The Auto-inhibitory Function of Importin α Is Essential in Vivo*

Revised for publication, October 25, 2002, and in revised form, December 10, 2002
Published, JBC Papers in Press, December 16, 2002, DOI 10.1074/jbc.M210951200

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Proteins that contain a classical nuclear localization signal (NLS) are recognized in the cytoplasm by a heterodimeric import receptor composed of importin/karyopherin α and β. The importin α subunit recognizes classical NLS sequences, and the importin β subunit directs the complex to the nuclear pore. Recent work shows that the N-terminal importin β binding (IBB) domain of importin α regulates NLS-cargo binding in the absence of importin β in vitro. To analyze the in vivo functions of the IBB domain, we created a series of mutants in the Saccharomyces cerevisiae importin α protein. These mutants dissect the two functions of the N-terminal IBB domain, importin β binding and auto-inhibition. One of these importin α mutations, A3, decreases auto-inhibitory function without impacting binding to importin β or the importin α export receptor, Cse1p. We used this mutant to show that the auto-inhibitory function is essential in vivo and to provide evidence that this auto-inhibitory-defective importin α remains bound to NLS-cargo within the nucleus. We propose a model where the auto-inhibitory activity of importin α is required for NLS-cargo release and the subsequent Cse1p-dependent recycling of importin α to the cytoplasm.

In eukaryotes, the nuclear envelope provides an essential barrier that separates the nuclear genome from the intermediary metabolism, signaling systems, and translation machinery of the cytoplasm. Selective bi-directional transport of macromolecules across this nuclear envelope regulates critical cellular processes such as gene expression (1, 2). All nucleocytoplasmic transport of macromolecules occurs through large proteinaceous structures, called nuclear pore complexes (NPC),1 that perforate the nuclear envelope (3, 4). These macromolecular cargoes are specifically targeted to and transported through NPCs by a family of soluble nuclear transport receptors (5, 6).

The small GTPase, Ran, governs the interactions between the nuclear transport receptors and macromolecular cargoes (5, 7). Import receptors bind cargo in the absence of RanGTP, whereas export receptors bind cargo in a trimeric complex with RanGTP (5, 8). This mode of regulation requires an asymmetric distribution of RanGTP, with more RanGTP in the nucleus than in the cytoplasm. To achieve this asymmetry, the Ran regulatory proteins are compartmentalized with the GTPase activating protein (RanGAP), which generates RanGDP, in the cytoplasm (9) and the guanine nucleotide exchange factor (RCC1), which generates RanGTP, in the nucleus (10).

The best-characterized nuclear import process occurs via receptor recognition of a classical nuclear localization signal (NLS). This classical NLS is typified by a cluster of basic amino acids (monopartite) or two clusters of basic amino acids separated by a 10–12 amino acid linker (bipartite) (11, 12). A heterodimeric import receptor, composed of importins α and β (also known as karyopherin α and β), mediates the nuclear import of proteins that contain a classical NLS (13–15). Over the last several years many studies have led to a detailed model for the individual steps in the classic nuclear transport cycle (5, 16): 1) importin α binds to the NLS-cargo to form a trimeric import complex with importin β; 2) this NLS-cargo/importin α/importin β complex is targeted to the NPC by importin β; 3) the complex then translocates into the nucleus where it encounters RanGTP; 4) upon binding RanGTP, importin β dissociates from NLS-cargo/importin α; 5) NLS-cargo is released from importin α; and 6) once cargo is released, importin α is recycled to the cytoplasm by its export receptor, Cse1p/CAS, in a trimeric complex with RanGTP. Thus, the directionality and efficiency of nuclear import of NLS-cargo is accomplished not only by the Ran GTPase cycle but also by an additional series of protein-protein interactions, occurring in defined locations, that result in changes in affinities of the transport receptor for NLS-cargo.

Dissociation of the NLS-cargo/importin α/importin β import complex is critical for delivery of NLS-cargo into the nucleus (7). RanGTP-mediated dissociation of importin β from the trimeric import complex (17) results in an NLS-cargo/importin α complex. Precisely how NLS-cargo is then released from importin α is unknown; however, a recent study shows that Cse1p and the nucleoporin, Nup2p, can facilitate cargo release in vitro (18). The release of cargo is important in the subsequent functions of both the cargo (7) and importin α (19–21). For example, a recent study identified a critical cargo, TPX2, of the importin α/β complex whose release from importin α is essential for mitotic progression (22). Although this study did not determine the mechanism of cargo release, it highlighted the importance of understanding how cargoes are efficiently dissociated from importin α within the nucleus to mediate their cellular function. Furthermore, Cse1p, the export receptor for importin α, can only interact with importin α that is not bound to NLS-

1 The abbreviations used are: NPC, nuclear pore complex; CEN, centromeric; DIC, differential interference contrast; 3-FOA, 3-fluoro- orotic acid; GFP, green fluorescent protein; GST, glutathione S-transferase; IBB, importin β binding; NLS, nuclear localization signal; RanGTP-SS, RanGTP regenerating system; PBS, phosphate-buffered saline; DAPI, 4′,6-diamidino-2-phenylindole; GTP-γS, guanosine 5′-3-O-(thio)triphosphate.
cargo (19–21). Thus, for importin α to be recycled to the cytoplasm, it must be dissociated from NLS-cargo within the nucleus. This ensures that importin α is recycled to the cytoplasm only when it has released NLS-cargo; and thereby provides a mechanism for assuring uni-directional transport of NLS-cargo into the nucleus.

The convergence of data from structural analyses and in vitro binding studies provides insight into how importin α binds to and regulates interactions with NLS-cargo (17, 23–26). Domain analysis has shown that importin α has an N-terminal domain that binds importin β (the importin β binding domain or IBB), a central armadillo domain that constitutes the NLS binding pocket, and a C-terminal region that appears to be important for binding to its export receptor Cse1p/CAS (21, 27). Structural studies have extended the knowledge obtained from this domain analysis. Conti et al. (25) solved the structure of truncated Saccharomyces cerevisiae importin α (a amino acid residues 89–530) in the presence of an NLS peptide revealing details of how the central armadillo domain of importin α creates specific binding pockets for NLS-cargo. More recently, the structure of full-length mouse importin α, solved in the absence of NLS-cargo, showed that the N-terminal IBB domain of importin α can form an intramolecular interaction with the NLS-binding pocket of importin β (23). This observation suggested that the IBB, in addition to mediating binding to importin β (28), could have a second role as an auto-inhibitory domain to regulate cargo binding (23). In support of this hypothesis, in vitro binding studies have shown that importin α lacking the proposed auto-inhibitory domain (ΔIBB-α), binds more tightly to NLS-cargo than full-length importin α (26), and that this auto-inhibition of full-length importin α binding to NLS-cargo is relieved in the presence of importin β (17, 26). Further analysis of the N-terminal IBB domain of importin α revealed a proposed internal NLS that could serve as an auto-inhibitory sequence to regulate NLS binding through intramolecular competition for the NLS binding site (23, 24). Taken together, these studies suggest a dual role for the N-terminal domain of importin α in 1) binding to importin β and 2) auto-inhibition of NLS-cargo binding.

To analyze the in vivo requirement for of the auto-inhibitory activity of importin α, we created a set of importin α mutants. These mutants dissect the two functions of the N-terminal domain, importin β binding and auto-inhibition. One of these mutants specifically decreases auto-inhibitory function without impacting RanGTP-regulated binding to importin β. Despite normal binding to both importin β and Cse1p, this importin α mutant is unable to function in vivo. These experiments demonstrate that the auto-inhibitory function of importin α is essential in vivo. Our data support the hypothesis that the auto-inhibitory mutant is deficient in NLS-cargo release from importin α and the subsequent recycling of importin α to the cytoplasm. Thus, we propose that the auto-inhibitory function of importin α is necessary for efficient release of NLS-cargo into the nucleus via intramolecular competition of the N-terminal IBB domain at the NLS-binding site.

EXPERIMENTAL PROCEDURES

Strains, Plasmids, and Chemicals—All chemicals were obtained from Sigma or USBiological unless otherwise noted. All DNA manipulations were performed according to standard methods (29), and all media was prepared by standard procedures (30). All yeast strains and plasmids used in this study are described in Table I. A complete deletion of the SRP1 open reading frame was created using a standard PCR-based strategy (31) in the wild-type diploid ACY247 to create the haploid ASRP1 strain (ACY249) maintained by an SRP1 URA3 plasmid (pAC876). Importin α-GFP and A3-importin α-GFP were integrated at the endogenous importin α locus of wild-type (ACY192) and cse1-1 (ACY671) cells using a standard integration strategy. This integration strategy creates a duplication at the endogenous importin α locus such that both endogenous importin α and importin α-GFP are expressed from an SRP1 promoter.

Generation of Importin α Mutants—Amino acid substitutions were introduced in the S. cerevisiae importin α (SRP1) coding region using PCR. For most mutants, mutagenesis was carried out on importin α in the bacterial expression vector pProEX-HTB (PerkinElmer Life Sciences). Mutations were subcloned from the bacterial expression vector into the yeast expression plasmid for in vivo studies. An 88-amino acid N-terminal deletion of importin α, ΔIBB-α (pAC959), was created using the following PCR-based strategy. An importin α plasmid (pAC876) was used as a template to amplify the importin α promoter and ΔIBB-α open reading frame starting at a PCR-introduced ATG start codon preceding leucine residue 89. These products were further amplified by PCR to introduce ΔIBB-α (amino acid residues 89–342) expressed from the endogenous importin α promoter. The resulting PCR product was cloned into the yeast centromeric (CEN) plasmid pRS315 (32). For all constructs generated, the presence of each desired mutation and the absence of any other mutations were confirmed by DNA sequencing.

In Vivo Functional Analysis—The in vivo function of each of the importin α mutants was tested using a plasmid shuffle technique (33). Plasmids encoding each of the importin α mutant proteins were individually transformed into SRP1 deletion cells (ACY254) containing the URA3 SRP1 maintenance plasmid, pAC876. Single transformants were grown in liquid culture to saturation, serially diluted (1:10), and spotted on minimal media plates lacking leucine as a control or on fluoro- orotic acid (FOA) plates. The URA3 SRP1 maintenance plasmid encoded wild-type importin α (pAC876) (33). Plates were incubated at 30 °C for 3 days.

Immunoblot Analysis—Immunoblot analysis was performed essentially as described previously (34). Briefly, cultures were grown to log phase in appropriate media at 30 °C. Cells were harvested by centrifugation and washed twice in water and once in PBSMT (100 mM KH2PO4, pH 7.0, 15 mM (NH4)2SO4, 75 mM KOH, 5 mM MgCl2, 0.5% Triton X-100). Cells were subsequently lysed in PBSMT with protease inhibitors (0.5 mM phenylmethylsulfonyl fluoride, 3 μg/ml each of aprotinin, leupeptin, chymostatin, and pepstatin) by glass bead lysis. Equal amounts of total protein (generally 10 μg) were resolved by SDS-PAGE and transferred to nitrocellulose. The Echerichia coli strain BL21(DE3) and purified by nickel affinity chromatography essentially as described previously (26, 38, 39). Importin β was expressed and purified as described previously (28). GST-importin α and GST-A3-importin α were expressed as described elsewhere (40).

Expression and Purification of Recombinant Proteins—Assays were performed with purified recombinant S. cerevisiae proteins Srp1p (importin α), Kap95p (importin β), Cse1p, and Gsp1p (Ran). Full-length His6-importin α (residues 1–542), His6-ΔIBB-importin α (residues 89–530), His6-IBB-GFP (importin α residues 1–88), His6-SV40 (SP-KKKKVEAS)-NLS-GFP, His6-Cse1p, and His6-Ran were expressed in the Echerichia coli strain BL21(DE3) and purified by nickel affinity chromatography essentially as described previously (26, 38, 39). Importin β was expressed and purified as described previously (28). GST-importin α and GST-A3-importin α were expressed as described elsewhere (40).

Fluorescence Depolarization Assay—Fluorescence anisotropy measurements were carried out using an ISS PC1 fluorometer fitted with polarization filters. The dissociation constants for the binding of SV40 NLS-GFP and IBB-GFP to importin α were measured essentially as described previously (26, 38). Briefly, SV40 NLS-GFP or IBB-GFP was diluted in PBS to the desired concentration (5–20 nM) in a total volume of 1 ml in a 1-cm quartz cuvette. Changes in the anisotropy of the GFP fluorophore were monitored as aliquots of the full-length wild-type or mutant importin α proteins were successively added to the volume. Changes in anisotropy were used to calculate the fraction of the GFP fluorophore bound, yielding a binding isotherm for the reaction. The binding isotherm was then fit through nonlinear regression to a simple binding equation to obtain dissociation constants. All Kd values were calculated as detailed at http://www.biochem.emory.edu/Hodel/FittingCurves/fitting_curves.htm. The dissociation constant for importin α binding to importin β was measured using a competition assay (41) with IBB-GFP. The assay was carried out as described above except changes in the anisotropy of IBB-GFP were monitored in the presence of increasing amounts of importin β. This yielded a dissociation constant for IBB-GFP binding to importin β. To measure the fraction of importin α to importin β, the binding of IBB-GFP was examined in the presence of three different concentrations of full-length wild-type or mutant importin α. The Kd values for wild-type and mutant importin α proteins binding to importin β were determined by fitting the resulting binding curves to an equation for the fraction of IBB-GFP.
Functional Dissection of Importin α

TABLE I
Strains and plasmids used in this study

| Strain/plasmid | Description |
|---------------|-------------|
| ACY192 (wild-type) | MaTA ara3-52 leu2Δ1 trp1 (51) |
| ACY247 (wild-type) | MaTaU ara3-52 ura3-52 leu2Δ1 thr1Δ1 his3Δ200 his3Δ200 ade2 ade2 ade3 ade3 lys2 lys2 LYS2 trp1/ TRP1 |
| ACY324 (ΔSRP1) | MaTaU ara3-52 leu2Δ1 lys2 his3Δ200 SRP1::HIS3 |
| ACY671 (car-1-1) | MaTaU ara3-52 leu2Δ1 trp1 (52) |
| pA3 (pRS315) | CEN, LEU2, AMPR (32) |
| pA8 (pRS426) | 2μ, URA3, AMPR (32) |
| pA8 (pRS303) | HIS3-integrating, AMPR (32) |
| pA366 | RSΔ1, AMPR, pET-based importin β bacterial expression vector (26g) |
| pAC492 | Srr1p, AMPR, pProEX-HTB importin α bacterial expression vector (25g) |
| pAC493 | ΔIBB-ΔIBB-AMP (32), pProEX-HTB bacterial expression vector (25) |
| pAC631 | GST-SRP1, AMPR, pGEX4T-1 bacterial expression vector |
| pAC480 | CSE1, 2μ, URA3, AMPR (56) |
| pAC835 | A2 (3KRKRKK→AAAA)-SRP1, CEN, LEU2, AMPR |
| pAC836 | A1 (3RRRRR→AAAA)-SRP1, CEN, LEU2, AMPR |
| pAC855 | A3 (3KKKR→AAAA)-SRP1, CEN, LEU2, AMPR |
| pAC856 | SRP1, CEN, LEU2, AMPR |
| pAC857 | SRP1, CEN, URA3, AMPR |
| pAC883 | SRP1-GFP, CEN, URA3, AMPR |
| pAC890 | A3-SRP1-GFP, CEN, URA3, AMPR |
| pAC891 | SRP1-c-myc (3X), CEN, URA3, AMPR |
| pAC892 | A1-SRP1-c-myc (3X), CEN, URA3, AMPR |
| pAC893 | A2-SRP1-c-myc (3X), CEN, URA3, AMPR |
| pAC894 | A3-SRP1-c-myc (3X), CEN, URA3, AMPR |
| pAC895 | ΔIBB-SRP1, CEN, LEU2, AMPR |
| pAC896 | ΔIBB-SRP1-c-myc (3X), CEN, URA3, AMPR |
| pAC899 | CSE1, KANΔ, pET-9d bacterial expression vector (40) |
| pAC1012 | A3-ED-SRP1-GFP, CEN, URA3, AMPR |
| pAC1045 | pGAL1-10-BPSV40-GFP, 2μ, LEU2, AMPR |
| pAC1156 | SRP1, integration, URA3, AMPR |
| pAC1157 | A3-SRP1, integration, URA3, AMPR |
| pAC1200 | GSP1, AMPR, pProEX-HTB bacterial expression vector (39) |
| pAC1206 | IBB-GFP, KANΔ, pET-28a bacterial expression vector (26) |
| pAC1207 | SV40 NLS-GFP, KANΔ, pET-28a bacterial expression vector (26) |
| pAC1210 | A1-SRP1, AMPR, pProEX-HTB bacterial expression vector |
| pAC1211 | A2-SRP1, AMPR, pProEX-HTB bacterial expression vector |
| pAC1212 | A3-SRP1, AMPR, pProEX-HTB bacterial expression vector |
| pAC1227 | GST-A3-SRP1, AMPR, pGEX4T-1 bacterial expression vector |
| pAC1286 | A3-IBB-GFP, KANΔ, pET-28a bacterial expression vector |

* The S. cerevisiae proteins Srp1p (importin α), Rs11p (importin β), and Gsp1p (Ran) were used for these experiments.

bound as a function of $K_D$ for IBB, $K_F$ for the full-length importin α protein, the total IBB-GFP concentration, the total importin β concentration, and the total concentration of the full-length importin α protein. Binding energies were calculated using $\Delta G = RT \ln K_D$ where $RT = -0.59 \text{ kcal/mol}$.

Solid Phase Binding Assay—As previously described (26), importin β was covalently coupled to epoxy-activated Sepharose beads (Amersham Biosciences) at a concentration of 1 mg/ml. Approximately 0.2 mg/ml purified full-length wild-type or full-length mutant importin α or ΔIBB-α was incubated with 0.5 ml of importin β-coupled beads in binding buffer (50 mM Tris-HCl, pH 7.8, 100 mM NaCl, 20 mM dithiothreitol) for 15 min on ice before addition to the beads at 4°C for 2 h. The supernatants from each reaction were subsequently saved as the eluant fractions. The beads were washed twice with binding buffer before elution with elution buffer. The unbound (2% of total unbound), bound (2% of total bound), and eluant (2% of total eluant) fractions were resolved on a 10% SDS-PAGE gel and visualized by Coomassie Brilliant Blue staining (42).

The GST-importin α proteins were bound to glutathione-Sepharose (Amersham Biosciences) and tested for interaction with Cse1p essentially as described previously (40), with the following modifications. The GST-importin α-conjugated beads were washed five times with PBSMT before incubation with purified Cse1p (10 mg) and Gsp1p (20 mg) in the presence or absence of the RanGTP-RS in a total volume of 500 µl. The bound (50% of total bound) fractions were resolved on a 5% SDS-PAGE gel and immunoblotted as described. For quantification, immunoblots were analyzed using a fluorescence imager and Quantity One Software (Bio-Rad).

Microscopy—Indirect immunofluorescence microscopy was performed as described elsewhere (37) with the following conditions. The myc antibody was used at 1:100 dilution, and the GFP antibody was used at 1:2000 for incubation with cells overnight at 4°C. The Texas Red-labeled anti-mouse secondary antibody (Jackson Immunoresearch, 1:1000 dilution) was incubated with cells for 2 h at room temperature. DNA was stained with DAPI (1 µg/ml). Samples were viewed through a Texas Red-optimized filter (Chroma Technology) using an Olympus BX60 epifluorescence microscope equipped with a Photometrics Quantix digital camera.

Direct fluorescence microscopy was used to localize GFP fusion proteins in live cells. For all experiments, cells were stained with DAPI to visualize the DNA and confirm the location of the nucleus. The localization of the fusion proteins was monitored by directly viewing the GFP signal in living cells through a GFP-optimized filter as described for indirect immunofluorescence microscopy.

Immunoprecipitation of Importin α-myc—Agarose-conjugated anti-myc antibody beads (9E-10 Santa Cruz Biotechnology) were used to immunoprecipitate importin α-myc proteins. Wild-type cells (ACY192) expressing importin α-myc (pAC891) or Δimportin α-myc (pAC894) from the endogenous Srp1p promoter were transformed with galactose-inducible plasmids encoding GFP (pAC1042) alone or a bipartite NLS (KRTA)D(SEFSKEK)KRRKVE-GFP (pAC1045) (38). Cells were grown to early-log phase in synthetic media containing 2% raffinose, induced with 2% galactose, and incubated at 30°C for 3 h. Lysates were prepared as described for immunoblot analysis.

Six milligrams of total protein lysate was incubated with myc antibody-coupled agarose beads (10 µl of volume of packed beads) for 5 h at 4°C in the absence or presence of a RanGTP-RS. Beads were washed twice in PBSMT and once in PBSM before elution with 30 µl of sample buffer (125 mM Tris-HCl, pH 6.8, 250 mM dithiothreitol, 5% SDS, 0.25% bromphenol blue, 25% glycerol). The bound (50% of total bound) fractions were resolved on a 10% SDS-PAGE gel and immunoblotted as described. For quantification, immunoblots were analyzed using a fluorescence imager and Quantity One Software (Bio-Rad).
RESULTS

Generation of Mutant Importin α Proteins—Previous structural and in vitro analyses identified an auto-inhibitory sequence within the N-terminal IBB domain of importin α (23, 24, 26). The structural studies suggested that this auto-inhibitory domain interacts with the NLS binding pocket by mimicking an NLS sequence (23, 24). Alignment of the N-terminal domains of importin α proteins from yeast, mouse, and human reveals three clusters of conserved basic amino acids that could serve as auto-inhibitory NLS sequences (Fig. 1). To identify the amino acid residues that are required for the auto-inhibitory function of importin α, we carried out an alanine scan by individually substituting each of these basic clusters in yeast importin α (Srp1p) to generate importin α mutants referred to as A1 (RRR→AAA), A2 (KKAR→AAA), and A3 (KKRR→AAA) (Fig. 1). The full-length mouse importin α crystal structure showed that residues 44–54 (corresponding to yeast importin α residues 49–59) interact with the larger NLS binding pocket (24). Although this suggests that our A3 mutant should target this intramolecular interaction, we completed a broader, unbiased alanine scan and analyzed the function of each of the mutant importin α proteins, A1, A2, and A3.

In Vivo Function of Importin α Mutants—To test the function of the mutant importin α proteins in vivo, each mutant was transformed into yeast cells deleted for the endogenous importin α gene (SRP1) and plasmid shuffle (see “Experimental Procedures”) was used to replace the functional wild-type copy of SRP1 (Fig. 2A). This results in ΔSRP1 cells that express each of the mutant importin α proteins from their own promoter on a low copy centromeric plasmid as the only copy of importin α. Controls demonstrate that a wild-type importin α plasmid can rescue ΔSRP1 cells, whereas neither a vector alone nor the N-terminally truncated importin α (ΔIBB-α) can functionally replace SRP1. Results shown in Fig. 2A indicate that none of the importin α mutant proteins, A1, A2, or A3, can function in vivo.

To confirm that each mutant protein is expressed at a similar level to wild-type importin α, we analyzed their expression using a C-terminal triple myc tag. Wild-type importin α-myc can functionally replace endogenous importin α (data not shown). Immunoblotting with the myc-tagged importin α proteins demonstrates that each of the mutant proteins is expressed at approximately the same level as wild-type importin α (Fig. 2B, compare lanes 3–6 with lane 2). This suggests that none of the mutations in the N-terminal IBB domain of importin α significantly affect the level of the protein within the cell, but rather that these mutations perturb the function of the importin α proteins. Importin α undergoes a series of defined protein–protein interactions during a nuclear transport cycle (16). Thus, to determine the basis for the loss of function of each of the importin α mutants, we investigated the interaction of the mutant importin α proteins with both NLS-cargo and nuclear transport factors essential for importin α function.

Identification of an Importin α Mutant with Decreased Auto-inhibitory Function—To examine both NLS binding and auto-inhibitory function for each importin α protein, we used a quantitative, fluorescence anisotropy, solution binding assay (26, 38). Full-length wild-type importin α binds weakly to a classical SV40-NLS due to the N-terminal auto-inhibitory IBB domain. However, when the same experiment was carried out in the presence of a stoichiometric amount of importin β, the N-terminal auto-inhibition of full-length importin α was relieved and much tighter binding to the SV40-NLS was observed. Thus, this assay actually examines three aspects of importin α function: binding to NLS-cargo (directly), auto-inhibitory function (directly, measured by the ability of full-length importin α to bind NLS-cargo in the absence of importin β), and binding to importin β (indirectly, based on the relief of auto-inhibition).

To examine NLS binding and the auto-inhibitory function for each importin α variant, we performed the fluorescence anisotropy assay using a monopartite SV40-NLS-cargo (26, 38). The assay was carried out with each importin α protein in the presence and absence of importin β (Fig. 3). Typical curves for binding of SV40-NLS-GFP to wild-type importin α in the absence (○) and presence (▲) of importin β are shown in Fig. 3A. As described under “Experimental Procedures,” these curves were used to calculate Kd values for the interaction between the NLS-cargo and importin α (Table II). The change in free energy (ΔG) for the binding of each importin α protein to NLS-cargo in the absence (■) and presence (▲) of importin β is shown in Fig. 3B. As previously demonstrated (24, 26), wild-type importin α binds to SV40-NLS-GFP weakly (Kd ~ 500 nM), but the affinity increases ~30-fold (Kd ~ 18 nM) in the presence of importin β. The A1-importin α and A2-importin α mutants show weak binding to...
SV40-NLS-GFP, comparable to wild-type importin α, suggesting that the N-terminal auto-inhibitory function is intact in these mutants. However, the binding to NLS is not enhanced in the presence of importin β, showing that there is no relief of auto-inhibition. This observation implies that the A1-importin α and A2-importin α mutants have decreased binding to importin β.

In contrast to the A1 and A2 mutants, the A3-importin α mutant binds to SV40-NLS-GFP more tightly (K_d = 73 nM) than full-length wild-type importin α (K_d = 500 nM), an ~7-fold increase in affinity. This suggests that A3-importin α has decreased auto-inhibitory function. When the assay is performed in the presence of importin β, the affinity of SV40-NLS-GFP for A3-importin α increases to approximately the same binding affinity (K_d ~ 20 nM) as measured for wild-type importin α (K_d ~ 18 nM), which shows that the residual auto-inhibition can be relieved by importin β and suggests that the A3-importin α mutant retains normal binding to importin β. In addition, these data show that the A3-importin α/β complex is able to bind NLS-cargo with approximately the same affinity as the wild-type importin α/β complex. Similar results were obtained when two different cargoes containing distinct NLS sequences were used (data not shown).

Our analysis of the auto-inhibition of A3-importin α suggests that this mutant IBB domain has a decreased affinity for the NLS binding pocket compared with a wild-type IBB domain. To directly examine this interaction, we analyzed binding of the IBB domain to the NLS binding pocket in trans. We compared the binding of wild-type and A3 IBB-GFP to ΔIBB-α using fluorescence anisotropy (Fig. 3C). Wild-type IBB-GFP binds to ΔIBB-α with a K_d of ~14 ± 5 μM. Binding of A3 IBB-GFP to ΔIBB-α was significantly less and was too weak to accurately measure in this assay.

**Interaction of Importin α Proteins with Importin β**—The interaction of importin α with importin β is essential for targeting the import complex to nuclear pores (5). Therefore, to evaluate the in vivo role of the auto-inhibitory function of importin α, it is critical to demonstrate that we have separated the two functions of the N-terminal IBB domain of importin α (importin β binding and auto-inhibition). Although the auto-inhibitory binding assay suggests that A3-importin α retains wild-type binding to importin β, it does not measure this binding directly. To quantitatively measure the binding of importin α to importin β we utilized two assays. First, we used a competition assay where we examined the ability of each full-length importin α protein to compete with the IBB-GFP for binding to importin β. Second, we performed a direct binding assay to measure binding of wild-type and A3 IBB-GFP to importin β.

For the competition binding assay, we first used fluorescence anisotropy to measure the binding of wild-type IBB-GFP to importin β. This yielded a K_d of ~17 ± 5 nM (Fig. 4). The competition experiment was then carried out in the presence of three concentrations of each importin α protein (wild-type, A1, A2, and A3) in competition with IBB-GFP. This analysis yields equilibrium binding constants for the interaction of wild-type and each of the mutant importin α proteins with importin β (Table III). Full-length importin α binds to importin β with a K_d of ~0.6 μM. Both A1-importin α and A2-importin α show decreased affinity for importin β with decreases of ~15-fold and ~28-fold, respectively. In contrast, as suggested by the NLS binding experiments, A3-importin α binds to importin β with approximately the same affinity (K_d ~ 0.4 μM) as wild-type importin α (K_d ~ 0.6 μM). To further confirm that the A3 mutation within the IBB domain does not affect the interaction between importin α and importin β, we performed the direct binding assay. This assay measures binding of wild-type and A3 IBB-GFP to importin β using fluorescence anisotropy. Binding curves for IBB-GFP and A3 IBB-GFP are shown in Fig. 4. A3 IBB-GFP binds to importin β with the same affinity (K_d ~ 20 ± 5 nM) as wild-type IBB-GFP (K_d ~ 17 ± 5 nM). These results indicate that the A3 mutant of importin α has compromised auto-inhibitory function but binds to importin β with wild-type affinity. Because this mutant dissects the two functions of the IBB domain, we focused our subsequent experi-
ments on the A3 mutant to analyze the in vivo contribution of the auto-inhibitory activity of importin α.

**Dissociation of the Importin α/Importin β Complex by RanGTP**—To determine whether the A3-importin α can be dissociated from importin β by RanGTP, we performed a bead dissociation assay. We covalently attached purified recombinant importin β to activated epoxy beads and then incubated the beads with purified importin α to pre-form the importin α/β complex. We have previously shown that full-length importin α specifically binds to the importin β-coupled beads (26). Both wild-type and A3-importin α can bind to the importin β beads (Fig. 5A, lanes 2 and 4). Fig. 5B shows that both wild-type and A3-importin α are released from importin β upon addition of an RanGTP-regenerating system (RanGTP-RS) (39). Similar results were obtained when the experiment was carried out using Ran loaded with 100 μM GTPγS in place of the RanGTP-RS to modulate the nucleotide bound state of Ran (data not shown). RanGDP, which does not bind with high affinity to importin β (43), did not dissociate any of the pre-formed complexes (data not shown).

**Localization of A3-importin α**—Our in vitro experiments demonstrated that the A3-importin α protein interacts with importin β in a RanGTP-dependent manner that is comparable to wild-type importin α. One prediction from these experiments is that the A3 protein should be efficiently targeted to the nucleus in vivo through a productive interaction with importin β. Wild-type S. cerevisiae importin α protein is localized throughout the cell with some accumulation at the nuclear rim and/or within the nucleus depending on the tag (37, 44). This steady-state localization reflects a dynamic state where importin α enters the nucleus and is then exported by Cse1p (19, 20, 44). To analyze the intracellular localization of importin α, we first utilized C-terminal triple myc-tagged fusion proteins. These tagged proteins were expressed from the importin α promoter on a centromeric plasmid and localized by indirect immunofluorescence (Fig. 6A). Wild-type importin α-myc is localized throughout the cell but accumulates within the nucleus (Fig. 6A, panel A). The A3-importin α-myc protein also accumulates within the nucleus (Fig. 6A, panel D). For comparison, the localization of A2-importin α, which has compromised binding to importin β, is also shown (Fig. 6A, panel G). It is clear that, in contrast to wild-type and A3-importin α, the A2 protein does not accumulate within the nucleus. Although the localization of wild-type and A3-importin α was very similar, we consistently noticed less cytoplasmic signal for the A3 protein. This raised the possibility that the A3 protein could have a more nuclear steady-state localization than wild-type importin α.

To address this possibility, we re-examined the localization using an assay that can more readily distinguish between importin α at the nuclear rim and importin α within the nuclear interior (44). This assay relies on visualization of an importin α-GFP fusion protein that is expressed from the endogenous importin α promoter. We integrated both wild-type and A3-importin α-GFP fusion proteins at the endogenous importin α locus as described under “Experimental Procedures.” As previously reported (44), wild-type importin α accumulates at the nuclear rim in wild-type cells when visualized in this manner (Fig. 6B, panel A). However, A3-importin α-GFP is localized within the nucleus (Fig. 6, B (panel C) and C) suggesting that this mutant protein is not efficiently recycled to the cytoplasm. The localization of A3-importin α-GFP is similar to that of importin α-GFP in cse1-1 mutant cells where importin α is not efficiently recycled to the cytoplasm (Fig. 6B, panel E) (20, 36). A3-importin α-GFP also accumulates within the nucleus of cse1-1 cells (Fig. 6B, panel G).

**Co-immunoprecipitation of Importin α and NLS-cargo**—Because A3-importin α has decreased auto-inhibitory activity and a more steady-state nuclear localization than wild-type importin α, it seems likely that this mutant protein accumulates within the nucleus bound to NLS-cargo. Thus, A3-importin α should bind to more NLS-cargo than wild-type importin α in cell lysates. To test this prediction, we performed a co-immunoprecipitation with cells expressing either myc-tagged wild-type importin α or myc-tagged A3-importin α together with a bipartite NLS-GFP cargo. The bipartite NLS-GFP protein is localized to the nucleus in vivo (data not shown).

Presumably, at least two major NLS-cargo/importin α complexes exist: 1) the trimeric import complex of NLS-cargo/importin α/ importin β complexes described under “Experimental Procedures.” Samples were resolved by SDS-PAGE and stained with Coomassie Brilliant Blue. For each protein, U (unbound) (lanes 1 and 3) and B (bound) (lanes 2 and 4) fractions are shown. B, the pre-formed importin α/importin β complexes were incubated with a RanGTP-RS. The bound (B) (lanes 1 and 3) and eluant (E) (lanes 2 and 4) fractions were analyzed as described for A. The positions of importin α, Ran, and importin β (which minimally leaches off the beads) are indicated by the arrows.
portin α/importin β, and 2) NLS-cargo/importin α, which may exist transiently after importin β is released. Immunoprecipitation of importin α could isolate both complexes; however, addition of RanGTP should dissociate the trimeric complex, because RanGTP causes a conformational change in importin β that results in importin β dissociation from importin α (17, 45). If A3-importin α is defective in NLS-cargo release from importin α, it should bind more NLS-cargo in cell lysates than wild-type importin α under the same conditions.

Agarose-conjugated anti-myc antibody beads were used to immunoprecipitate importin α-myc from lysates (see “Experimental Procedures”). The bound fractions were analyzed for co-immunoprecipitation of the bipartite NLS-GFP reporter protein and importin β. As a control, GFP alone was not co-immunoprecipitated with either of the importin α proteins (data not shown). The experiment was carried out in the presence of a RanGTP-RS to isolate NLS-cargo/importin α complexes in the absence of importin β. The RanGTP-RS dissociates importin β from the complex and decreases the amount of NLS-GFP co-immunoprecipitated (compare lanes 1 and 2). This confirms that in the presence of RanGTP the primary co-immunoprecipitated complex isolated is not the trimeric import complex of NLS-cargo/importin α/importin β but a complex of NLS-cargo/importin α under the same conditions. Results of the experiment indicate that, in the presence of RanGTP, ~3-fold more NLS-GFP co-immunoprecipitated with A3-importin α-myc than wild-type importin α-myc (Fig. 7A, compare lanes 2 and 3). Expression of the importin α-myc proteins and the NLS-cargo protein was similar in each lysate (data not shown). Protein bands were compared by fluorescence imaging under conditions where none of the bands were saturated. For each sample the amount of NLS-cargo present was calculated relative to the amount of importin α precipitated in that experiment.

Interaction of A3-importin α with Cse1p—For importin α to be functional in vivo, it must be efficiently recycled to the cytoplasm by its export receptor, Cse1p/CAS. Formation of this export complex requires both the direct interaction between importin α and Cse1p and the dissociation of NLS-cargo (19–21). To examine the physical interaction of A3-importin α with Cse1p, we used a bead binding assay. Both wild-type and A3-importin α were expressed in E. coli as GST fusion proteins as described under “Experimental Procedures.” These proteins were bound to glutathione beads and incubated with purified His6-tagged Cse1p (10 μg) and His-tagged Gsp1p (20 μg). Lanes are designated “−” or “+” to indicate whether binding was carried out in the absence or presence of a RanGTP-RS. B, in vitro interaction of importin α and Cse1p. Wild-type and A3-importin α were expressed in E. coli as GST fusion proteins. The importin α GST fusion proteins (~30 μg) were bound to glutathione beads and incubated with purified His-tagged Cse1p (10 μg) and His-tagged Gsp1p (20 μg). Lanes are designated “−” or “+” to indicate whether binding was carried out in the absence or presence of a RanGTP-RS. C, in vivo interaction of importin α and Cse1p. Wild-type cells expressing importin α-GFP (panels A and C), A3-importin α-GFP (panels E and G), or A3-ED-importin α-GFP (panels I and K) were transformed with either a 2μ plasmid expressing Cse1p (pAC640) or a vector control (pAC8). Cells were grown to early-log phase, and the GFP signal was viewed directly in living cells. Corresponding DIC images are shown. His6-tagged Cse1p in the absence or presence of a RanGTP-RS. As shown in Fig. 7B, A3-importin α binds to Cse1p in a RanGTP-dependent manner. Furthermore, in the presence of RanGTP, Cse1p binding to A3-importin α is comparable to binding to wild-type importin α (Fig. 7B, compare lanes 2 and 3). Protein
bands were compared by immunoblotting and fluorescence imaging. Results shown are typical of three independent experiments. In addition, both wild-type importin α and A3-importin α interacted with Cse1p to the same extent in a two-hybrid assay (data not shown).

As a complement to the *in vitro* binding experiments, we examined importin α recycling by Cse1p *in vivo*. We took advantage of the C-terminal GFP fusion protein, importin α-GFP, expressed from the importin α promoter on a centromeric plasmid. Previous work demonstrates that fusion of GFP to the C terminus of wild-type importin α results in a protein that can function *in vivo* when expressed as the only copy of importin α (44). However, plasmid expression results in increased amounts of importin α-GFP and thus a more nuclear localization than observed for integrated importin α-GFP protein (as shown in Fig. 6B). When expressed on a centromeric plasmid, both wild-type importin α-GFP and A3-importin α-GFP show primarily nuclear localization (Fig. 7C, panels A and E). Overexpression of Cse1p decreases the nuclear localization of wild-type importin α-GFP presumably by facilitating export (44). Thus, cells that overexpress Cse1p show a more diffuse (throughout the cell) localization pattern for importin α-GFP. As previously reported, we find that wild-type importin α-GFP is recycled to the cytoplasm and diffusely localized throughout the cell when Cse1p is overexpressed on a high copy plasmid (Fig. 7C, compare panels A and C). This diffuse localization is observed in ~80% of cells examined. In contrast, A3-importin α-GFP remains in the nucleus even when Cse1p is overexpressed (Fig. 7C, compare panels E and G). There are two possible explanations for why A3-importin α-GFP is not recycled to the cytoplasm in cells that overexpress Cse1p. First, A3-importin α-GFP may be bound to NLS-cargo and therefore unable to efficiently interact with Cse1p. Several lines of experimentation have shown that importin α cannot interact simultaneously with NLS-cargo and Cse1p (19–21), which presumably prevents recycling of importin α with NLS-cargo still bound. Thus, overexpression of Cse1p should not cause redistribution of a mutant importin α-GFP protein if it is still bound to NLS-cargo. Second, the A3 mutation in importin α could directly interfere with the physical interaction between importin α and Cse1p. However, our results shown in Fig. 7B demonstrate that A3-importin α is still able to bind to Cse1p. This strongly suggests that the lack of recycling of A3-importin α to the cytoplasm by Cse1p is due to a tighter interaction between A3-importin α and NLS-cargo in the nucleus.

To examine whether the lack of A3-importin α recycling was in fact due to bound NLS-cargo, we utilized a known importin α mutant, ED-importin α (D203K/E402R), that has significantly reduced binding to NLS-cargo (22). The interaction between *S. cerevisiae* ED-IIBB-α and SV40-NLS is too weak to detect in the anisotropy assay implying that the binding constant is at least in the millimolar range (data not shown). Because this mutant protein cannot bind to NLS-cargo, it can be combined with the A3 mutation, to create an A3-importin α mutant protein (A3-ED-importin α) that cannot bind NLS-cargo. If this protein can be efficiently recycled to the cytoplasm, it confirms that bound cargo prevents recycling of A3-importin α. Alternatively, if the reason that A3-importin α is not recycled to the cytoplasm is independent of binding to NLS-cargo, then regardless of the ED mutation, A3-ED-importin α would not be recycled and would remain in the nucleus.

When the ED mutations are combined with A3-importin α to create A3-ED-importin α-GFP, the steady localization of the double-mutant protein is within the nucleus (Fig. 7C, panel I) indistinguishable from the localization of wild-type or A3-importin α-GFP. However, in contrast to the result obtained with A3-importin α-GFP, overexpression of Cse1p causes relocalization of A3-ED-importin α-GFP to the cytoplasm in ~60% of cells (Fig. 7C, panel K). Immunoblotting demonstrates that each of the importin α-GFP proteins is expressed at approximately the same level as wild-type importin α-GFP and that cells transformed with the CSE1 plasmid all have a similar increase in expression of Cse1p (data not shown). Results of these experiments suggest that A3-importin α accumulates in the nucleus bound to NLS-cargo and thereby support our hypothesis that the auto-inhibitory function of importin α is required for efficient NLS-cargo release in the nucleus. In addition, these results show that A3-importin α can interact with Cse1p *in vivo* when its ability to bind NLS-cargo is abrogated.

**DISCUSSION**

This study demonstrates that the N-terminal IBB domain of importin α has two essential functions *in vivo*. This domain is known to bind importin β for targeting of the import complex to the nuclear pore (14, 15, 46). In addition, previous structural studies demonstrated that the IBB domain contained an NLS-like sequence that could compete directly with NLS binding to importin α through an intramolecular interaction (23, 24). Investigations of the *in vitro* behavior of importin α confirmed that the N-terminal domain inhibited binding of NLS-cargo to importin α (24, 26). This auto-inhibitory function was relieved when importin β bound to sequences of the N-terminal domain of importin α just upstream of the NLS-like sequence (47). Here we create mutants of importin α that dissect the two functions of the N-terminal domain, importin β binding and auto-inhibition. We demonstrate that mutations within the NLS-like sequence in importin α (mutant A3) specifically decrease the auto-inhibitory function of the N terminus without affecting binding to importin β and provide evidence that this auto-inhibitory function is required for efficient NLS-cargo release.
in the nucleus. Yeast cells expressing this mutant of importin α as the sole copy of importin α are not viable, demonstrating that the NLS-like sequence mediates an essential function of importin α in vivo.

An in vivo analysis of the specific defect of A3-importin α shows that this mutant protein accumulates in the nucleus. This nuclear accumulation suggests that A3-importin α is not efficiently recycled from the nucleus to the cytoplasm by the export receptor, Cse1p. Our in vitro data showing that the A3 mutations do not appear to have a direct impact on the binding of Cse1p to importin α suggest that this defect is due to impaired release of cargo in the nucleus. Previous work has shown that the Cse1p/CAS association with importin α does not depend on the N-terminal domain (40, 48). In fact, the Cse1p/CAS binding site has been mapped to the C terminus of human importin α (21). Our in vivo data support these in vitro analyses, because a variant of the A3 mutant of importin α, where the NLS-binding function was destroyed (A3-ED), could be efficiently recycled to the cytoplasm by Cse1p. Taken together, these analyses suggest that the accumulation of A3-importin α in the nucleus is due to the persistence of an NLS-cargo/importin α complex in the nucleus, which inhibits the interaction between importin α and Cse1p. This strongly supports the hypothesis that an essential function of the NLS-like sequence in the N-terminal domain of importin α is the efficient release of NLS-cargo from importin α within the nucleus.

The release of NLS-cargo from importin α is necessary for both the function of NLS-cargoes within the nucleus and for recycling of importin α to the cytoplasm. The accumulation of A3-importin α in the nucleus also demonstrates in vivo the intimate link between cargo release and recycling of importin α. Because NLS-cargo release is required for binding to Cse1p/CAS (19–21), it has been proposed that Cse1p/CAS may facilitate release of NLS-cargo (7, 18). Unfortunately, the molecular details of the interaction of importin α with Cse1p/CAS have not yet been elucidated. Furthermore, it is not known how Cse1p/CAS distinguishes between the NLS-cargo bound and free importin α proteins. One possibility is that a conformational change in importin α signals cargo release to Cse1p.

Although our data suggest that auto-inhibition is essential for efficient NLS-cargo release from importin α, it does not exclude the possibility that additional factors may facilitate this dissociation. Specifically, a recent study demonstrated that in vitro both Cse1p and the nucleoporin Nup2p facilitate dissociation of NLS-cargo from importin α (18). It is not yet clear how Nup2p may function in cargo release, although the observation that Nup2p interacts with both full-length and IBB-α (44, 49) leads us to suspect that this function is either independent of, or complementary to, the auto-inhibitory function characterized in this study. Further work will be necessary to determine how importin α, Cse1p, and Nup2p interact as well as how Cse1p and Nup2p contribute to NLS-cargo delivery into the nucleus in vivo.

Although our study suggests that the essential role of importin α auto-inhibition is within the nucleus for release of NLS-cargo from importin α, we also present quantitative data that suggests a secondary role in recognition/targeting in the cytoplasm. Indeed, a cytoplasmic role would provide a mechanism by which formation of the import complex is cooperative, leading from free importin α to a trimeric import complex consisting of NLS-cargo, importin α, and importin β (24). We have determined that full-length importin α binds to importin β with low affinity ($K_d \sim 0.6 \mu M$) in the absence of any NLS-cargo, presumably because the N-terminal domain forms an intramolecular interaction with the NLS-binding pocket. In contrast, the IBB domain alone binds more tightly to importin β ($K_d \sim 17$ nm). This quantitative analysis of protein-protein interactions shows that, in the cytoplasm, NLS-cargo, importin α, and importin β have a low affinity for each other (micromolar $K_d$) unless they are coincident, in which case they form a fairly tight trimeric complex (nanomolar $K_d$). This cooperative complex formation would presumably prevent nonproductive permutual Ran-dependent cycling of an importin α/importin β complex without NLS-cargo. Thus importin α is a dynamic adaptor protein that has evolved to couple NLS-cargo import complex formation in the cytoplasm and NLS-cargo import complex dissociation in the nucleus through regulated protein-protein interactions.

We note that the absolute dissociation constants measured in our studies differ from previously reported values (18, 24), particularly in the binding of importin α to importin β. The values presented in this study are unique in that they are taken from a measurement of binding at equilibrium in solution. Previously reported values were derived from assays that depended on the separation of bound ligand from unbound (i.e. solid phase pull-down (18) or surface plasmon resonance assays (24)). The dependence of these assays on the kinetics of the binding reaction may play a role in the disparity in the published values. Further experimentation will be needed to determine the origin of the variance in measured binding constants.

Although RanGTP binding to importin β is the primary signal that initiates import complex disassembly, our data suggest that the IBB is critical for delaying this signal to the NLS binding pocket on importin α. Thus, this study incorporates an NLS-cargo release step into the classic nuclear transport pathway. A schematic is shown in Fig. 8: 1) NLS-cargo/importin α/importin β cooperatively associate to form the trimeric import complex in the cytoplasm; 2) in the nucleus RanGTP mediates dissociation of importin β from the import complex to release an NLS-cargo/importin α complex; 3) the N-terminal domain of importin α competes with NLS-cargo to bind the NLS-binding pocket; and 4) NLS-cargo is released into the nucleus, and the auto-inhibitory N-terminal domain is bound to the NLS-binding site. This renders importin α incapable of binding nuclear NLS-containing proteins prior to export to the cytoplasm by Cse1p. This model for protein import requires precise tuning of the thermodynamic interactions between the various species for the reaction to proceed efficiently in a single direction. In addition, it will be interesting to determine what contribution other release factors, such as Cse1p and Nup2p (18, 50), make to cargo release in vivo.

In summary, we present experimental evidence for the auto-inhibition of importin α in vivo and provide a mechanistic understanding of the essential nature of the auto-inhibitory function in NLS-cargo delivery into the nucleus as well as a potential role in NLS-cargo recognition/targeting in the cytoplasm. We propose that changes in the binding affinities of the nuclear transport components underlie and define the mechanism of regulation of nuclear transport. A complete understanding of nuclear transport requires a quantitative model for the nuclear transport process that correlates structural analyses, in vitro interaction energies, and in vivo functionality. Such analyses to determine the energetics of individual mechanisms in the nuclear transport process will be required to fully understand the rapid, selective, and highly regulated processes of nuclear transport.

Acknowledgments—We thank Dr. Maureen Powers for experimental suggestions and D. M. Green, A. Lange, and Dr. S. W. Leung for their critical review of the manuscript.
The Auto-inhibitory Function of Importin α Is Essential in Vivo
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J. Biol. Chem. 2003, 278:5854-5863.
doi: 10.1074/jbc.M210951200 originally published online December 16, 2002

Access the most updated version of this article at doi: 10.1074/jbc.M210951200

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