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Karyopherin β2 mediates nuclear import of a mRNA binding protein

(cDNA-deduced sequence/recombinant β2/Ran/GTP hydrolysis/repeat nucleoporins)

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ABSTRACT **We have cloned and sequenced cDNA for human karyopherin β2, also known as transportin. In a solution binding assay, recombinant β2 bound directly to recombinant nuclear mRNA-binding protein A1. Binding was inhibited by a peptide representing A1's previously characterized M9 nuclear localization sequence (NLS), but not by a peptide representing a classical NLS. As previously shown for karyopherin β1, karyopherin β2 bound to several nucleoporins containing characteristic peptide repeat motifs. In a solution binding assay, both β1 and β2 competed with each other for binding to immobilized repeat nucleoporin Nup98. In digitonin-permeabilized cells, β2 was able to dock A1 at the nuclear rim and to import it into the nucleoplasm. At low concentrations of β2, there was no stimulation of import by the exogenous addition of the GTPase Ran. However, at higher concentrations of β2 there was marked stimulation of import by Ran. Import was inhibited by the nonhydrolyzable GTP analog guanylyl imidodiphosphate by a Ran mutant that is unable to hydrolyze GTP and also by wheat germ agglutinin. Consistent with the solution binding results, karyopherin β2 inhibited karyopherin α/β1-mediated import of a classical NLS containing substrate and, *vice versa*, β1 inhibited β2-mediated import of A1 substrate, suggesting that the two import pathways merge at the level of docking of β1 and β2 to repeat nucleoporins.**

Import of proteins containing a nuclear localization sequence (NLS) into digitonin-permeabilized cells is mediated by soluble transport factors. A heterodimer, termed karyopherin or importin, recognizes the NLS protein in the cytoplasm via its α subunit and, via its β subunit, docks the complex to a subset of peptide repeat containing nucleoporins (1–10). The GTPase Ran (11, 12) and a Ran interacting protein, termed p10 (or NTF2) (13, 14), then mediates release and GTP-hydrolysis-dependent transport of the NLS protein and karyopherin α into the nucleus with karyopherin β staying behind at the nuclear pore complex (10, 15–17). Homologs of these transport factors also have been identified in yeast and recombinant Kap60p/Srp1p (karyopherin α) and Kap95p (karyopherin β) can substitute for their mammalian homologs in docking NLS protein to the nuclear rim of digitonin-permeabilized mammalian cells (18).

Studies in yeast have revealed the existence so far of three proteins that are both structurally and functionally related to Kap95p (ref. 19; M. P. Rout, G.B., and J. D. Aitchison, unpublished data) and therefore have been classified as members of the yeast β karyopherin family. All four yeast β karyopherins [Kap95p, Kap104p, Pse1p (20), and Kap123p] have been shown to serve as transport factors for nuclear protein import (ref. 19; M. P. Rout, G.B., and J. D. Aitchison, unpublished data).

A detailed characterization of the yeast Kap104p was recently reported (19). A cytosolic complex could be isolated that contained Kap104p and two abundant nuclear mRNA binding proteins, Nab2p and Nab4p, with Nab4p being the likely homolog of the vertebrate nuclear mRNA-binding protein A1. This complex did not contain karyopherin α. Thus, unlike Kap95p, Kap104p bound directly to transport substrate, without an adaptor. Like Kap95p, Kap104p bound directly to a subset of peptide repeat containing nucleoporins. Most importantly, a mutant Kap104p was rapidly degraded at the nonpermissive temperature resulting in concomitant failure to import Nab2p but still allowing import of a protein containing a classical NLS. Hence yeast Kap104p is a signal recognition and docking factor for at least Nab2p, whose NLS still has to be identified. Nab2p or Nab4p do not contain a region of close similarity to the previously mapped M9 nuclear localization sequence of the abundant human nuclear mRNA binding protein A1 (21). A human homolog of Kap104p, termed transportin, has recently been described and shown to be necessary for the nuclear import of an M9-carrying reporter protein (22).

Here we describe the further functional characterization of Kap104p's human homolog that we have termed karyopherin β2.

MATERIALS AND METHODS

Cloning of Karyopherin β2 cDNA. The GenBank cDNA clone 224297 (accession number R54232) containing a sequence that was similar to yeast karyopherin β2 (Kap104p) was obtained from Genome Systems (St. Louis). This clone coded for the C-terminal region of karyopherin β2. To obtain the full-length coding sequence, a human liver 5′-Stretch Plus cDNA library (CLONTECH) was screened according to the manufacturer's instructions using a single-stranded antisense oligonucleotide (corresponding to bases 105–155 of the Expressed Sequence Tag database) that was labeled with [γ-^{32}P]-ATP by polynucleotide kinase (23). Three partial overlapping clones were isolated and cloned in pBlue Script II SK (Stratagene). The full-length coding sequence for karyopherin β2 was determined from these overlapping clones and cDNA clone 224297 and has been deposited in the GenBank database (accession no. U72069).

Expression and Purification of Recombinant Proteins. To obtain a full-length cDNA for karyopherin β2, a fragment of the clone 224297 was generated by digestion with *Bst*EII and *Not*I. This fragment was ligated between the *Bst*EII and *Not*I sites of pBluescript II SK containing the longest clone previ-

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Abbreviations: NLS, nuclear localization sequence; GST, glutathione *S*-transferase.

Data deposition: The sequence reported in this paper has been deposited in the GenBank database (accession no. U72069).

ously isolated from the human liver cDNA library. To obtain a cDNA coding for a glutathione *S*-transferase (GST)-karyopherin β2 fusion protein, the full-length karyopherin β2 cDNA was amplified by the PCR using the primers 5′CACCTCAGGCCCCGGGCCAAGAAGGAG3′ and 5′GGGACTGCAGCTCGAGTGTATTAGAATAAAA3′ (introducing an *Xma*I and an *Xho*I site at the 5′ and 3′ ends, respectively), and then subcloned in frame in pGEX-4T-3 (Pharmacia). The GST-karyopherin β2 fusion protein was purified from *Escherichia coli* BL21/LysS by binding to glutathione Sepharose 4B beads (Pharmacia). The recombinant protein was recovered from the beads by cleavage with thrombin (Sigma) followed by inactivation of thrombin by hirudin (Sigma).

To obtain a cDNA for the fusion protein GST-human nuclear mRNA binding protein A1, the cDNA clone 81773 (Genome Systems) coding for full-length A1 was amplified by the PCR primers 5′-AAAGTCTCTCTTCACCCCCCGGGTCAAGTCTAA-3′ and 5′-CTCCTGCTAAGCTTTGTTCTCGAGTTAAAATCT-3′ introducing an *Xma*I and a *Xho*I site at the 5′ and 3′ ends, respectively. The PCR product was subcloned in frame into the *Xma*I/*Xho*I. sites of pGEX-4T-3. The GST-A1 fusion protein expressed in *E. coli* BL21/LysS was purified by binding to glutathione beads and recovered by elution with 10 mM reduced glutathione. The GST-A1 fusion protein was labeled with fluorescein isothiocyanate (24).

Recombinant human karyopherin α2, human wild-type Ran, mutant Ran, p10, and Nup98 (residues 43–824) were prepared as described (9, 10, 25–28). Recombinant GST-karyopherin β1 was prepared as described (6).

Synthetic Peptides. Peptides corresponding to the NLS of simian virus 40 T antigen (24) and the M9 sequence of the human nuclear mRNA binding protein A1 (21) were synthesized with an N-terminal Cys for chemical coupling reactions.

Solution Binding Assay. The assay was performed essentially as described (16). GST-A1 (1.5 μg) was immobilized on 10 μl of glutathione beads, and 1 μg of karyopherin β2 was added alone or together with 25× or 100× molar excess of the A1 NLS or simian virus 40 NLS synthetic peptides. Recombinant Nup98 (5 μg) immobilized on 10 μl of Affigel beads (Bio-Rad) was incubated with 1 μg of GST-β1 or 1 μg of β2 or with 1 μg of GST-β1 and 10× molar excess of β2 or with 1 μg of β2 and 10× molar excess of GST-β1.

Overlay Blot Assay. Proteins of rat liver nuclear envelopes (29) or *E. coli* lysates expressing Nup98 fragments (28) were separated by SDS/PAGE and transferred to nitrocellulose. The blot was blocked for 1 h at room temperature in 5% milk/0.2% Tween 20 in transport buffer [20 mM Hepes·KOH, pH 7.3/110 mM KOAc/2 mM $Mg(OAc)_2$/1 mM EGTA/2 mM DTT] and then incubated for 1 h at room temperature in the same buffer containing β2 (1 μg/μl) or β1 (1 μg/μl) previously metabolically labeled with ^{35}S-Express Protein Labeling Mix (NEN). The blot was washed three × 10 min in the same buffer and 3 min in transport buffer, then dried and exposed for autoradiography.

Nuclear Import Assay. Import assays were performed on digitonin-permeabilized HeLa cells essentially as described (24), except that GTP was added at 1 mM. Where specified, GTP was substituted by 2 mM guanylyl imidodiphosphate.

When included, proteins were added to give the following final concentrations per assay: 0.5 μg of karyopherin α2; 0.5 μg

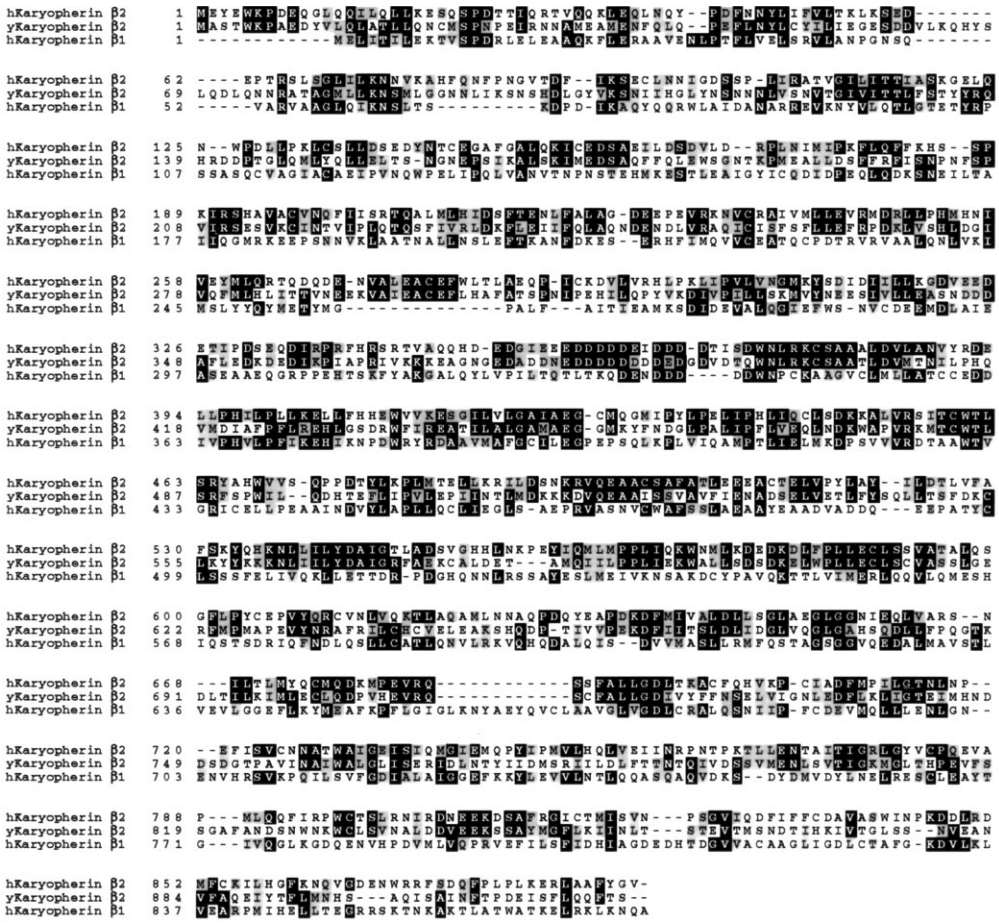

FIG. 1. Comparison of amino acid sequences of human karyopherin β2, yeast karyopherin β2 (Kap104p), and human karyopherin β1. Sequences were aligned using the CLUSTALW v.1.6 program and analyzed with BOXSHADE. Identical amino acids are indicated by black boxes and similar amino acids by gray boxes.

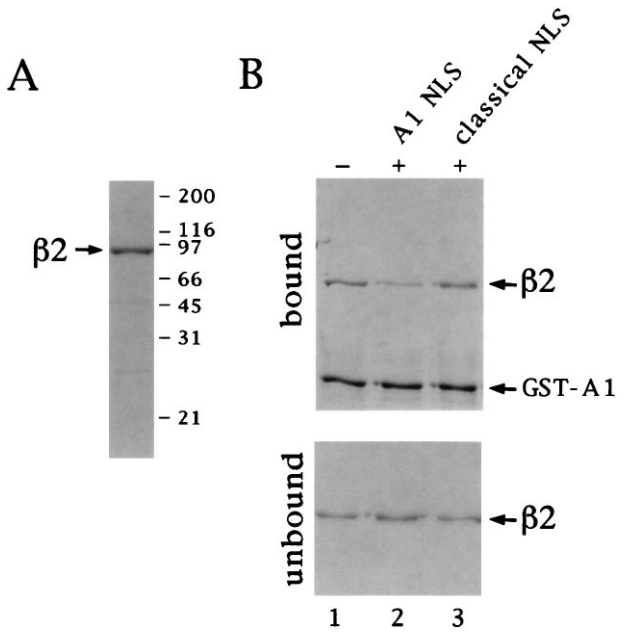

FIG. 2. Karyopherin β2 binds to a fusion protein (GST-A1) containing the nuclear mRNA binding protein A1 via a specific sequence. (*A*) Purified recombinant karyopherin β2 analyzed by SDS/PAGE and visualized by Coomassie blue staining. (*B*) Immobilized GST-A1 was incubated with karyopherin β2 alone (lane 1) or with a 25× molar excess of a synthetic peptide representing the NLS of the A1 protein (lane 2) or with a 100× molar excess of the classical NLS peptide (lane 3). Bound and unbound fractions were analyzed by SDS/PAGE and Coomassie blue staining.

of karyopherin β1; 4 μg of Ran (wild type or mutant); 60 ng of p10; 0.4 μg of NLS-human serum albumin; 1 μg of GST-A1; and 4 μg of wheat germ agglutinin.

RESULTS

We searched the Expressed Sequence Tag database and found several sequences that were similar to yeast Kap104p. Using an antisense oligonucleotides derived from one of these sequences (see *Materials and Methods*), we screened a human liver cDNA library and obtained several clones. From overlapping cDNA clones we determined the DNA sequence and obtained a complete, cDNA-deduced amino acid sequence for the protein that we termed human karyopherin β2 (Fig. 1). Its calculated molecular mass is 101,321 daltons. Over its entire

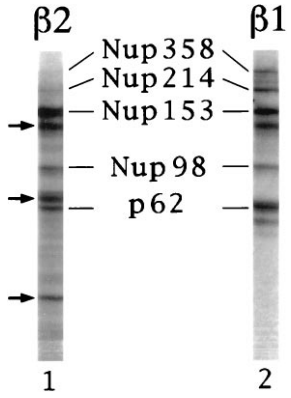

FIG. 3. Karyopherin β2 binds to peptide repeat-containing nucleoporins. Proteins from purified rat liver nuclear envelopes were separated by SDS/PAGE, transferred to nitrocellulose, and incubated with ^{35}S-labeled karyopherin β2 (lane 1) or ^{35}S-labeled karyopherin β1 (lane 2). The arrows indicate positions of unidentified bands that interact with karyopherin β2.

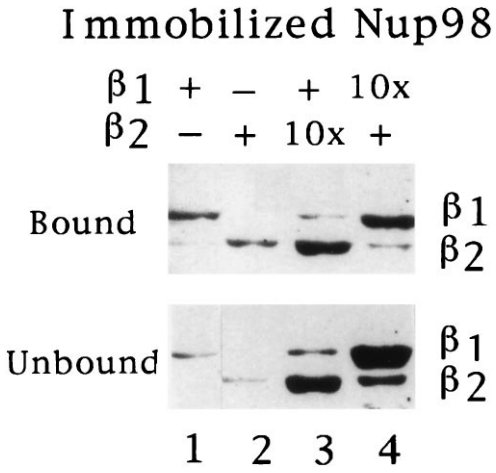

FIG. 4. Karyopherin β2 and β1 compete for binding to Nup98. Immobilized Nup98 was incubated with GST-β1 (lane 1), β2 (lane 2), GST-β1 and 10× molar excess of β2 (lane 3), or β2 and 10× molar excess of GST-β1 (lane 4). Bound and unbound fractions were analyzed by SDS/PAGE and Coomassie blue staining.

sequence, human β2 is 34% identical and 50% similar to yeast Kap104p (Fig. 1). Human β2 is 17% identical and 30% similar to human β1 (Fig. 1). While this work was in progress, Pollard *et al.* (22) reported similar data and proposed the term transportin for karyopherin β2. The two sequences were identical, except for an isoleucine at position 217 in transportin that is substituted with a threonine in karyopherin β2.

We assembled a cDNA coding for full-length β2 and expressed it in *E. coli* as a GST fusion protein with a thrombin cleavage site between the GST and the β2 moiety of the fusion protein. Thrombin cleavage generated a full-length recombinant β2 (Fig. 2*A*). Recombinant β2 bound to an immobilized fusion protein consisting of GST and the A1 protein (Fig. 2*B*, lane 1). The NLS of A1 has been sublocalized to its C-terminal region and termed M9 (21). A synthetic peptide representing this region inhibited binding of recombinant β2 to A1 (Fig. 2*B*, lane 2) whereas a classical NLS peptide had no effect (Fig. 2*B*, lane 3). We conclude that karyopherin β2 is a signal recognition factor that specifically recognizes the A1 type NLS, but not the classical NLS, confirming previous data (22).

Karyopherin β2 is also a docking factor as it binds to a subset of nucleoporins. This was shown by using SDS/PAGE-separated nuclear envelope proteins that were transferred to nitrocellulose and were probed in overlay blots with metabolically labeled [^{35}S]karyopherin β2 (Fig. 3*A*, lane 1). For comparison, the same blot was also probed with metabolically labeled [^{35}S]karyopherin β1 (lane 2) that was previously shown in this assay to bind to a subset of peptide repeat containing nucleoporins (10). Like karyopherin β1, karyopherin β2 bound to several proteins that comigrated with known peptide repeat-containing nucleoporins.

The binding site for karyopherin β1 has previously been mapped to the peptide repeat-containing region of the nucleoporin Nup98 (26). To determine whether β2 also bound to repeat regions we used *E. coli* lysates that contained recombinant regions of Nup98 (26) and probed them in an overlay blot with ^{35}S-labeled β2. As previously reported for β1, β2 bound to the N-terminal fragment of Nup98 that contains the peptide repeat region, but not to the repeat-lacking C-terminal region of Nup98 (data not shown). When Nup98 was purified and immobilized, it bound GST-β1 or β2 in a solution binding assay (Fig. 4*B*, lanes 1 and 2). Interestingly, both β1 and β2 competed with each other for binding (lanes 3 and 4). Together these data suggest that β1 and β2 bound to similar or overlapping sites in the peptide repeat region of Nup98.

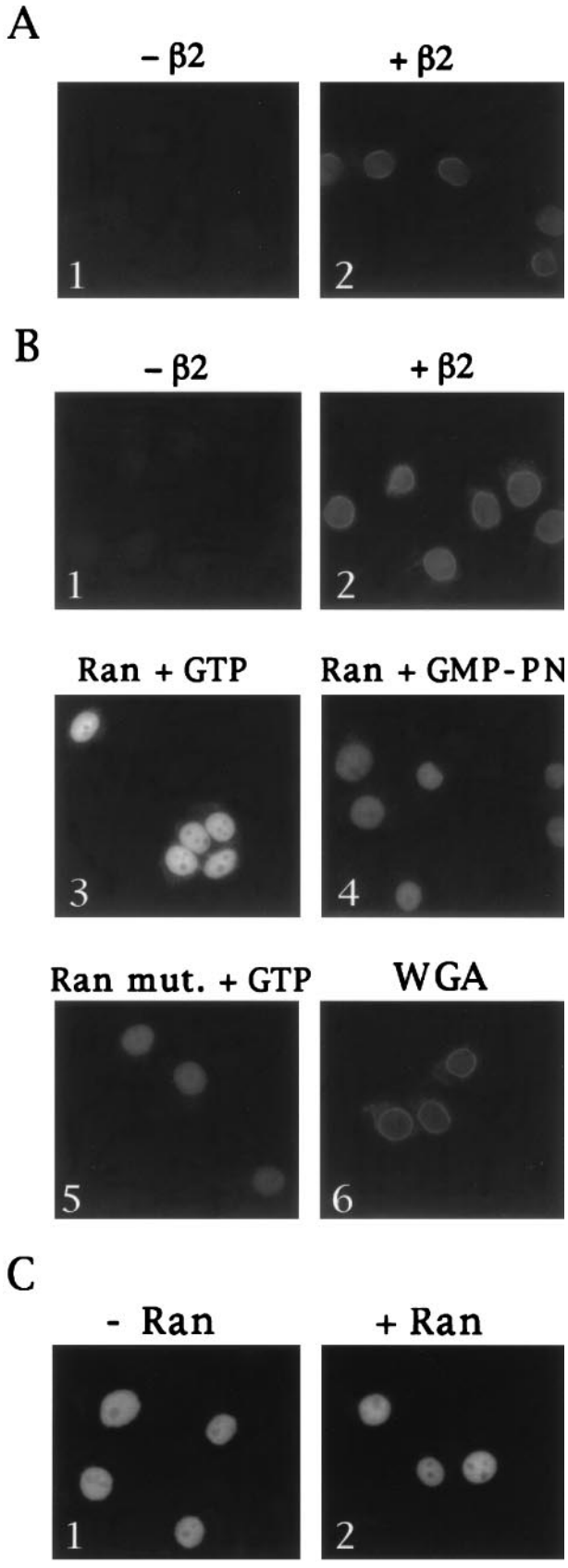

FIG. 5. GST-A1 import into the nucleus requires karyopherin β2 and Ran. (*A*) Digitonin-permeabilized HeLa cells were incubated at 4°C with fluorescently labeled GST-A1, in the presence or absence of karyopherin β2 (2 μg/assay) as indicated. (*B*) Permeabilized cells were incubated at 20°C, with fluorescently labeled GST-A1 in the absence (panel 1) or in the presence of karyopherin β2 (2 μg/assay) (panels 2–6). Wild-type Ran (panels 3, 4, and 6) or a GTPase-deficient mutant Ran (panel 5) were added. Wheat germ agglutinin (WGA) was added in panel 6. (*C*) Permeabilized cells were incubated at 20°C with fluorescently labeled GST-A1 and karyopherin β2 (0.5 μg/assay) in the presence or absence of Ran as indicated.

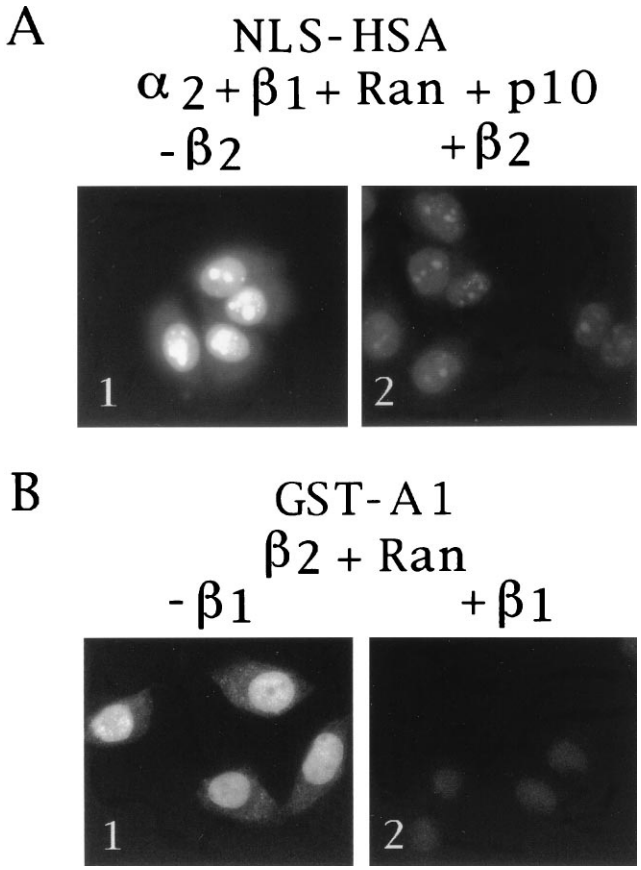

FIG. 6. Karyopherin β1 and karyopherin β2 compete for nuclear import. (*A*) Digitonin-permeabilized HeLa cells were incubated at 20°C with fluorescently labeled NLS-human serum albumin, karyopherin α2, karyopherin β1, Ran, and p10 in the absence (panel 1) or in the presence of 10× molar excess of karyopherin β2 (panel 2). (*B*) Digitonin-permeabilized HeLa cells were incubated with fluorescently labeled GST-A1, karyopherin β2, and Ran in the absence (panel 1) or in the presence of 10× molar excess of karyopherin β1 (panel 2).

To assay for docking to the nuclear rim, digitonin-permeabilized cells were incubated on ice in transport buffer containing fluorescently labeled GST-A1, with or without recombinant β2. Nuclear rim staining was observed only in the presence of β2 (Fig. 5 *A*, 1 and 2) indicating that β2 is required for docking of GST-A1 at nucleoporins. In contrast, there was no β2-mediated docking of fluorescently labeled, classical NLS-containing substrate [NLS-human serum albumin (24)], either in the absence or presence of karyopherin α (data not shown). Hence, karyopherin β2 is both a signal recognition and docking factor that specifically recognizes A1's NLS and docks A1 to repeat containing nucleoporins without requiring an energy generating system.

To assay for import, digitonin-permeabilized cells were incubated at room temperature with fluorescently labeled GST-A1, an ATP-generating system, GTP, in the absence or presence of various concentrations of β2, and in the absence or presence of recombinant Ran. In the absence of both β2 and Ran there was no readily detectable import of GST-A1 into nuclei (Fig. 5*B*1). Together with the docking data this result indicates that the digitonin-permeabilized cells contained either little endogenous β2 or that endogenous A1-type NLS

substrate was inefficiently displaced by GST-A1. In the presence of low concentrations of β2 (0.5 μg/assay) there was import (Fig. 5*C*1) which was not noticeably stimulated by exogenously added Ran (Fig. 5*C*2). The imported GST-A1 was distributed throughout the nucleoplasm but apparently was excluded from nucleoli. These negatively stained nucleoli served as a useful criterion for β2-mediated GST-A1 import. At higher concentrations of β2 (2 μg/assay) and in the absence of added Ran there was primarily docking at the nuclear rim and little, if any, import based on the absence of negatively stained nucleoli (Fig. 5*B*2), whereas in the presence of Ran there was a striking stimulation of import with the appearance of negatively stained nucleoli (Fig. 5*B*3). Similar results were obtained at still higher concentrations of β2 (4 μg/assay)(data not shown). These data indicate that at low levels of added β2 (0.5 μg/assay) the endogenous Ran of the digitonin-permeabilized cells may suffice for maximal import, whereas exogenously added Ran is required to achieve maximal levels of import at higher concentrations of exogenously added β2. Import was inhibited in the presence of the nonhydrolyzable guanylyl imidodiphosphate (Fig. 5*B*4) or by the exogenous addition of mutant Ran that is unable to hydrolyze GTP (Fig. 5*B*5). Wheat germ agglutinin inhibited import but still allowed β2-mediated docking (Fig. 5*B*6).

Our finding (see above) that β1 and β2 competed with each other for binding to the repeat containing domain of Nup98 (and likely also to those of other repeat-containing nucleoporins), suggested that β1 and β2 also may compete with each other in nuclear import by competing for common or overlapping nucleoporin docking sites. Indeed, import of NLS-human serum albumin, mediated by karyopherin α2, β1, Ran, and p10 (10) (Fig. 6*A*1) was inhibited by β2 (Fig. 6*A*2) and *vice versa*, import of GST-A1, mediated by β2 and and Ran (Fig. 6*B*1) was inhibited by β1 (Fig. 6*B*2). Hence, the distinct α/β1- and β2-mediated pathways of nuclear import for their substrates appear to at least partially merge at the level of docking to nucleoporins.

DISCUSSION

From this and other recent studies (refs. 19, 22, 30; M. P. Rout, G.B., and J. D. Aitchison, unpublished data) it appears that the import of nuclear proteins occurs by at least three different pathways in mammalian cells (or yeast). Proteins are directed into these pathways by distinct NLSs and by cognate NLS recognition and docking factors of the karyopherin family. As a more general nomenclature we suggest the generic terms karyopherin β1, β2, β3, and β4 for Kap95p, Kap104p, Pse1p, and Kap123p, respectively. It seems likely that each of the karyopherins recognizes its own type of NLS. Should this be the case then, as a further simplification of nomenclature, the corresponding NLSs might be termed NLS-1, NLS-2, NLS-3, and NLS-4, respectively. It appears that karyopherin β2, β3, and β4 bind directly their cognate NLSs (ref. 19; M. P. Rout, G.B., and J. D. Aitchison, unpublished data). In contrast, karyopherin β1 uses karyopherin α as an adaptor for binding to the NLS-1 substrate. In yeast there is only one α karyopherin, whereas in mammalian cells there are at least two α karyopherins, which may have distinct, but overlapping, substrate specificities (10). All β karyopherins bind directly to similar (but not always identical) repeat nucleoporins. Therefore, all three (or four) presently known import pathways appear to merge at the level of docking of the various β karyopherins to similar or overlapping repeat domains of some, but not necessarily identical, repeat nucleoporins. These repeat nucleoporins are distinct components of the fibers emanating from the nucleoplasmic and the cytoplasmic side of the nuclear pore complex (reviewed in ref. 30). Hence the karyopherins function to concentrate transport substrate at multiple docking sites of the nuclear pore complex fibers. This fibrous zone would serve as an atrium to the central opening of the nuclear pore complex. Cytoplasmic proteins lacking NLSs for karyopherin-mediated docking (or lacking sites for direct docking) to repeat nucleoporins might be sterically excluded from this atrium and therefore would be prevented from entering the nucleus. Steric exclusion from the atrium would be more efficient for large cytoplasmic proteins and less efficient for small cytoplasmic proteins, which therefore may enter the nucleus without an NLS or without a docking site to repeat nucleoporins.

In this paper we have focused on the characterization of a mammalian karyopherin β2. Similar to yeast β2 (19), this mammalian β2 bound directly to NLS-2 substrate (the mRNA-binding protein A1) and to nucleoporins. In solution binding assays, binding to the NLS-2 substrate could be competed for by NLS-2 peptide, but not by NLS-1 peptide, indicating that β2 binds specifically to NLS-2, but does not bind to NLS-1 (see also ref. 22). In overlay blots, mammalian β2 apparently bound to some of the same repeat nucleoporins, which previously had been shown to bind to β1 (10), though there were distinct differences in the binding affinity. For example, although binding to the nucleoplasmically exposed nucleoporins Nup98 and Nup153 was similar for β2 and β1, binding to Nup358 and Nup 214, cytoplasmically exposed nucleoporins, was much weaker for β2 than it was for β1. The significance of these affinity differences for both nuclear import and export remains to be elucidated. For one nucleoporin, Nup98, we have localized β2 binding to the N-terminal repeat motif containing region of Nup98. It is likely that binding to other repeat motif-containing nucleoporins is also to their repeat regions, although this remains to be shown.

Recombinant β2 was able to dock the fluorescently labeled GST-A1 protein at the nuclear rim of digitonin-permeabilized cells at 0°C. At 20°C and in the presence of GTP and an energy-generating system, fluorescently labeled GST-A1 was not imported into nuclei unless β2 was present in the import reaction. At low concentration of β2, exogenously added Ran did not stimulate import. However, at higher concentrations of β2 there was primarily docking and, based on the absence of negatively stained nucleoli, virtually no import. Strikingly, the addition of Ran greatly stimulated import and diminished docking. These data suggested that Ran is required for β2-mediated import and that at lower concentration of β2 the endogenous Ran is sufficient for import but that at higher concentration of β2 exogenously added Ran is required for maximal import. It is presently not clear why the import that is likely to be mediated by endogenous Ran appears to be inhibited at high concentrations of β2.

GTP hydrolysis is required for β2-mediated import as nonhydrolyzable guanylyl imidodiphosphate or the addition of a Ran mutant that is unable to hydrolyze GTP inhibited import. The exact function of Ran and GTP hydrolyisis in β2-mediated import remains to be elucidated. Unlike β1, which binds Ran-GTP with high affinity in a solution binding assay (16, 27), high affinity binding of Ran-GTP to β2 could not be detected (data not shown). Moreover, Ran-GTP does not appear to dissociate β2 bound to immobilized GST-A1 and p10 does not stimulate β2-mediated import into nuclei of digitonin-permeabilized cells as it does in the case of α/β1-mediated import of NLS-1 protein (data not shown). One possibility is that Ran stimulation of β2-mediated import is indirect and is due to Ran-mediated clearance of endogenous α/β1 and of β3 [which also binds Ran-GTP (31)] from nucleoporin docking sites. However the finding that preincubation of digitonin-permeabilized cells with Ran-GTP did not abolish Ran stimulation of GST-A1 import at the higher concentration of β2 (2 μg/assay) (data not shown) argues against this possibility.

In summary, karyopherin β2 functions as a signal recognition and docking factor that binds directly to an NLS-2

containing substrate and to peptide repeat-containing nucleoporins. Karyopherin β2- mediated docking of NLS-2 substrate at the nuclear rim of digitonin-permeabilized cells occurs on ice and does not require energy. Import is stimulated by Ran and GTP hydrolysis. Not all nuclear mRNA binding proteins are imported via the NLS-2 pathway (32). Moreover, proteins other than nuclear mRNA binding proteins may be imported via the NLS-2 pathway.

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