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Mammalian karyopherin $\alpha_1\beta$ and $\alpha_2\beta$ heterodimers: α_1 or α_2 subunit binds nuclear localization signal and β subunit interacts with peptide repeat-containing nucleoporins

(digitonin-permeabilized cells/recombinant transport factors/docking and import reaction/immunofluorescence localization/nuclear envelope blot)

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ABSTRACT **Although only 44% identical to human karyopherin α_1, human karyopherin α_2 (Rch1 protein) substituted for human karyopherin α_1 (hSRP-1/NPI-1) in recognizing a standard nuclear localization sequence and karyopherin β-dependent targeting to the nuclear envelope of digitonin-permeabilized cells. By immunofluorescence microscopy of methanol-fixed cells, karyopherin β was localized to the cytoplasm and the nuclear envelope and was absent from the nuclear interior. Digitonin permeabilization of buffalo rat liver cells depleted their endogenous karyopherin β. Recombinant karyopherin β can bind directly to the nuclear envelope of digitonin-permeabilized cells at 0°C (docking reaction). In contrast, recombinant karyopherin α_1 or α_2 did not bind unless karyopherin β was present. Likewise, in an import reaction (at 20°C) with all recombinant transport factors (karyopherin α_1 or α_2, karyopherin β, Ran, and p10) import depended on karyopherin β. Localization of the exogenously added transport factors after a 30-min import reaction showed karyopherin β at the nuclear envelope and karyopherin α_1 or α_2, Ran, and p10 in the nuclear interior. In an overlay assay with SDS/PAGE-resolved and nitrocellulose-transferred proteins of the nuclear envelope, ^{35}S-labeled karyopherin β bound to at least four peptide repeat-containing nucleoporins—Nup358, Nup214, Nup153, and Nup98.**

Bidirectional transport of proteins, ribonucleoproteins (RNPs), and deoxyribonucleoproteins into and out of the nucleus proceeds through the nuclear pore complex (NPC). Using digitonin-permeabilized mammalian cells and a nuclear localization sequence (NLS)-containing protein, a number of transport factors have been isolated from cytosol and shown to be required for import into nuclei. So far these factors are a heterodimeric protein complex (1–4), termed karyopherin (3), which is required for targeting NLS substrate to NPCs, and two proteins, the small GTPase Ran (5, 6) and p10 (7), which are required for transport into the nucleus.

Considerable progress has been made in identifying and characterizing NPC proteins (collectively referred to as nucleoporins) (for review, see ref. 8). Of particular interest is a subgroup of nucleoporins that share a variety of peptide repeats. Some of these repeat-containing nucleoporins are situated exclusively on the cytoplasmic side, such as Nup358 (9) and Nup214 (10), most likely as constituents of 50-nm-long fibers emanating from the NPC into the cytoplasm. Other repeat-containing nucleoporins, such as Nup153 (11) and Nup98 (12), are located exclusively on the nucleoplasmic side, as components of the nuclear basket structure. Still another repeat-containing nucleoporin, p62, appears to be located in the center of the NPC (for review, see ref. 8). Some of these nucleoporins have previously been implicated in transport (import and export across the NPC) by showing that a variety of monoclonal antibodies or wheat germ agglutinin inhibit transport (for review, see ref. 8), although the possibility that the observed inhibition was due to nonspecific steric effects cannot be ruled out by this type of experiment. Recently, however, a direct biochemical interaction between the isolated transport factors and peptide repeat-containing nucleoporins has been demonstrated. Thus, Nup358, located at or near the tip of the cytoplasmic fibers, has been shown to contain four Ran-binding sites (9). Moreover, docking of NLS substrate to repeat-containing nucleoporins was found to be mediated by a cytosolic subfraction A (13) that contains the targeting activity and whose active component is karyopherin (3, 4). Indeed, mapping of the docking site(s) for Nup98 showed it (them) to be located in the repeat-containing N-terminal half of Nup98 (12). Finally, the repeat-containing nucleoporins also contain binding sites for p10 (M. Matunis, G.B., and M.H., unpublished data). It has been proposed that the repeat-containing nucleoporins serve as a stationary phase and the transport factors as the mobile phase in transport across the NPC (12).

We showed previously that the α subunit serves as an NLS receptor and proposed that the β subunit serves as an adaptor that binds to the α subunit–NLS substrate complex and to the repeat-containing nucleoporins (4). Here we show that the previously used karyopherin α subunit [corresponding to hSRP-1/NPI-1 (14, 15)], now termed karyopherin α_1, can be replaced by karyopherin α_2 [corresponding to the hSRP-1-related Rch1 (16)]. Although karyopherin α_1 and α_2 are only 44% identical, we did not detect any functional difference. We also show that karyopherin β is localized in the cytoplasm and at the nuclear rim and is largely lost after digitonin permeabilization of cells. Unlike karyopherin α, which alone cannot bind to the nuclear envelope of digitonin-permeabilized cells, karyopherin β can bind without karyopherin α being present. In an import reaction with all recombinant transport factors, the karyopherin α subunits, Ran, and p10 are shown to enter the nucleus, whereas the β subunit remains at the nuclear envelope. This result suggests that the heterodimeric karyopherin complex is dissociated during transport. Overlay binding assays showed that the β subunit binds to the repeat-containing nucleoporins. These data are consistent with the previously proposed function of karyopherin β as an adaptor between karyopherin α/NLS substrate complex and repeat-containing nucleoporins.

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Abbreviations: HSA, human serum albumin; NLS, nuclear localization sequence; NPC, nuclear pore complex; FITC, fluorescein isothiocyanate.

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MATERIALS AND METHODS

The nuclear docking or import assay using digitonin-permeabilized buffalo rat liver (BRL) cells was by described procedures (4, 13).

Recombinant Proteins. Preparation of recombinant karyopherin β was as described (3). Recombinant human Ran was prepared and loaded with GTP as described (17). The cDNA open reading frame of human p10 was obtained by PCR using as template the pTacT7L-PP15 clone (18) (from U. Grundmann, Behringwerke AG SGE Therapeutika, Marburg, Germany) and primers containing *Nde* I and *Ava* I restriction sites. The PCR product was subcloned in the pET21 vector (Novagen) containing a C-terminal histidine tag, expressed in *Escherichia coli* strain BL21(DE3)pLysE and purified on a Ni-nitrilo-triacetic acid (NTA) column. A functional active dimer of p10 was isolated by fast protein liquid chromatography (FPLC) on a Mono Q column followed by FPLC Superose 75 gel filtration; details of the procedure will be described elsewhere. Purification of recombinant karyopherin α_1/hSRP and karyopherin α_2/Rch1 was as follows. A DNA segment containing the full coding sequence for hSRP (NPI-1) was obtained by PCR with hSRP cDNA as template and primers containing *Bal* I and *Eco*NI restriction sites. The PCR product was subcloned in the pSE420 vector containing a 6-histidine C-terminal tag. A DNA segment coding for human Rch1 (minus 33 amino acid residues from the N-terminal end) was obtained by PCR with Rch1 cDNA as template and primers containing *Nco* I and *Bam*HI restriction sites. The PCR product was digested and ligated in the pQE-60 vector containing a C-terminal histidine tag (Qiagen, Chatsworth, CA). Expression was induced with 1 mM isopropyl β-D-thiogalactoside for 3 hr at 30°C in *E. coli* DH5α for karyopherin α_1 and in *E. coli* M15 (pREP4) for karyopherin α_2. The cells were harvested and resuspended in 50 mM sodium phosphate, pH 8.0/300 mM NaCl/1 mM phenylmethylsulfonyl fluoride/pepstatin (10 μg/ml)/leupeptin (10 μg/ml)/aprotinin (10 μg/ml) (Boehringer Mannheim). Cells were subjected to two freeze-thaw cycles and finally disrupted by ultrasonic sound. The insoluble material was pelleted by centrifugation at 20,000 $\times g$ for 20 min at 4°C. The supernatant was adjusted to 10 mM imidazole/10% glycerol and incubated with 1 ml of Ni-NTA Sepharose (Qiagen) for 2 hr at 4°C. The mixture was placed into a column, washed with 30 mM imidazole in the same buffer, and eluted with 500 mM imidazole. The eluates were dialyzed overnight at 4°C against buffer A (3). ^{35}S-labeled karyopherin β was prepared by growing *E. coli* at 30°C in 100 ml of M9 medium. When cells reached an OD_{600} value of 0.9, isopropyl β-D-thiogalactoside was added to 0.1 mM; then after 15 min, rifampicin (Boehringer Mannheim) was added to 0.25 mg/ml, and after another 15 min, 10 mCi (1 Ci = 37 GBq) of the labeling mix Expre^{35}S^{35}S (NEN) was added to the culture. After 3 hr bacteria were harvested, and karyopherin β was purified as described (3).

Raising Antibodies. Purified recombinant rat karyopherin β was subjected to SDS/PAGE; the karyopherin β band was cut from the dried gel, rehydrated, homogenized, and used to immunize rabbits. A Rch1 peptide containing amino acid residues 499–515 was synthesized and conjugated to keyhole limpet hemagglutinin (Genosys Biotechnologies, The Woodlands, TX) and injected into rabbits.

Immunoblotting. Recombinant purified karyopherin β, recombinant karyopherin α_2, digitonin-extracted BRL cells, and the digitonin extract were separated by SDS/PAGE and transferred to nitrocellulose. After blocking with 2% nonfat dry milk in phosphate-buffered saline the blots were incubated with anti-karyopherin β antibodies (1:1000) or anti-karyopherin α_2 antibodies (1:500) and with ^{125}I-labeled protein A.

Immunofluorescence. BRL cells in culture were fixed with cold methanol (6 min at −20°C) before or after digitonin permeabilization (digitonin at 50 μg/ml, 5 min at room temperature) or after completion of the docking or import reaction. Cells were incubated with nonfat dry milk as a blocking agent and further probed with different antibodies: anti-karyopherin β (1:100), anti-karyopherin α_1 (1:100) (15), anti-karyopherin α_2 (1:100), and anti-human Ran (1:100) (7). The bound antibodies were visualized by fluorescein isothiocyanate (FITC)-labeled goat anti-rabbit IgG. Anti-human karyopherin α_1, anti-human karyopherin α_2, and anti-human Ran do not cross-react with their corresponding antigens in BRL cells. Recombinant p10 was labeled with FITC as described (19).

Blot Overlays. Proteins of rat liver nuclear envelopes (3) were subjected to SDS/PAGE, transferred to nitrocellulose, and incubated as described (3) with the import substrate NLS–human serum albumin (HSA) in the presence of *Xenopus* fraction A. The substrate was detected by anti-HSA antibodies followed by ^{125}I-labeled protein A (3). For the karyopherin β-binding assay, the nitrocellulose blot was blocked for 1 hr at room temperature in buffer A/2% nonfat dry milk/0.2% Tween 20, incubated 1 hr at room temperature with ^{35}S-labeled recombinant karyopherin β (2 μg/ml) in blocking buffer, washed three times for 10 min in the same blocking buffer and 3 min in buffer A, then dried, and exposed for radioautography.

RESULTS

Karyopherin α_2 Substitutes for Karyopherin α_1. To test whether human karyopherin α_2 (Rch1) can substitute for karyopherin α_1 (hSRP-1/NPI-1) in NLS substrate binding, as well as in the targeting and import reactions in digitonin-permeabilized cells, we purified recombinant karyopherin α_2 (lacking 33 N-terminal residues and containing a C-terminal histidine tag) from *E. coli* (Fig. 1, lane 1). We also raised rabbit antibodies against a synthetic peptide corresponding to the C-terminal region of human karyopherin α_2. The antipeptide antiserum reacted with recombinant human karyopherin α_2 (Fig. 1, lane 2) but did not react with its rat counterpart in BRL cells (data not shown). As previously demonstrated for recombinant karyopherin α_1 (4), the recombinant karyopherin α_2 bound import substrate containing a wild-type NLS (Fig. 1, lane 3) but not a mutant NLS-containing substrate (Fig. 1, lane 4).

As reported (4) for recombinant karyopherin α_1 in a targeting reaction in digitonin-permeabilized cells, the recombinant karyopherin α_2 subunit by itself does not target NLS substrate to the nuclear envelope (Fig. 2*A*, *a*) but does so only in the presence of recombinant karyopherin β (Fig. 2*A*, *b*).

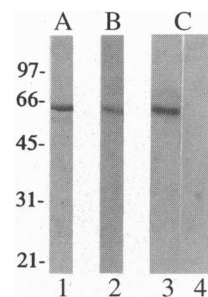

FIG. 1. Karyopherin α_2 binds the NLS–HSA import substrate. (*A*) Coomassie blue staining of recombinant karyopherin α_2. (*B*) Immunoblotting with anti-karyopherin α_2 antibody of recombinant karyopherin α_2. (*C*) Karyopherin α_2 (200 ng) was subjected to SDS/PAGE and transferred to nitrocellulose; the strips were then incubated with wild-type NLS–HSA (lane 3) or mutant NLS–HSA (lane 4) (13). Ligand binding was detected by incubation with anti-HSA antibodies, ^{125}I-labeled protein A, and autoradiography (4). M_r markers ($\times 10^{-3}$) are indicated at left.

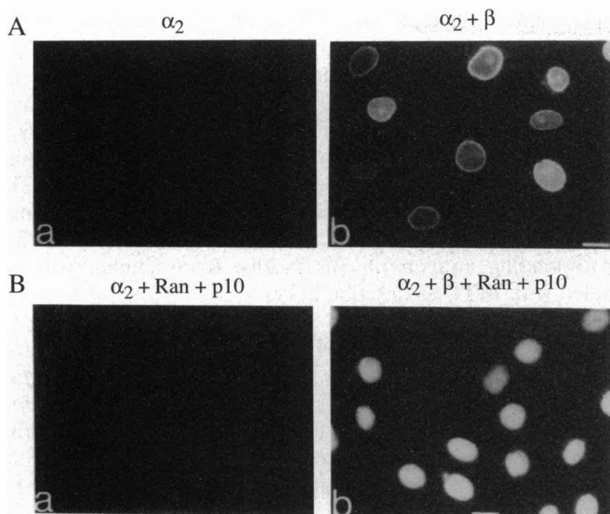

FIG. 2. Karyopherin α_2 substitutes for karyopherin α_1 in karyopherin β-dependent docking and import. (*A*) Docking. Digitonin-permeabilized cells were incubated for 30 min on ice with import substrate in the presence of 100 nM karyopherin α_2 (*a*) or 100 nM karyopherin α_2 plus 100 nM karyopherin β (*b*). (Bar = 10 μm.) (*B*) Nuclear import. Digitonin-permeabilized cells were incubated for 30 min at room temperature in the presence of Ran at 100 μg/ml, p10 at 3 μg/ml (7), and either 100 nM karyopherin α_2 alone (*a*) or together with 100 nM karyopherin β (*b*). (Bar = 10 μm.)

Likewise, recombinant karyopherin α_2 can substitute for recombinant karyopherin α_1 in an import reaction into digitonin-permeabilized BRL cells using recombinant β, Ran, and p10 (Fig. 2*B, b*). When recombinant β was omitted, there was no import (Fig. 2*B, a*). We conclude that recombinant karyopherin α_2 can substitute for karyopherin α_1 in NLS substrate binding; karyopherin β-dependent targeting; and karyopherin β-, Ran-, and p10-dependent import.

Karyopherin β Binds to the Nuclear Envelope. We immunized rabbits with recombinant rat karyopherin β and obtained an antiserum that reacted with the antigen (Fig. 3, lane 1). In indirect immunofluorescence of cold methanol-fixed BRL cells with anti-karyopherin β antibodies we observed some staining in the cytoplasm and strong staining at the nuclear envelope (Fig. 4, *a*). When the BRL cells were first permeabilized by digitonin, the cytoplasmic and nuclear rim staining was lost (Fig. 4*b*), suggesting that digitonin-permeabilized cells retain little, if any, of the endogenous karyopherin β. This result was confirmed when blots of SDS/PAGE-resolved proteins of digitonin-permeabilized cells (Fig. 3, lane 2) and of the digitonin extract (Fig. 3, lane 3) were

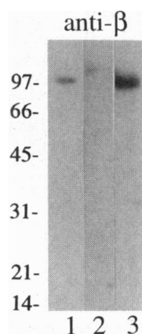

FIG. 3. Karyopherin β is extracted from BRL cells by digitonin permeabilization. Recombinant rat karyopherin β (lane 1), digitonin-extracted BRL cells (lane 2), and the digitonin extract (lane 3) were separated by SDS/PAGE, transferred to nitrocellulose, and incubated with anti-karyopherin β antibodies (1:100 dilution) followed by ^{125}I-labeled protein A. Molecular mass markers in kDa are indicated at left.

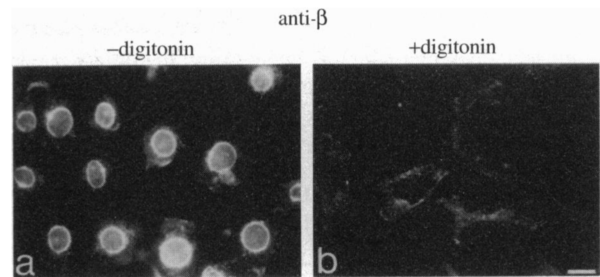

FIG. 4. Karyopherin β localized at the nuclear envelope and in the cytoplasm is lost during digitonin permeabilization. BRL cells in culture were fixed with methanol before (*a*) or after digitonin permeabilization (*b*) and immunostained with anti-karyopherin β antibodies followed by FITC-labeled goat anti-rabbit antibodies. (Bar = 10 μm.)

probed with anti-karyopherin β antibodies. The extraction by digitonin of endogenous karyopherin β explains the requirement for exogenous karyopherin β in both the docking and import reaction in digitonin-permeabilized cells. It should be noted here that digitonin permeabilization of BRL cells also leads to extraction of karyopherin α_1 and α_2 (data not shown) from the cytoplasm, consistent with the finding that each of these subunits forms a heterodimeric complex with karyopherin β. However, nuclear karyopherin α_1 (19) and α_2 (data not shown) are not extracted by digitonin.

We had previously proposed that the β subunit of the karyopherin heterodimer functions as an adaptor, binding to karyopherin α and to the NPC (4). To test this hypothesis we incubated digitonin-permeabilized cells with either recombi-

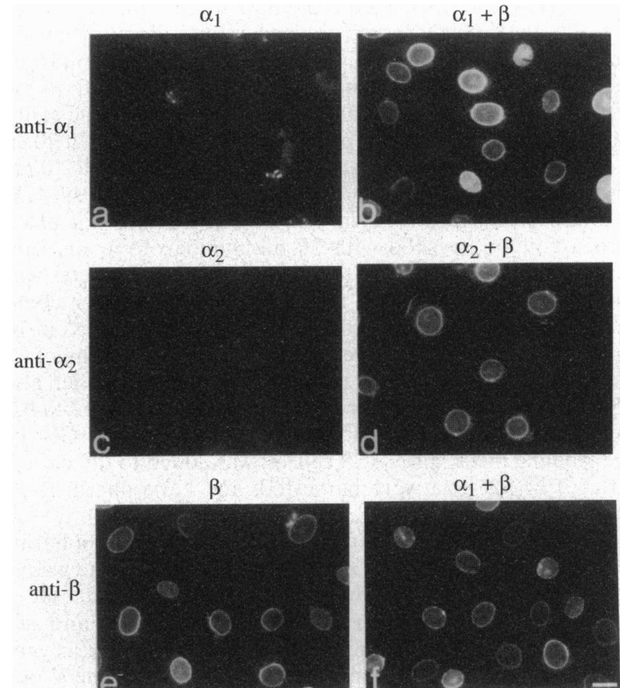

FIG. 5. Karyopherin β functions as an adaptor between karyopherin α/import substrate complex and the NPC. Digitonin-permeabilized cells were incubated with the import substrate in the presence of either karyopherin α_1 alone (*a*), karyopherin α_2 alone (*c*), karyopherin β alone (*e*), karyopherin α_1 and β (*b* and *f*), or karyopherin α_2 and β (*d*). After the docking reaction the cells were washed in transport buffer, fixed with methanol, and immunostained with anti-karyopherin α_1 antibodies (*a* and *b*), anti-karyopherin α_2 antibodies (*c* and *d*), or anti-karyopherin β antibodies (*e* and *f*). (Bar = 10 μm.)

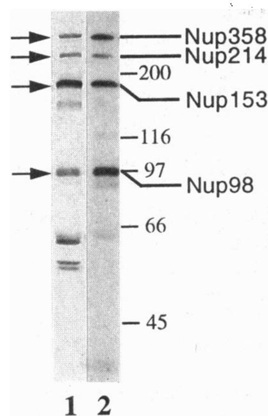

FIG. 6. Karyopherin β binds to peptide repeat-containing nucleoporins. SDS/PAGE-resolved proteins of rat liver nuclear envelopes were transferred to nitrocellulose and incubated with either ^{35}S-labeled recombinant karyopherin β (detected by radioautography) (lane 1) or the import substrate NLS–HSA in the presence of *Xenopus* cytosolic fraction A (12) (lane 2). The import substrate was detected by anti-HSA as described (3). The upper band in lane 2, previously referred to as p270 (3), was characterized as nucleoporin Nup358 (9). Arrows point to nucleoporins labeled in both lanes. Molecular mass markers in kDa are indicated at right.

nant karyopherin α_1, α_2, or β subunit alone or with one of the two α subunits and the β subunit and then used indirect immunofluorescence with monospecific antibodies to detect binding (Fig. 5). Consistent with our proposal we found that neither karyopherin α_1 (Fig. 5*a*) nor karyopherin α_2 (Fig. 5*c*) bound by itself to the nuclear envelope. However, both karyopherin α_1 (Fig. 5*b*) and karyopherin α_2 (Fig. 5*d*) bound when incubated in the presence of karyopherin β. Most importantly, karyopherin β could bind by itself (Fig. 5*e*).

Karyopherin β Binds to Peptide Repeat-Containing Nucleoporins. We have previously shown in a blot assay that the cytosolic subfraction A mediates binding of NLS substrate to peptide repeat-containing nucleoporins present in a rat liver nuclear envelope fraction (3, 12) (Fig. 6, lane 2). When an identical blot was probed with ^{35}S-labeled recombinant karyopherin β, we found that similar nucleoporins (Nup358, Nup214, Nup153, Nup98, and perhaps p62) were decorated (Fig. 6, lane 1). We conclude that karyopherin β binds to peptide repeat-containing nucleoporins on both the cytoplasmic (Nup358, Nup214) and nucleoplasmic side (Nup153, Nup98).

Karyopherin β Does Not Enter Nucleoplasm, Whereas Karyopherin α, Ran, and p10 Do. We performed an import reaction into nuclei of digitonin-permeabilized BRL cells with an NLS substrate and recombinant transport factors and then localized each of the exogenously added transport factors by fluorescence microscopy (Fig. 7). Both karyopherin α_1 and α_2 entered the nucleus (Fig. 7 *a* and *c*). So did Ran (Fig. 7*e*) and FITC-labeled p10 (Fig. 7*f*) and, of course, the import substrate (Fig. 7*d*). The only transport factor that did not enter the nucleus was karyopherin β (Fig. 7*b*). This result suggested that during import the karyopherin heterodimer was dissociated.

DISCUSSION

Our data here indicate that recombinant karyopherin α_2 [corresponding to Rch1 (16)] can functionally substitute for recombinant karyopherin α_1 [corresponding to hSRP/NPI-1 (14, 15)]. Either of these two proteins bound an NLS substrate (ref. 4 and Fig. 1); either of them, together with karyopherin β, docked the NLS substrate to the nuclear envelope of digitonin-permeabilized cells; and either of them imported the NLS substrate into nuclei of digitonin-permeabilized cells in the presence of recombinant karyopherin β, recombinant Ran, and recombinant p10. Thus, although human karyopherin α_1 and α_2 are only 44% identical, they appear functionally indistinguishable. However, there are probably differences between karyopherin α_1 and α_2—e.g., in the recognition of NLSs and

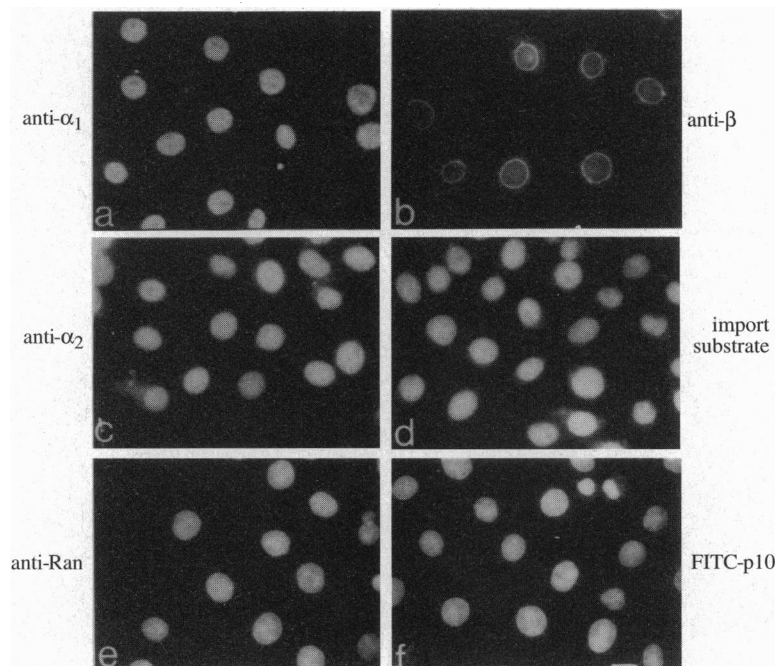

FIG. 7. Karyopherin α_1/α_2, Ran, and p10 enter the nucleus, whereas karyopherin β remains at the nuclear envelope during nuclear import of the substrate. The import assay was done in digitonin-permeabilized cells in the presence of karyopherin α_1 (100 nM), karyopherin β (100 nM), Ran (100 μg/ml), and either p10, at 3 μg/ml (*a, b, c, d,* and *e*) or FITC–p10 (*f*). In *c*, karyopherin α_1 was substituted by karyopherin α_2. After the import reaction the cells were washed, fixed with methanol, and probed either with anti-karyopherin α_1 antibodies (*a*), with anti-karyopherin β antibodies (*b*), with anti-karyopherin α_2 antibodies (*c*), or with anti-Ran antibodies (*e*) and stained with FITC-labeled goat anti-rabbit IgG or directly examined for rhodamine-labeled import substrate (*d*) or FITC-labeled p10 (*f*). (Bar = 10 μm.)

surrounding structures or in other parameters that our assays have not detected. An important result is that karyopherin β binds directly to a subgroup of nucleoporins that share various peptide repeats and that are located on the cytoplasmic side (Nup358, Nup214) and the nucleoplasmic side (Nup98, Nup153). This result indicates a division of labor; the α subunit of the karyopherin heterodimer serves in NLS recognition, and the β subunit mediates docking to the peptide repeat-containing nucleoporins. Most interesting is the finding that the karyopherin heterodimer dissociates at some point during transport; karyopherin α enters the nucleus and is retained there, whereas karyopherin β either does not enter the nucleoplasm or enters but is not retained there, being rapidly exported back into the cytoplasm (19). Immunofluorescence localization with anti-karyopherin β antibodies showed primarily cytoplasmic staining and a strong nuclear rim staining but no significant intranuclear staining, arguing against an intranuclear presence of karyopherin β. However, this cellular distribution may simply result from kinetic partitioning of karyopherin β between a short intranuclear transport phase, a longer transport phase across the NPC (in both directions), and a cytoplasmic pool.

The retention of either karyopherin α_1 or α_2 in the nucleus, even after digitonin permeabilization (ref. 19; data not shown for α_2), is another point of interest. Is intranuclear karyopherin α_1 or α_2 still docked to intranuclear sites (20), and is docking mediated by a karyopherin β-like protein? There are several proteins in GenBank that show some homology to karyopherin β. Although the function and location of these proteins are unknown, they might function in nuclear retention of various karyopherin α subunits and/or transport out of the nucleus.

We are greatly indebted to Robert E. O'Neill and Peter Palese for their generous gift of antibodies against karyopherin α_1, karyopherin α_1-expressing vector pSE420, and the cDNA for Rch1 and to Philip Bernstein for recombinant human Ran.

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