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The binding site of karyopherin α for karyopherin β overlaps with a nuclear localization sequence

(nuclear protein import/liquid phase binding assays/nuclear localization sequence recognition domain/presence of ligand and receptor in one molecule)

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ABSTRACT **By using proteolysis, recombinant mutant proteins, or synthetic peptides and by testing these reagents in liquid phase binding or nuclear import assays, we have mapped binding regions of karyopherin α. We found that the C-terminal region of karyopherin α recognizes the nuclear localization sequence (NLS), whereas its N-terminal region binds karyopherin β. Surprisingly, karyopherin α also contains an NLS. Thus, karyopherin α belongs to a group of proteins that contain both a ligand (NLS) and a cognate receptor (NLS recognition site) in one molecule with a potential for autologous ligand–receptor interactions. The NLS of karyopherin α overlaps with the binding site of karyopherin α for karyopherin β. Hence, binding of karyopherin β to karyopherin α covers the NLS of karyopherin α. This prevents autologous ligand receptor interactions and explains the observed cooperative binding of karyopherin α to a heterologous NLS protein in the presence of karyopherin β.**

The karyopherin heterodimer functions as a signal recognition and targeting factor in nuclear protein import (1–12). The α subunit of karyopherin recognizes the nuclear localization sequence (NLS) (3, 4, 9, 13) and the β subunit of karyopherin binds to peptide repeat-containing nucleoporins (4, 13–16). Interestingly, binding of recombinant yeast karyopherin α to NLS proteins was previously observed to be cooperatively enhanced in the presence of karyopherin β (13). However, the reason for this cooperativity is unknown.

In this paper, we report the identification of the binding regions for NLS and karyopherin β of karyopherin α. Fragments of karyopherin α were generated by proteolytic digestion of recombinant α, by expression in *Escherichia coli* of recombinant α mutants lacking specific regions, or by synthetic peptides representing regions of α. These reagents were tested in liquid phase binding and nuclear import assays. Karyopherin α's NLS binding site was localized to a C-terminal region and its β binding site to an N-terminal region. Surprisingly, the α subunit also contains an NLS. Hence, α contains both a ligand (NLS) and a cognate NLS recognition site within one molecule with the potential for self-interaction. Interestingly, karyopherin α's NLS overlaps with the binding site for karyopherin β. Thus, binding of karyopherin β to karyopherin α to form a heterodimer would cover karyopherin α's NLS. This would prevent karyopherin α's NLS from competing with that of a heterologous NLS protein for karyopherin α's NLS recognition site and thus would explain the previously observed cooperative binding of karyopherin α to a heterologous NLS protein in the presence of karyopherin β (13).

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MATERIALS AND METHODS

Preparation of Recombinant Transport Factors. Recombinant, full-length, human karyopherin α1 (referred to as α), rat karyopherin β (lacking 12 N-terminal residues), and full-length human p10 were expressed as His6 tagged proteins and purified from *E. coli* BL21(DE3) lysates on a Ni-nitrilotriacetic acid (NTA) column as previously described (3, 4). Recombinant human Ran was prepared as described (17).

Preparation of Mutants of Karyopherin α. Three karyopherin α mutants (αΔ49, lacking 49 N-terminal residues; αΔ115, lacking 115 N-terminal residues; α(50-456), comprising residues 50–456) were prepared by using PCR with primers containing restriction sites for insertion into the pET21b vector; expression in *E. coli* BL21(DE3) was induced with 1 mM isopropyl β-D-thiogalactoside. The recombinant His6 tagged proteins were purified as described (4) except that cells were lysed in a French pressure cell. The purified proteins were dialyzed against transport buffer (20 mM Hepes·KOH, pH 7.3/110 mM potassium acetate/2 mM magnesium acetate/1 mM EGTA/2 mM dithiothreitol), aliquoted, and stored at −70°C until use.

Synthetic Peptides. The following peptides were synthesized: EHYFGTEDED (aa residues 501–510 of karyopherin α; CEMRRRREEEGLQLRKQKREEQLFKRRN (aa residues 23–49 of karyopherin α with an N-terminal Cys added for coupling); ATAEEETEEE (aa residues 51–60 of karyopherin α); and CYTPPKKKRKV, the NLS of simian virus 40 T antigen as well as CYTPPKTKRKV, an NLS mutant form (18), each with an N-terminal Cys added for coupling.

Peptide Coupling. The import substrate NLS–human serum albumin (HSA) was prepared by coupling rhodamine-labeled HSA to the NLS of simian virus 40 T antigen as described (19). The peptide representing aa residues 23-49 of karyopherin α was coupled to HSA using the same procedure (19).

Solution Binding Assay. Assays were performed with one of the proteins immobilized. Immobilization was carried out as follows: NLS–HSA or karyopherin β was incubated for 4 hr at 4°C with AffiGel 10 (2 μg of each protein per 10 μl of packed beads) in binding buffer [20 mM Hepes·KOH, pH 7.3/110 mM KOAc/2mM $Mg(OAc)_2$/1 mM EGTA/2 mM dithiothreitol/0.1% Tween-20]. Under these conditions, the coupling reaction was complete, as no proteins could be detected upon SDS/PAGE analysis of the soluble fraction. To block remaining active sites, the gels were incubated with 1 M ethanolamine·HCl, pH 8.0, for 1 hr at 4°C. The beads were collected by centrifugation at 2000 × *g* for 30 s, and washed by 3 cycles of resuspension in 1 ml binding buffer and subsequent centrifugation. For the solution binding assay, washed beads were resuspended in a 50% slurry by adding one volume of binding buffer. The bead slurry (20 μl) was incubated in a final volume

Abbreviations: HSA, human serum albumin; NLS, nuclear localization sequence.

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of 40 μl with various components (see figure legends) in binding buffer for 30 min at 20°C. Beads were collected by centrifugation at 2000 × *g* for 30 s. The supernatant (29.5 μl) was collected and represented the unbound fraction. Beads were washed 2× by resuspension in 0.5 ml binding buffer at 20°C followed by sedimentation. Proteins that remained associated with the beads represented the bound fraction. Bound and unbound fractions were analyzed by SDS/PAGE and Coomassie brilliant blue staining as described (13).

Isolation of a Tryptic Fragment of Karyopherin α that Binds NLS. Karyopherin αΔ49 (200 μg) was incubated with 20 μl immobilized TPCK Trypsin (Pierce) for 18 hr at 20°C. The immobilized TPCK Trypsin was removed from the digestion mixture by centrifugation at 2000 × *g* for 1 min. The supernatant was incubated with NLS–HSA immobilized on AffiGel 10 (100 μl packed beads containing 200 μg of NLS–HSA) for 30 min at 20°C. After centrifugation at 2000 × *g* for 1 min, the supernatant was removed. After two cycles of resuspension in 1 ml of binding buffer and sedimentation, beads were eluted with 200 μl of 2M NaCl. The eluate was analyzed by SDS/PAGE and Coomassie brilliant blue staining. For sequence analysis, the isolated tryptic fragment was subjected to SDS/PAGE, electrophoretically transferred to polyvinylidine difluoride (PVDF) membrane and stained with Amido Black. The band was excised, digested with endoproteinase Lys-C and one of the peptides was subjected to sequence analysis (20).

Nuclear Import Assays. Assays were performed using Buffalo rat liver (BRL) cells permeabilized with digitonin as described (4). Import of fluorescently labeled NLS-HSA was quantitatively analyzed as previously described (19).

RESULTS

The C-Terminal Domain of Karyopherin α Contains the NLS Binding Site. Human karyopherin α1 (4), also known as h-Srp1 (21) or NPI-1 (22) and referred to here as karyopherin α, consists of three distinct structural domains (Fig. 1*a*): an N-terminal hydrophilic domain (residues 1–115) that is rich in charged residues; a central hydrophobic domain (residues 116–456) that contains eight arm (for armadillo) repeats of 42 residues each (23); and a C-terminal hydrophilic domain (residues 457–538). We have previously shown that recombinant human αΔ49 (α1 lacking 49 N-terminal residues; Fig. 1*b*) functions in import of the transport substrate NLS-HSA into nuclei of digitonin-permeabilized mammalian cells (3). Therefore, αΔ49 can be expected to retain both hitherto known functions of α: (*i*) binding to NLS and (*ii*) binding to karyopherin β. Indeed, like full-length α (Fig. 2*A*, lane 1), αΔ49 retained its ability to bind to AffiGel-immobilized NLS–HSA (Fig. 2*A*, lane 2) (for the ability of αΔ49 to bind β, see below). Neither α nor αΔ49 bound to AffiGel-immobilized mutant NLS–HSA (data not shown).

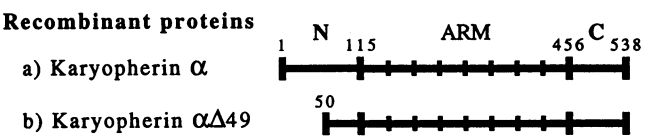

FIG. 1. Recombinant karyopherin α mutants and synthetic peptides of karyopherin α used in this study. ARM, armadillo repeats of 42 residues. Boldface letters indicate acidic residues in *e* and *g* and basic residues in *f*.

To isolate an α fragment with NLS binding activity, we subjected karyopherin αΔ49 to digestion with immobilized trypsin, incubated the tryptic digest with immobilized NLS–HSA, eluted with 2.0 M NaCl, and analyzed the eluate by SDS/PAGE and Coomassie blue staining. The isolated tryptic fragment, referred to as α*, had an estimated M_r of about 7 (Fig. 2*A*, lane 3). Microsequencing of an internal peptide obtained from α* by endopeptidase Lys-C cleavage yielded a sequence that is identical to residues 481-495 of α. As there is no Lys downstream of residue 495, α* extends to the C-terminal residue 538 of α. However, we do not know how far α* extends upstream as we were unable to obtain its N-terminal sequence. When αΔ49 and α* were coincubated with immobilized NLS–HSA, α* interfered with αΔ49 binding to

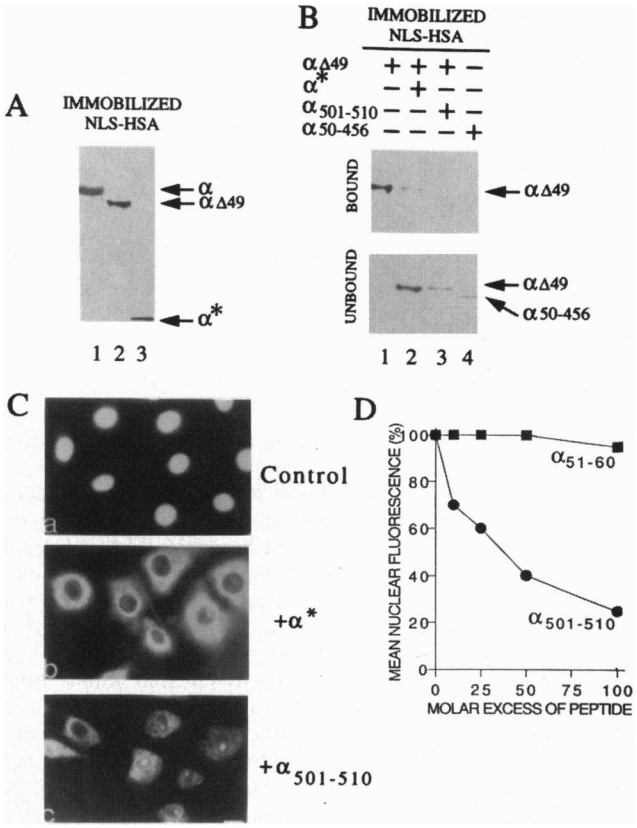

FIG. 2. The C-terminal domain of karyopherin α contains the NLS binding region. (*A*) Analysis by SDS/PAGE and Coomassie blue staining of proteins bound to immobilized NLS–HSA: lane 1, full-length α (1 μg); lane 2, αΔ49 (1 μg); lane 3, a tryptic fragment of αΔ49 that was eluted from immobilized NLS–HSA by 2 M NaCl and is referred to as α*. (*B*) Analysis by SDS/PAGE and Coomassie blue staining of NLS–HSA bound and unbound fractions. Immobilized NLS–HSA was incubated with (lane 1) αΔ49 at 0.5 μg alone, (lane 2) together with 1 μg of α*, (lane 3) together with a 100× molar excess of synthetic peptide $\alpha_{501\text{-}510}$, or (lane 4) with 0.5 μg of the $\alpha_{50\text{-}456}$ mutant alone. (*C*) Competitive inhibition of nuclear import of NLS–HSA in digitonin-permeabilized cells by the tryptic fragment α* and by the synthetic peptide $\alpha_{501\text{-}510}$. (*Ca*) Import of NLS–HSA (200 nM) in the presence of karyopherin α and β (each at 200 nM), Ran (3 μM), and p10 (6 μM). (*Cb* and *Cc*) Import reactions contain in addition either 0.5 μg of the tryptic fragment α* or a 100× molar excess of the synthetic peptide $\alpha_{501\text{-}510}$, respectively. (Bar = 10 μm.) (*D*) Quantitative analysis of nuclear import in the presence of increasing amounts of the synthetic peptides $\alpha_{501\text{-}510}$ and $\alpha_{51\text{-}60}$.

NLS–HSA (Fig. 2*B*, compare lanes 1 and 2), indicating that α* competed with αΔ49 for binding to NLS–HSA. Moreover, α* inhibited import of NLS–HSA into nuclei of digitonin-permeabilized cells (Fig. 2*Cb*). We also constructed a derivative of αΔ49 that lacked the C-terminal region. This derivative, $\alpha_{50\text{-}456}$ (Fig. 1*d*), was unable to bind to immobilized NLS–HSA (Fig. 2*B*, lane 4). Taken together, these data indicate that the C-terminal domain of karyopherin α contains the NLS binding site.

To further map the NLS binding site in the C-terminal region of α, we synthesized a decapeptide comprising residues 501–510 (Fig. 1*e*). This peptide, $\alpha_{501\text{-}510}$, was chosen because it contains five acidic residues that might constitute part of the binding site for the basic residue-rich NLS. Indeed, when αΔ49 and a 100-fold excess of $\alpha_{501\text{-}510}$ were coincubated with immobilized NLS–HSA, there was greatly diminished binding of αΔ49 to NLS–HSA (Fig. 2*B*, lane 3). Moreover, $\alpha_{501\text{-}510}$ inhibited import of NLS–HSA into nuclei of digitonin-permeabilized cells (Fig. 2*Cc*) in a concentration dependent manner (Fig. 2*D*). The inhibition by $\alpha_{501\text{-}510}$ was specific, as another highly acidic peptide from the N-terminal domain of karyopherin α, $\alpha_{51\text{-}60}$ (Fig. 1*g*), had no effect on import of NLS–HSA into nuclei of digitonin-permeabilized cells (Fig. 2*D*). These data indicate that residues 501–510 of karyopherin α constitute a major determinant of its NLS binding site.

The N-Terminal Domain of Karyopherin α Contains the Binding Site for Karyopherin β. To test whether the N-terminal domain of α contains the β binding site, we made a construct that lacked 115 N-terminal residues. This α mutant, αΔ115 (Fig. 1*c*), did not bind to immobilized karyopherin β (Fig. 3*A*, lane 3), whereas full-length α or αΔ49 did bind (Fig. 3*A*, lanes 1 and 2, respectively). The binding of αΔ49 to β is consistent with our previous data showing that αΔ49 functioned in NLS–HSA import into nuclei of digitonin-permeabilized cells (3). As expected, αΔ115 was unable to substitute for full-length α in import of NLS–HSA into nuclei of digitonin-permeabilized cells (Fig. 3*C*). Taken together, these data suggested that a major determinant for α's binding site for β is localized between residues 50–115 (see below).

Karyopherin α's Binding Site to Karyopherin β Overlaps with an NLS. One interesting aspect of the N-terminal region of α is the presence of a potential bipartite NLS (24) that appears to be conserved in all known karyopherin α homologues. To determine whether this sequence could function as an NLS, we synthesized a peptide, $\alpha_{23\text{-}49}$ (Fig. 1*f*), containing the potential bipartite NLS. When crosslinked to HSA and immobilized on AffiGel, this peptide was indeed able to bind both α and αΔ49 (Fig. 3*B*, lanes 2 and 3, respectively), but not $\alpha_{50\text{-}456}$, which lacks the NLS recognition site (data not shown). Moreover, binding of α or of αΔ49 to immobilized $\alpha_{23\text{-}49}$ was competed for by wt-NLS (Fig. 3*B*, lanes 5 and 6) but not by mutant NLS (data not shown). These data suggest that $\alpha_{23\text{-}49}$ does indeed contain an NLS.

Interestingly, the peptide also bound karyopherin β (Fig. 3*B*, lane 1). Unlike peptide binding to α and αΔ49 that was competed for by NLS, peptide binding to karyopherin β was not inhibited by NLS (Fig. 3*B*, lane 4). These data suggest that α's binding site for β is not restricted to residues 50–115 of α (see above) but extends upstream to include residues 23–49 of α. Indeed, when we tested whether quantitative differences could be detected in nuclear import between full-length α and αΔ49, we found that the extent of nuclear import, as a function of concentration, was higher for full-length α than it was for αΔ49 (Fig. 3*C*). We conclude that α's residues 23–49 not only constitute part of α's binding site to β, but also contain an NLS.

The presence in karyopherin α of both a ligand (NLS) and a receptor function (NLS recognition site) suggests a potential for self-interaction. Indeed, gel filtration experiments indicated that α forms dimers (2). Binding of α's NLS to α's NLS recognition site might explain α's relatively low affinity for

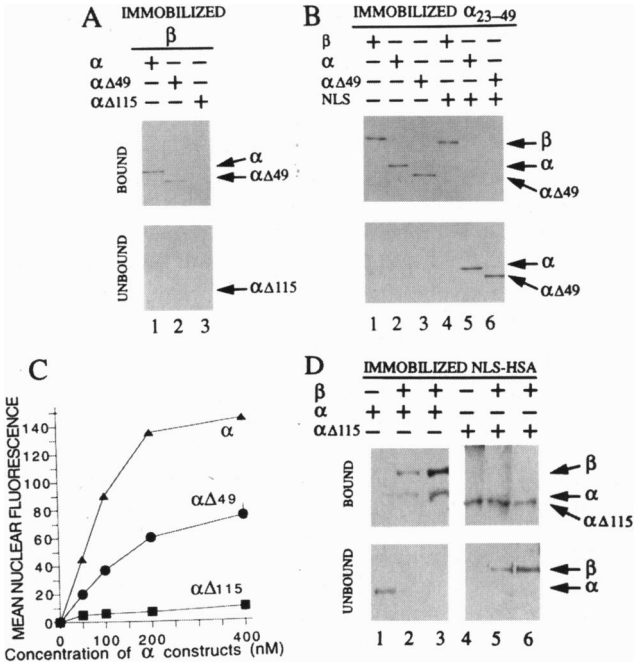

FIG. 3. The N-terminal domain of karyopherin α contains the binding site for karyopherin β. (*A*) Analysis by SDS/PAGE and Coomassie blue staining of karyopherin β bound and unbound fractions. Immobilized karyopherin β was incubated with (lane 1) 0.5 μg of α, (lane 2) 0.5 μg of αΔ49, or (lane 3) 0.5 μg of αΔ115. (*B*) Analysis by SDS/PAGE and Coomassie blue staining of $\alpha_{23\text{-}49}$ peptide bound and unbound fractions. Immobilized synthetic peptide $\alpha_{23\text{-}49}$ was incubated with karyopherin β, karyopherin α, or αΔ49 (each at 0.5 μg) in the absence (lanes 1–3) or presence (lanes 4–6) of a 100× molar excess of the NLS of simian virus 40 T antigen. (*C*) Quantitative analysis of the nuclear import of NLS–HSA in digitonin-permeabilized cells in the presence of karyopherin β (200 nM), Ran (3 μM), p10 (6 μM), and increasing concentrations of karyopherin α, αΔ49, or αΔ115. (*D*) Analysis by SDS/PAGE and Coomassie blue staining of NLS–HSA bound and unbound fractions. Immobilized NLS–HSA (0.5 μg per 10 μl packed beads) was incubated (lane 1) with 0.3 μg α alone, (lanes 2 and 3, respectively) together with 0.3 or 0.6 μg of β, (lane 4) with 0.3 μg of αΔ115 alone, or (lanes 5 and 6, respectively) together with 0.3 or 0.6 μg of β.

NLS–HSA (Fig. 3*D*, lane 1) because α's own NLS might compete with that of the heterologous NLS–HSA for binding to α's NLS recognition site. However, in the presence of β, α's NLS, overlapping with α's site for β, would be covered by bound β, thereby preventing α's NLS from competing with NLS–HSA for α's NLS recognition site. This would explain the cooperative effect of β on α binding to NLS (Fig. 3*D*, lanes 2 and 3), previously reported for the corresponding proteins of yeast (13). If competition between α's NLS and NLS–HSA for α's NLS recognition site occurred, then αΔ115 (Fig. 1*c*) that lacks the NLS but still contains the NLS recognition site should bind as well to NLS–HSA as full-length α did in the presence of β; indeed, it did (Fig. 3*D*, lane 4; compare with lane 3). As expected, the addition of β did not enhance αΔ115 binding to NLS–HSA (Fig. 3*D*, lanes 5 and 6) as αΔ115 lacked the β binding site.

DISCUSSION

Using proteolytic fragmentation, recombinant mutant proteins, or synthetic peptides representing regions of karyopherin α and assaying these reagents in liquid phase binding and nuclear import reactions, we have mapped karyopherin α's binding sites for karyopherin β and for the NLS substrate. Our results are summarized in Fig. 4. We found that karyopherin α contains both an NLS and an NLS recognition site; hence,

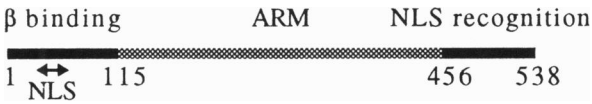

FIG. 4. Schematic representation of karyopherin α and its binding regions. For details, see text and Fig. 1*a*.

karyopherin α contains both a ligand (NLS) and a cognate ligand recognition site in one molecule, potentially allowing for autologous reactions. Moreover, karyopherin α's NLS overlaps with its β binding site.

Karyopherin α's Binding Site for NLS. A tryptic fragment of karyopherin α representing part of its C-terminal region was found to contain the binding site for NLS. This tryptic peptide was isolated by affinity chromatography on immobilized NLS–HSA and was eluted at high salt, indicating that its interaction with NLS might be electrostatic in nature. A synthetic decapeptide located near the C terminus of this region and containing five acidic residues was found to substitute for the effects of the tryptic peptide. These acidic residues might interact with the basic residues of the NLS and therefore might constitute a determinant of α's recognition site for NLS. However, the C-terminal region of karyopherin α contains other acidic residues and it is possible that these contribute to NLS recognition as well.

We also obtained a karyopherin α mutant that lacked the entire hydrophilic C-terminal domain. As expected, this α mutant was unable to bind to NLS–HSA. It could still bind to karyopherin β indicating that removal of α's C-terminal domain had no adverse effects on α's other known function, namely binding to karyopherin β.

Karyopherin α's Binding Site for Karyopherin β Overlaps with an NLS. Surprisingly, karyopherin α's N-terminal hydrophilic region contained two overlapping sites: an NLS and a binding site for karyopherin β. An α mutant lacking 49 N-terminal residues, αΔ49, still functioned in binding to karyopherin β, consistent with previously reported results (3) that αΔ49 functions in import of NLS–HSA into nuclei of digitonin-permeabilized cells. However, an α mutant lacking 115 N-terminal residues, αΔ115, no longer functioned in binding to β and in nuclear import. These results indicate that a determinant for α binding to β is located between residues 50 and 115 of α and that this determinant is sufficient for binding to β. A candidate for this determinant is a sequence, K_{105}FRKLLSKE$_{113}$, that is rich in basic residues. This basic cluster contains a Ser that is conserved in all karyopherin α homologs so far described. A conditional mutation of yeast karyopherin α, where this Ser is changed to Phe, inhibits docking of NLS substrate at the nuclear envelope (25), consistent with this region's involvement in binding karyopherin β.

However, it is clear that the region between residues 50 and 115 of karyopherin α is not the only determinant for α binding to karyopherin β. We found that a synthetic peptide, $\alpha_{23\text{-}49}$, was also able to bind β indicating that α's binding site for β extends further upstream. Hence, α's binding site for β comprises regions both upstream and downstream of residue 50. Although the downstream region is clearly sufficient for binding to β, the upstream region may contribute to the strength of binding. Indeed, we found that the extent of nuclear import into nuclei of digitonin-permeabilized cells as a function of α or αΔ49 concentration was 2–3 times higher for full-length α than it was for αΔ49. This is consistent with a more efficient α binding to β in the presence of both upstream and downstream determinants.

Interestingly, the $\alpha_{23\text{-}49}$ peptide also contained an NLS as it bound to α or αΔ49. This binding was competed for by synthetic wt-NLS but not by a mutant NLS. Most importantly, binding of $\alpha_{23\text{-}49}$ to β was not competed for by wild-type (or mutant) NLS peptide. Hence, α's N-terminal β binding site overlaps with an NLS. The presence in karyopherin α of both a ligand (NLS) and a cognate receptor function (NLS recognition site) would allow for autologous reactions. In the absence of β, α's own NLS would compete with the heterologous NLS of another protein, such as NLS–HSA, for binding to α's NLS recognition site. Such competition would explain the relatively low affinity of α for an NLS protein. However, in the presence of β, binding of β to its cognate site on α would cover the overlapping NLS. This would prevent autologous reactions and competition from α's NLS for binding to α's NLS recognition site, consistent with the observed cooperativity of β for binding of α to an NLS protein (Fig. 3*D*; ref. 13). Likewise, the Ran–GTP induced dissociation of the karyopherin heterodimer and the resulting release of NLS protein from karyopherin α (13) can be explained by competition between karyopherin α's NLS, no longer covered by karyopherin β, with the heterologous NLS protein for binding to karyopherin α's NLS recognition site. In agreement with this interpretation are the data obtained with αΔ115. This mutant lacks the NLS and the overlapping binding region for β but retains the NLS recognition site. Hence, binding of αΔ115 to NLS–HSA was much more efficient than that of full-length α, and was as efficient as that of full-length α in the presence of β. As expected, αΔ115 binding to β was not enhanced in the presence of β.

There are precedents for the presence in one molecule of both ligand and cognate receptor. In c-Src, for example, a C-terminal phosphotyrosine (ligand) may bind to the SH2 domain (receptor). Such an autologous intramolecular ligand–receptor interaction yields inactive c-Src as an important means to regulate c-Src activity (26). Likewise, in the case of karyopherin α, autologous or heterologous ligand–receptor interactions may operate for up- or down-regulating nuclear import.

The function of the central arm repeats of karyopherin α remains to be elucidated. A protein consisting essentially of arm motifs and termed smgGDS has been implicated in GDP/GTP exchange for a family of ras-related GTPases (23). As Ran–GTP can be generated locally at the nuclear pore complex from Ran–GDP in a karyopherin and p10-dependent fashion (27), the arm region of α may function in exchanging GDP for GTP in Ran.

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