherin β3, whereas binding of Ran-GDP was minimal.

Karyopherin β3 Binds to Ribosomal Proteins in an Overlay Assay. As discussed below, data from yeast (M. Rout, G.B., and J. Aitchison, unpublished data) indicate that Kap123p is involved in the nuclear import of ribosomal proteins and that Kap121p can overtake that function in the absence of Kap123p. As karyopherin β3 is homologous to both Kap121p and Kap123p, and no other closer mammalian homologue for Kap123p has so far been detected, it was of interest to determine whether karyopherin β3 can bind to ribosomal proteins. Ribosomal proteins from rat liver were separated by reverse-phase HPLC, electrophoresed, transferred to nitrocellulose, and an overlay assay was performed with recombinant karyopherin β3 (Fig. 6). The two ribosomal proteins that reacted with karyopherin β3 were identified by internal peptide sequencing after preparative electrophoresis and transfer to poly(vinylidene difluoride) membrane. The higher molecular mass band (lane 4) was identified as rat ribosomal protein RL13 (33) and the lower molecular mass band (lane 8) as rat ribosomal protein RL23 (34).

DISCUSSION

Recent evidence points to the presence of more than one distinct pathway for the nuclear import of proteins. It appears
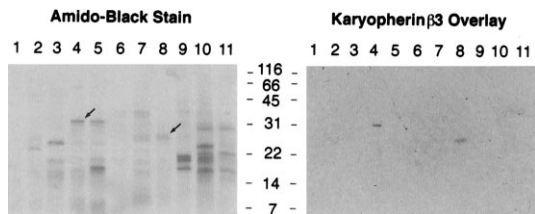

FIG. 6.   Karyopherin β3 binds to ribosomal proteins in an overlay assay. Ribosomal proteins were separated by reverse-phase HPLC, electrophoresed, transferred to nitrocellulose membrane, stained with amido black (*Left*), and subjected to overlay assay with recombinant karyopherin β3 (*Right*). Fractions 6–16 are shown; the remaining fractions were negative by overlay assay (not shown). Small arrows indicate the positive bands on the amido black-stained gel.

that different subclasses of proteins use different transport systems to gain entry into the nucleus. As discussed earlier, traditional nuclear localization sequence-bearing proteins are transported by a heterodimer consisting of karyopherin α and karyopherin β1. In this system one member of the pair (karyopherin α) binds to the nuclear localization sequence of the import substrate while the other (karyopherin β1) binds to the repeat regions of nucleoporins, thus docking the substrate at the nuclear pore complex. In a subsequent energy-dependent step, Ran-GTP binds to karyopherin β1 and displaces karyopherin α, which then enters the nucleus along with the import substrate.

A second transport system is used by the mRNA binding proteins Nab2 and Nab4 in yeast and hnRNP A1 in mammalian cells (23–25). In yeast, one protein of the karyopherin β family (Kap104p/karyopherin β2) mediates nuclear import of certain mRNA-binding proteins by binding both to the import substrate and to repeat nucleoporins. Likewise, the mammalian homologue of Kap104p (transportin or karyopherin β2) is capable of mediating nuclear import by binding directly to substrate and repeat-containing nucleoporins.

A third pathway appears to be involved in the nuclear import of ribosomal proteins (M. Rout, G.B., and J. Aitchison, unpublished data). A yeast member of the karyopherin β family, Kap123p (karyopherin β4), binds several ribosomal proteins *in vivo* and in overlay assays. Deletion of *KAP123* results in failure of nuclear import of a reporter gene linked to a ribosomal nuclear localization sequence. In the absence of Kap123p, another karyopherin β homologue, Kap121p (karyopherin β3), can bind to ribosomal proteins in an overlay assay and may partially take over its function in ribosomal protein transport. On the other hand, deletion of *KAP121* is lethal; therefore Kap121p must have functions in yeast other than those shared with Kap123p.

The similarity of karyopherin β3 to other members of the karyopherin β family (Fig. 1), its cytoplasmic and nuclear rim localization (Fig. 3), its binding to repeat nucleoporins (Fig. 4) and to Ran-GTP (Fig. 5), and data from its yeast homologues strongly suggest that karyopherin β3 is a nuclear import factor. In addition to Kap121p, karyopherin β3 shows significant similarity to yeast Kap123p. As mentioned above, recent data indicate that these yeast homologues of karyopherin β3, particularly Kap123p, are involved in the nuclear import of ribosomal proteins. However, no other close human homologues of Kap123p have been identified in the GenBank database at this writing. In contrast, many EST hits have been recorded to date with similarity to yeast Kap121p, some of which were used in the cloning and expression of human karyopherin β3 as discussed in *Results*. This is somewhat surprising, because the human counterpart of Kap123p would be expected to be a relatively abundant and ubiquitous protein as it presumably would serve the function of ribosomal protein import. One way to interpret these findings is that human karyopherin β3 might be the counterpart of both yeast Kap123p and Kap121p. In this context, it is of significance that karyopherin β3 binds to two ribosomal proteins (RL13 and RL23) in an overlay assay (Fig. 6). These considerations suggest that karyopherin β3 may be involved in the nuclear import of ribosomal proteins in mammalian cells. However, *in vitro* import assays are needed to determine whether this indeed is the case.

The three pathways of nuclear import appear to converge at the level of binding to repeat-containing nucleoporins. The different members of the karyopherin β family seem to bind to a similar site on nucleoporins because they can compete with each other for binding (Figs. 4 and 5). However, there seems to be a difference in the relative binding affinity of karyopherin β1 and β3 to different nucleoporins (Fig. 4). Whether this reflects on the mechanisms of nuclear import used by these different karyopherins remains to be deter-

mined. Another interesting difference between karyopherin $\beta1$ and $\beta3$ is that the stretch of amino acids between position 383 and 498 in karyopherin $\beta3$, which is very well conserved between the yeast and human karyopherin $\beta3$ (Fig. 1*A*), is virtually nonexistent in human karyopherin $\beta1$ (Fig. 1*B*). This site may be relevant to the functional subspecialization of karyopherin $\beta3$.

To summarize, in this paper we report the cloning and characterization of karyopherin $\beta3$, the human homologue of yeast Kap121p, and provide evidence that it is a nuclear transport factor. The transport substrate(s) of karyopherin $\beta3$ remain to be fully characterized, but our preliminary results suggest that it might be involved in the nuclear import of some ribosomal proteins.

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