Characterization of the Auto-inhibitory Sequence within the N-terminal Domain of Importin $\alpha$

Michelle T. Harreman‡, Pamela E. Cohen, Mary R. Hodel, Glyn J. Truscott, Anita H. Corbett§, and Alec E. Hodel

Department of Biochemistry and $\dagger$Graduate Program in Biochemistry, Cell and Developmental Biology, Emory University School of Medicine, Atlanta, GA 30322

§Corresponding author: Anita H. Corbett
Department of Biochemistry
Emory University School of Medicine
1510 Clifton Rd, NE
Atlanta, GA 30322

Tel: 404-727-4546
FAX: 404-727-3954
E-mail: acorbe2@emory.edu

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SUMMARY

Protein cargoes that contain a classical nuclear localization signal (NLS) are transported into the nucleus through binding to a heterodimeric receptor comprised of importin/karyopherin α and β. An evolutionarily conserved auto-inhibitory sequence within the N-terminal importin β binding (IBB) domain of importin α regulates NLS-cargo binding to the NLS binding pocket on importin α. In this study, we have used site-directed mutagenesis coupled with *in vitro* binding assays and *in vivo* analyses to investigate the intramolecular interaction of the N-terminal IBB domain and the NLS binding pocket of *Saccharomyces cerevisiae* importin α, Srp1p. We find that mutations within the IBB domain that decrease the binding affinity of the auto-inhibitory sequence for the NLS binding pocket impact importin α function *in vivo*. In addition, the severity of the *in vivo* phenotype is directly correlated to the reduction of auto-inhibition measured *in vitro* suggesting that the *in vivo* phenotypes are directly related to the loss of auto-inhibitory function. We exploit a conditional auto-inhibitory mutant, *srp1-55*, to study the *in vivo* functional overlap between the N-terminal IBB domain of importin α and other factors implicated in NLS-cargo release, Cse1p and Nup2p. We propose that the N-terminal IBB domain of importin α and Cse1p function together in NLS-cargo release while Nup2p contributes to cargo release/importin α recycling through a distinct mechanism.
INTRODUCTION

All macromolecules that move into and out of the nucleus are transported through nuclear pore complexes, large proteinaceous channels that are embedded in the nuclear envelope (1,2). Soluble factors are required to recognize, target, and transport most macromolecules through the nuclear pores (3,4). The best characterized nuclear transport process occurs via receptor recognition of classical nuclear localization signals (NLSs)¹ on protein cargoes targeted for nuclear import (3,5). These classical NLSs are typified by a single cluster of basic amino acids (monopartite) or two clusters of basic amino acids separated by a 10-12 amino acid linker (bipartite) (6,7).

Protein cargoes that contain classical NLSs are recognized in the cytoplasm by a heterodimeric receptor composed of importin/karyopherin α and importin/karyopherin β (3,8-10). Importin α recognizes and binds the NLS and importin β translocates the trimeric import complex through the nuclear pore (3). Delivery into the nucleus is dependent on the small GTPase Ran, which governs the interactions between the nuclear transport receptors and macromolecular cargoes and thus confers directionality to nucleocytoplasmic transport (3,11). Once the cargo is delivered into the nucleus, the transport receptors are recycled to the cytoplasm (3).

Release of NLS-cargo into the nucleus is essential for both the function of the cargo and for recycling of the nuclear transport receptors to the cytoplasm (11-15). Thus, once the NLS-cargo/importin α/importin β complex reaches the nuclear face of the pore, the complex must be disassembled to deliver the cargo to the nuclear interior and to recycle the import receptors in an NLS-free state. The best characterized signal for the dissociation of the import complex is RanGTP-dependent dissociation of importin β from the trimeric complex (16-19). In the
nucleus, RanGTP binds to importin β, which causes a conformational change in importin β that results in the release of importin α (20). RanGTP triggered dissociation of importin β generates a transient NLS/importin α complex. The mechanism of NLS-cargo dissociation from importin α is not as well characterized, although recent studies suggest that the importin α export receptor, Cse1p, the nucleoporin, Nup2p, and the N-terminal auto-inhibitory domain of importin α could all play a role (19,21-23). Gilchrist et al. demonstrated that Cse1p and Nup2p can facilitate release of NLS-cargo in vitro (19). Furthermore, our recent data support a model where the auto-inhibitory domain of importin α couples the Ran-dependent dissociation of importin β with the subsequent release of NLS-cargo from importin α (23). Thus, these factors may cooperate or act sequentially to facilitate NLS-cargo release. It is not yet clear, however, how this occurs or how these factors functionally interact in vivo.

Domain analysis shows 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 the export receptor Cse1p (14,18). The structure of mouse importin α revealed that the N-terminal IBB domain of importin α can form an intramolecular interaction with the NLS-binding pocket of importin α (22). This observation suggested that the IBB, in addition to mediating binding to importin β, could also regulate cargo binding through an auto-inhibitory mechanism (22).

This may occur through competition between the IBB and NLS-cargo for binding to the NLS-binding pocket of importin α, which could facilitate the release of cargo into the nucleus. Additionally, the IBB could prevent NLS-cargo rebinding once it has been released. Thus, the IBB appears to act as a regulatory switch between the cytoplasmic form of importin α, which has
a high affinity for NLS-cargo due to binding to importin β, and the nuclear form, which has a low affinity for NLS cargo.

Importin α structural studies suggest that monopartite NLSs and the auto-inhibitory sequence bind to the NLS-binding pocket of importin α in a similar manner (22,24,25). Indeed, a conserved cluster of basic amino acids, that resembles a classical basic NLS, serves as the auto-inhibitory sequence (yeast importin α residues 54KRR56) (22,23). In vitro analysis of the energetic landscape of NLS binding to importin α revealed details of the specific interactions required for NLS binding to the pocket of importin α (26). In particular, Hodel et al. defined the requirements for specific amino acid residues within an NLS that are critical for high affinity interaction with importin α (26). We hypothesize that the auto-inhibitory sequence depends on similar energetic molecular interactions with the NLS binding pocket. This would be consistent with its role in NLS-cargo release through a direct competition mechanism.

The present study further characterizes the auto-inhibitory sequence within the N-terminal domain of S. cerevisiae importin α. We have utilized site-directed mutagenesis, in vitro binding assays and in vivo analyses to investigate the intramolecular interaction between the N-terminal IBB domain and the NLS binding pocket of importin α. Through these analyses we demonstrate that the auto-inhibitory sequence binds to the NLS binding pocket through energetic interactions that are analogous to those for a monopartite NLS. We present data in support of our hypothesis that the auto-inhibitory function of the IBB domain is responsible for essential in vivo functions. Our experiments demonstrate that the severity of the in vivo phenotypes are directly correlated to the reduction of auto-inhibition measured in vitro, suggesting that the in vivo phenotypes are directly related to the loss of auto-inhibitory function. Furthermore, we present data in support of functional overlap between the N-terminal domain of importin α,
Cse1p and Nup2p *in vivo*. We propose that the auto-inhibitory N-terminal domain of importin α and Cse1p function together in NLS-cargo release, whereas Nup2p functions through a different mechanism in this essential process.
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 (27), and all media was prepared by standard procedures (28). All yeast strains and plasmids used in this study are described in Table I.

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 (Life Sciences). DNA containing the mutations was subcloned from the bacterial expression vector into the yeast expression plasmid for in vivo studies. For all constructs generated, the presence of each desired mutation and the absence of any other mutations was confirmed by DNA sequencing.

Expression and Purification of Recombinant Proteins- Assays were performed with purified recombinant S. cerevisiae proteins Srp1p (importin α) and Rsl1p (importin β). Full-length His6-importin α (residues 1-542), His6-SV40 (SPKKKRKVEAS)-NLS-GFP and His6-Myc (PAAKRVKLD)-NLS-GFP were expressed in the E. coli strain BL21 (DE3) and purified by nickel affinity chromatography essentially as described (26,29). Importin β was expressed and purified as described previously (29).

Fluorescence Anisotropy Assay- Fluorescence anisotropy measurements were carried out using an ISS PC1 fluorimeter fitted with polarization filters. The dissociation constants for the binding of SV40 NLS-GFP and Myc NLS-GFP to importin α were measured essentially as described previously (26,29). Briefly, SV40-NLS-GFP or Myc-NLS-GFP was diluted in PBS to the desired concentration (~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 increasing amounts of wild-type or mutant importin α proteins were added to the assay 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 $K_d$ values are calculated as detailed at http://www.biochem.emory.edu/Hodel/Research/BindingCurves/fitting_curves.htm. Binding energies were calculated using $\Delta G = RT \ln K_d$ where $RT = -0.59 \text{ kcal/mol}$.

**In Vivo Functional Analysis** - The in vivo function of each of the importin α variants was tested using a plasmid shuffle technique (30). Plasmids encoding each of the importin α mutant proteins were individually transformed into SRP1 deletion cells (ACY324) containing the URA3 maintenance plasmid, pAC876 (23). 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 fluoroorotic acid (5-FOA) plates. The drug 5-FOA eliminates the URA3 plasmid-encoded wild-type importin α (pAC876) (30). Plates were incubated at the indicated temperatures for 3-5 days.

**Immunoblot Analysis** - Immunoblot analysis was performed by standard methods as described (31). 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 KH$_2$PO$_4$ pH 7.0, 15 mM (NH$_4$)$_2$SO$_4$, 75 mM KOH, 5 mM MgCl$_2$, 0.5% TritonX-100). Cells were subsequently lysed in PBSMT with protease inhibitors (0.5 mM PMSF, 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 immunoblotted with either
monoclonal anti-myc antibody (1:2000 dilution; Oncogene) or polyclonal anti-GFP antibody (1:10,000 dilution) (32).

**Microscopy**- Direct fluorescence microscopy was used to localize GFP fusion proteins in live cells. For all experiments, cells were stained with DAPI (1 µg/ml) 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 (Chroma Technology) using an Olympus BX60 epifluorescence microscope equipped with a Photometrics Quantix digital camera.

**Integration into the Yeast Genome**- The importin α-GFP mutants were integrated at the endogenous importin α locus of wild-type (ACY192) cells using a standard integration strategy (23). The integration created a duplication at the endogenous importin α locus such that both endogenous importin α and importin α-GFP were each expressed from SRP1 promoters. TUB1-GFP was integrated at the URA locus as described previously (33).

In contrast to the importin α-GFP integrations, the A55-importin α mutant replaced the wild-type copy of importin α. To integrate A55-importin α, the A55 mutation was subcloned into the SRP1 open reading frame cloned in the LEU2 integrating plasmid, pRS305 (34), to create srp1-55-pRS305 (pAC1128). A55-importin α was then integrated at the endogenous SRP1 locus by linearization of srp1-55-pRS305 and transformation into the wild-type diploid ACY247. Transformants that grew on plates lacking leucine were selected and the presence of the A55-importin α mutation was confirmed by PCR and sequencing. The heterozygous diploid was subsequently sporulated and tetrads were dissected to generate the haploid A55-importin α strain, srp1-55 (ACY642). This integration strategy is designed to make A55-importin α the only copy of importin α expressed in the haploid strain.
Yeast Genetic Analyses- Synthetic interactions between A55-importin α (srp1-55) and other nuclear transport factors were tested by creating double and triple mutant strains. These strains were made by crossing the various haploid mutant strains [srp1-55, cse1-1 (35), ∆nup2 (Research Genetics)]. The srp1-55 strain was covered by a SRP1 URA3 plasmid (pAC876) and mated to each of the mutants to be tested. The resultant heterozygous diploids were sporulated and dissected to generate the appropriate double and triple mutant haploid strains. For suppression analysis, high copy plasmids (2µ) expressing various nuclear transport factors were transformed into srp1-55 (ACY642) covered by a SRP1 URA3 plasmid (pAC876). Genetic interactions (synthetic growth defects/lethality and suppression) were assessed by growing single colonies in liquid culture to saturation, serially diluting (1:10) and spotting on minimal media plates as a control or on fluoroorotic acid (5-FOA) plates. Plates were incubated at the indicated temperatures for 3-6 days.

FACS Analysis- Cells were prepared for FACS analysis by staining with propidium iodide (36). Briefly, cells were ethanol fixed at 4°C, washed and resuspended in 1ml of 50 mM sodium citrate, pH 7.0. Cells were then treated with 0.08 mg/ml Rtase A for 1 h at 50°C, followed by 0.25 mg/ml proteinase K for 1 h at 50°C, before incubation in 8 µg/ml propidium iodide. Each sample was analyzed with a FACS Caliber cytometer.
RESULTS

Generation of Mutant Importin α Proteins- Previous analyses of the N-terminal domain of importin α protein revealed a conserved cluster of basic amino acids that serves as an auto-inhibitory sequence (mouse importin α residues 49KRR51 corresponding to yeast importin α residues 54KRR56) (22,23). Structural studies suggested that the auto-inhibitory sequence resembles an NLS and binds to the NLS-binding pocket in a manner similar to the binding of a monopartite NLS (22,24,25). In order to test this prediction, we individually substituted each of the basic amino acids to alanine in the 54KRR56 cluster of yeast importin α (Srp1p) to generate importin α mutants referred to as A54 (K54A), A55 (R55A) and A56 (R56A) (Fig. 1).

Auto-inhibitory Function of Importin α Mutants- The A54 and A55 mutations are in residues that correspond to NLS residues that are critical for a high affinity interaction with the NLS binding pocket (26). Thus, we would predict that the auto-inhibitory domains of the A54 and A55 proteins should have weaker binding to the NLS binding pocket and should therefore have a decrease in auto-inhibitory function when compared to wild-type importin α. To examine both NLS binding and auto-inhibitory function for each importin α variant, we used a quantitative, fluorescence anisotropy, solution binding assay (23,26,29). 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 is carried out in the presence of a stoichiometric amount of importin β, the N-terminal auto-inhibition of full-length importin α is relieved and much tighter binding (~ 30-fold) to the SV40-NLS is observed. In contrast, our previous work demonstrates that the A3-importin α mutant (54KRR56 → AAA) has decreased auto-inhibitory function without impacting importin β binding (23). Due to this decreased auto-inhibition, the
A3 protein binds to SV40-NLS-GFP ~7-fold more tightly than full-length wild-type importin α (23). This assay actually examines three aspects of importin α function: binding to NLS cargo, auto-inhibitory function (measured by the ability of full-length importin α to bind NLS cargo in the absence of importin β), and binding to importin β (based on the relief of auto-inhibition).

To examine NLS binding and the auto-inhibitory function for each importin α protein that contains a single amino acid change, we performed the fluorescence anisotropy assay using a monopartite SV40-NLS cargo. The assay was carried out with each importin α protein in the presence and absence of importin β (Fig. 2). Typical curves for binding of SV40-NLS-GFP to the mutant importin α proteins in the absence of importin β are shown in Fig. 2A. As described in Experimental Procedures, these curves are used to calculate Kd values for the interaction between NLS-cargo and importin α (see Table II). The Kd values can be used to determine the change in free energy (ΔG) to compare the wild-type and mutant proteins.

Thus, to assess the impact of each amino acid change within the auto-inhibitory sequence, we compare the Kd values and calculate the change in free energy, ΔG, for the binding of each importin α protein to NLS-cargo. As previously demonstrated, 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 (23). As a control, A3-importin α, which has decreased auto-inhibitory function, binds to SV40-NLS-GFP more tightly (Kd ~ 73 nM) than full-length wild-type importin α (Kd ~ 500 nM). A54-importin α binds to SV40-NLS-GFP with a similar affinity (Kd ~ 83 nM) to A3-importin α suggesting that K54 is the most critical residue in the 54KRR56 auto-inhibitory sequence. A55-importin α binds to SV40-NLS-GFP more tightly (Kd ~240 nM) than wild-type importin α, an ~2-fold increase in affinity. A56-importin α binds to SV40-NLS-
GFP with a similar affinity (Kd ~ 1300 nM) to wild-type importin α. To compare each of the importin α proteins, the change in free energy for the binding of each importin α protein to NLS cargo in the absence (□) and presence (■) of importin β (Fig. 2B). In the presence of importin β, the affinity of each of the mutant importin α proteins is similar to wild-type importin α suggesting that each mutant retains normal binding to importin β (Table II). These binding results also demonstrate that each of the mutant importin α/β complexes binds NLS-cargo with approximately the same affinity as the wild-type importin α/β complex.

**Correlation of Importin α Function with NLS Binding**- Previous structural studies suggested that the auto-inhibitory sequence, KRR, resembles an NLS and binds to the NLS-binding site in a similar way to a monopartite NLS (sites P2-P4) (22,24). Alignment of NLS sequences with the auto-inhibitory sequence shows that residue K54 of importin α binds to importin α at the same position (P2) as the essential lysine of a monopartite NLS (Fig. 2C) (26). Mutation of K54 to A54 significantly decreases the auto-inhibitory function of importin α (Fig. 2A). The decrease in auto-inhibition can be expressed as a change in free energy (ΔΔG) for each importin α mutant when compared to wild-type protein (Fig. 2C). As shown in Fig. 2C, residues critical for auto-inhibitory function correlate with residues that are critical for an NLS binding to the NLS binding pocket of importin α, where mutation of the P2 binding residue of the NLS to alanine causes the largest decrease in binding affinity (26). The NLS residue that binds importin α in position 3 (P3) has an intermediate contribution and the position 4 (P4) residue has a fairly weak contribution to the NLS binding energy (Fig. 2C). The in vitro auto-inhibitory behavior of A55-importin α and A56-importin α correlates with the energetic contributions of the corresponding residues within the SV40 and Myc monopartite NLSs (Fig. 2C). Thus, the auto-
inhibition of each of the importin α mutants correlates with the energetic contribution of each residue to a functional NLS.

**In Vivo Function of Importin α Mutants-** Our *in vitro* experiments demonstrate that variants of importin α with point mutations within the $^{54}$KRR$^{56}$ auto-inhibitory sequence exhibit a range of auto-inhibitory functions (Table II). To test the effect of different levels of auto-inhibition on importin α function *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 a plasmid borne wild-type copy of *SRP1* (Fig. 3A). This results in ∆*SRP1* 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 complement ∆*SRP1* cells while neither a vector alone nor the auto-inhibitory defective importin α (A3) can functionally replace *SRP1* (Fig. 3A, compare the control and 5-FOA plates). Results shown in Fig. 3A indicate that cells expressing A54-importin α have a pronounced cold-sensitive phenotype (no growth at 16°C) and grow extremely slowly even at 30°C. Cells expressing A55-importin α grow more slowly than wild-type at 30°C and are cold-sensitive at 16°C. Cells expressing A56-importin α grow similar to cells expressing wild-type importin α at all temperatures.

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. Immunoblotting of 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. 3B, 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 the A54 and A55 mutations perturb the function of the importin α proteins.

**Localization of the Mutant Importin α Proteins**- We have previously found that mutations that decrease the auto-inhibitory function of importin α result in its accumulation within the nucleus (23). This accumulation is presumably due to the persistence of an NLS-cargo/importin α complex within the nucleus. In order to further examine the importin α variants, we analyzed the localization of each protein using C-terminal GFP tagged fusion proteins. These importin α-GFP fusion proteins were expressed from the endogenous importin α promoter. We integrated each importin α-GFP fusion protein at the endogenous importin α locus and visualized them as described previously (23). Wild-type importin α localizes to the nuclear rim and the cytoplasm in wild-type cells when visualized in this manner (Fig. 3C, panel E). However, A3-importin α-GFP accumulates within the nucleus (Fig. 3C, panel A). The localization of A54-importin α-GFP (Fig. 3C, panel B) is similar to that of A3-importin α-GFP. A55-importin α-GFP also localizes to the nucleus but shows some cytoplasmic signal, suggesting that its localization is intermediate between A54-importin α and wild-type importin α (Fig. 3C, panel C). The localization of A56-importin α-GFP is similar to that of wild-type importin α-GFP (Fig. 3C, compare panel D to panel E).

**Analysis of R54-importin α**- Even conservative mutation of the P2 lysine to arginine in SV40 NLS results in an ~ 94-fold decrease in binding to the NLS binding pocket (26). To further test the hypothesis that the auto-inhibitory sequence interacts with importin α in the same manner as an NLS, we mutated K54 to an arginine residue to create R54-importin α (Fig. 1) and assessed the impact of this conservative mutation. R54-importin α binds to SV40-NLS-GFP
with ~5.5-fold higher affinity than wild-type importin α (Table II) as assessed by fluorescence anisotropy. This demonstrates that R54-importin α is defective in auto-inhibition and provides additional evidence that a monopartite NLS and the auto-inhibitory sequence bind to the NLS binding pocket of importin α with similar energetics. In the presence of importin β the binding of SV40-NLS-GFP to R54-importin α is similar to wild-type importin α (Table II).

To test the function of the R54-importin α protein in vivo, we used the plasmid shuffle technique and the importin α-GFP localization assay (see Experimental Procedures). R54-importin α was transformed into yeast cells deleted for the endogenous importin α gene (SRP1) and plasmid shuffle was used to replace the plasmid-borne wild-type copy of SRP1 (Fig. 4A). Results show that cells expressing R54-importin α as the only copy of importin α grow slowly at 30°C and are not viable at 16°C. The R54-importin α protein was expressed at similar levels to wild-type importin α (data not shown). In vivo localization analysis shows that the R54-importin α-GFP protein accumulates within the nucleus similar to A54-importin α-GFP (Fig. 4B).

In Trans Localization of the Auto-inhibitory Sequence- The IBB domain of importin α, which contains the auto-inhibitory sequence, binds weakly to the NLS binding pocket in trans (K_d~ 14 μm) (23). This suggests that the auto-inhibitory sequence should not bind to the NLS binding pocket with sufficient affinity to act as an NLS and direct a heterologous protein into the nucleus when expressed in vivo. To test whether the auto-inhibitory sequence can act like an NLS to direct a heterologous protein to the nucleus, we fused importin α residues 49-60 in frame with GFP-GFP to create IBB^{49-60}-GFP-GFP. The localization of this fusion protein was compared both to GFP-GFP, which lacks any NLS, and to SV40-NLS-GFP-GFP, a positive control which contains a canonical monopartite NLS. As shown in Fig. 5, the control GFP-GFP protein is diffusely localized throughout the cell. In contrast, SV40-GFP-GFP accumulates in
the nucleus. The IBB\textsuperscript{49-60}-GFP-GFP is localized throughout the cell similar to GFP-GFP. This demonstrates that the auto-inhibitory sequence cannot efficiently act as an NLS and direct a protein to the nucleus when expressed \textit{in trans}.

\textit{Genetic Analysis of NLS Release Factors}--Through analysis of the auto-inhibitory defective mutant of importin \(\alpha\), A3, we suggested a model where the auto-inhibitory activity of importin \(\alpha\) is required for NLS-cargo release and the subsequent Cse1p-dependent recycling of importin \(\alpha\) to the cytoplasm (23). Cse1p and Nup2p have also been reported to affect NLS-cargo release from importin \(\alpha\) (19,21). It has been suggested that these factors may cooperate or act sequentially to facilitate NLS-cargo release and may therefore functionally overlap. As shown in Fig. 3A, the A55 mutation in importin \(\alpha\) causes a conditional growth phenotype which correlates with its defect in auto-inhibitory function. This cold-sensitive phenotype can be exploited for genetic analysis. As a genetic test for functional overlap between the auto-inhibitory function of the IBB domain of importin \(\alpha\), Cse1p, and Nup2p, we tested for any exacerbated growth defects (synthetic lethality) in double or triple mutant cells.

The A55-importin \(\alpha\) allele (srp1-55) was integrated into the genome to avoid copy number effects (Experimental Procedures). Since \textit{CSE1} is essential, we used a well characterized cold-sensitive allele, \textit{cse1-1} (35). \textit{NUP2} is not essential (37) and therefore we utilized a complete deletion of the open reading frame, \(\Delta\text{nu}p2\). If Cse1p and Nup2p are involved in NLS-cargo release in the nucleus, cells with combined mutations in the importin \(\alpha\) auto-inhibitory function, \textit{CSE1} and/or \textit{NUP2} might be more growth compromised than any of the single mutants. We therefore generated each of the double mutants (srp1-55 \textit{cse1-1}, srp1-55 \(\Delta\text{nu}p2\), and \textit{cse1-1} \(\Delta\text{nu}p2\)) and the triple mutant (srp1-55 \textit{cse1-1} \(\Delta\text{nu}p2\)) maintained by a plasmid borne wild-type importin \(\alpha\) (\textit{SRP1}) as described in Experimental Procedures. As previously
reported (21,35,38) and shown in Fig. 6A, cse1-1 cells are cold-sensitive at 16°C and Δnup2 cells do not have any detectable growth defect. Results shown in Fig. 6A demonstrate that the srp1-55 cse1-1 double mutant is inviable (compare the control and 5-FOA plates). This synthetic lethal phenotype was observed at all temperatures tested. In addition, the srp1-55 Δnup2 and cse1-1 Δnup2 double mutants grow more slowly than either single mutant. As shown in Fig. 6A, these phenotypes are more pronounced at cold temperatures.

As a second genetic test for functional overlap, we assessed high copy suppression of the srp1-55 cold-sensitive phenotype. Importin β, Cse1p and Nup2p were expressed from a high copy plasmid and their ability to suppress the cold-sensitive phenotype of srp1-55 was examined as described in Experimental Procedures. Controls demonstrate that a wild-type importin α plasmid can complement the cold-sensitive phenotype of srp1-55 cells, while a vector alone cannot (Fig. 6B, compare the control and 5-FOA plates). Results shown in Fig. 6B demonstrate that importin β cannot complement the cold-sensitive phenotype of the srp1-55 cells. This is consistent with our in vitro analyses, Fig. 2B, where we show that the mutant importin α proteins bind to importin β with the same affinity as wild-type importin α. Nup2p also does not suppress the srp1-55 cold-sensitive phenotype. In contrast, overexpression of Cse1p partially suppresses the cold-sensitive phenotype of srp1-55 cells.

srp1-55 Cells Accumulate in G2/M at 18°C- Importin α is required for the execution of mitosis as cells with conditional mutations of importin α arrest with a G2/M phenotype (39). Therefore, to further characterize the srp1-55 cells, we assessed the morphology of cells grown at either 30°C or 18°C and compared them to wild-type cells grown under the same conditions. Microscopic analysis revealed that the srp1-55 cells have similar morphology to wild-type cells when grown at 30°C. Interestingly, at 18°C the srp1-55 cells were larger and there was a greater
proportion of large budded cells within the population as compared to wild-type cells (for example see Fig. 7A, compare panels G and H). To determine if these large budded cells were arrested uniformly within the G2/M phase of the cell cycle, we examined the microtubules by integrating TUB1-GFP into wild-type and srp1-55 cells (Experimental Procedures). The TUB1-GFP integrated wild-type and srp1-55 cells were grown to log phase at both 30°C and 18°C and compared by microscopic analysis (Fig. 7A). As shown in Fig. 7A, panel D, there is a variation in spindle length in the large budded srp1-55 cells, therefore these cells are not uniformly arrested at the same point within G2/M of the cell cycle. This was also confirmed by staining the DNA with DAPI (data not shown). These data suggest that the auto-inhibitory defective cells are not arrested, but rather pass through G2/M phase more slowly than wild-type cells.

The microscopic analysis of the srp1-55 cells suggests that they spend more time in the G2/M phase of the cell cycle, thus we predict that a higher percentage of cells should have replicated (2N) DNA than wild-type cells. To examine the DNA content of the srp1-55 cells, we performed FACS analysis of log phase cultures grown at both 30°C and 18°C and compared it to wild-type cells grown under the same conditions. Cells were stained with propidium iodide, and the DNA content was analyzed by flow cytometry. The FACS profile of wild-type cells grown at either 30°C or 18°C are indistinguishable in the distribution of cells with 1N and 2N DNA content (data not shown). Cells expressing the A55-importin α protein have a similar profile to wild-type cells at 30°C (data not shown). As shown in Fig. 7B, the 2N DNA peak of srp1-55 cells grown at 18°C is markedly broader than that of srp1-55 cells grown at 30°C, which is consistent with an increased proportion of cells in G2/M, and hence slower progression through this phase of the cell cycle.
This G2/M phenotype of srp1-55 cells provides another assay to characterize the genetic interaction between the auto-inhibitory function of importin α and Cse1p. As shown in Fig. 6B, expression of Cse1p from a high copy plasmid suppresses the cold-sensitive phenotype of srp1-55 cells. Therefore, we tested whether Cse1p expressed from a high copy plasmid could also suppress the G2/M phenotype of srp1-55 cells. Controls demonstrate that a wild-type importin α plasmid can suppress the srp1-55 G2/M phenotype while a vector alone cannot (Fig. 7B). Overexpression of Cse1p also suppresses the G2/M cell cycle phenotype of the srp1-55 cells.
DISCUSSION

Here we create single point mutations within the auto-inhibitory sequence of importin α and through analysis of these mutants determine the energetic contribution of each residue to the auto-inhibitory function of importin α. We demonstrate that the severity of the auto-inhibitory defect of each importin α protein measured \textit{in vitro} correlates with the \textit{in vivo} function and localization of each mutant protein. Taken together these analyses suggest that the auto-inhibitory sequence of the N-terminus of importin α can compete with an NLS-cargo for binding to the NLS binding pocket and support a model where this function is essential \textit{in vivo} for efficient NLS-cargo delivery into the nucleus.

Our previous analysis of auto-inhibitory function utilized a variant of importin α, A3, where all of the basic amino acids within the 54KRR56 sequence were mutated to alanine (23). In the current study, we show that amino acid K54 is the most important residue within the auto-inhibitory sequence. Indeed, the \textit{in vitro} and \textit{in vivo} phenotypes of the K54 protein are similar to those of the A3 protein. Even conservative mutation of K54 to arginine significantly decreases auto-inhibition. Mutation of residue R55 has an intermediate effect on auto-inhibition, whereas mutation of R56 appears to have no affect on importin α function. This graduated effect on auto-inhibitory function within the 54KRR56 sequence is consistent with the molecular interactions revealed by structural studies and is analogous to the energetic landscape of a monopartite NLS binding to the NLS binding pocket of importin α (22,25,26). Furthermore, we show that the severity of the \textit{in vivo} phenotype is directly correlated to the reduction of \textit{in vitro} auto-inhibition, suggesting that the \textit{in vivo} phenotypes are directly related to the loss of auto-inhibitory function.
The ability of the IBB domain to regulate NLS-cargo binding to the NLS binding pocket is presumably dependent on the energy the IBB gains from the intramolecular interaction versus an intermolecular interaction. Indeed, our data support a model where the energy for the binding of the auto-inhibitory sequence to the NLS binding pocket of importin α is obtained from the \textit{in cis} intramolecular interaction. We have previously shown that \textit{in trans} the IBB binds with low affinity (µM) to the NLS binding pocket of importin α (23). Consistent with this previous result, we demonstrate that the auto-inhibitory sequence is not able to target a heterologous protein into the nucleus of yeast cells when expressed \textit{in trans}, suggesting that the binding affinity for importin α is not sufficient for nuclear localization. Although the auto-inhibitory sequence can bind to the NLS binding pocket like an NLS, it would not be energetically favorable for it to interact with importin α with an affinity comparable to a functional NLS. However, the auto-inhibitory sequence can compete with an NLS for binding to the NLS binding pocket due to the physical tethering of the IBB to the NLS binding pocket domain of importin α, which significantly increases its local concentration. This requirement for an \textit{in cis} interaction between the IBB domain and the NLS binding pocket of importin α also presumably prevents the N-terminal domains of adjacent importin α proteins from interacting with NLS-cargo pockets intermolecularly and forming dimers.

Although RanGTP is the major determinant of import complex dissociation (16,17,19), the N-terminal auto-inhibitory domain of importin α, Cse1p and Nup2p also play a role in the release of NLS-cargo into the nucleus (19,21-23,25,29). The trimeric import complex is disassembled by the RanGTP-triggered dissociation of importin β to release a dimeric NLS-cargo/importin α complex into the nucleus. The importin α auto-inhibitory function is essential to efficiently dissociate this dimeric intermediate (23). In this study we use genetic analyses of
the auto-inhibitory domain of importin α, CSE1 and NUP2 to provide data in support of a model where these factors have overlapping functions in vivo that facilitate release of NLS-cargo from importin α. This in vivo data is consistent with a recent study by Gilchrist et al. where they demonstrated that each of these interactions is able to increase the dissociation rate of the dimeric NLS-cargo/importin α intermediate in vitro (19).

Our genetic data suggest that Cse1p and Nup2p have distinct functions in NLS-cargo release. This conclusion is supported by two lines of genetic evidence. First, cse1-1 and srp1-55 are synthetically lethal, but Δnup2 and srp1-55 show only a modest synthetic interaction. Second, the cold-sensitive phenotype of srp1-55 cells is suppressed by Cse1p but not by Nup2p. Independent biochemical experiments support the idea that Cse1p and Nup2p act through distinct mechanisms in NLS-cargo release (Gilchrist and Rexach, personal communication). As shown here and suggested previously (40), Cse1p and Nup2p demonstrate only minor genetic interactions. Thus, although the evidence suggests that both Cse1p and Nup2p participate in NLS-cargo release and/or importin α recycling, these proteins appear to function through distinct mechanisms.

A previous study also demonstrated a genetic interaction between importin α and Nup2p (41). This study utilized the srp1-31 allele of importin α (42). It should be noted that the srp1-31 allele has not been functionally characterized and therefore it is not known what step within the importin α transport cycle is affected. In contrast, importin β binding, NLS binding and auto-inhibitory function have all been analyzed for the srp1-55 mutant. Analysis of the genetic interactions of srp1-55 can therefore be interpreted in terms of the auto-inhibitory function of importin α. In srp1-31, serine residue 116 is substituted with a phenylalanine residue (42). This residue is outside of the NLS binding pocket and is not within the IBB domain of importin α.
In the future it may be interesting to determine whether the srp1-31 mutant is specifically defective in a particular step in the importin α transport cycle so that it is possible to more definitively interpret studies with srp1-31 cells.

The ability of Cse1p to suppress the cold-sensitive phenotypes of srp1-55 cells suggests an intimate relationship between NLS-cargo dissociation and recycling of importin α to the cytoplasm. There is also evidence that NLS-cargo and Cse1p cannot bind to importin α simultaneously (13,14). This presumably prevents the recycling of importin α that is still bound to NLS-cargo in the nucleus and thus futile cycles of nuclear transport. It is not known how importin α and Cse1p interact, although we have shown that an auto-inhibitory defective mutant of importin α still interacts with Cse1p (23). This suggests that high copy suppression of the srp1-55 cold-sensitive phenotype by CSE1 is not due to a change in the binding affinity of the two proteins, but rather an overlap of function between the two proteins. Future studies of the importin α/Cse1p interaction should allow us to dissect the mechanism of NLS-cargo release and importin α recycling.

Mutant srp1-55 cells accumulate in the G2/M phase of the cell cycle. This observation is consistent with a previous report where the conditional srp1-31 mutation causes mitotic cell cycle defects (39). Loeb et al. suggested that the importin α dependent transport of cell cycle regulators into the nucleus is critical for cell cycle progression. Indeed, the slow growth phenotype of srp1-55 cells could be due to the inefficient release of NLS-cargoes within the nucleus, in particular, specific cargoes required for mitosis. 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 in Xenopus (15). This highlights the importance of understanding how cargoes are efficiently dissociated from importin α within the nucleus to
mediate their cellular function. Future studies of the A55-importin α protein may allow us to identify these mitotic cargoes.

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FOOTNOTES

1. The abbreviations used are: CEN, centromeric; DAPI, 4’, 6-diamidino-2-phenylindole dihydrochloride; DIC, differential interference contrast; FACS, fluorescent activated cell sorter; 5-FOA, 5-fluoroorotic acid; GFP, green fluorescent protein; IBB, importin β binding; NLS, nuclear localization signal.
FIGURE LEGENDS

FIG. 1. **Mutations in the N-terminal auto-inhibitory sequence of *S. cerevisiae* importin α.**
The basic amino acid residues in the importin α auto-inhibitory sequence are boxed. Each residue of the auto-inhibitory sequence was individually mutated to alanine to generate the A54, A55 and A56 mutants or mutated to arginine to generate the R54 mutant in *S. cerevisiae* importin α.

FIG. 2. **Analysis of the auto-inhibitory function of importin α mutants.**

A, Binding of an NLS-GFP cargo to full-length importin α was measured by anisotropy. Binding curves for A54, A55 and A56 importin α proteins are shown. The anisotropy is plotted vs. the concentration of importin α on a logarithmic scale. B, Binding of an NLS-GFP cargo to each full-length importin α protein indicated was measured by fluorescence anisotropy in the absence (□) or presence (■) of a stoichiometric amount of importin β. For each importin α protein, the data were fit to an ideal binding curve to yield a value for Kₐ. The calculated binding energy (ΔG, kcal/mol) is shown on the left axis. Standard deviations are indicated by error bars. C, Comparison of the change in free energy (ΔΔG) when specific amino acids are mutated to alanine. The alignment of SV40 and Myc NLS sequences and the auto-inhibitory sequence of importin α are shown aligned along the X-axis with the position (P2-P4) that each amino acid binds within the NLS binding pocket. The energy profiles for each NLS sequence binding to importin α and the variant IBB importin α proteins binding to SV40 NLS-cargo were determined by anisotropy (26). The change in the free energy of binding (ΔΔG) between the NLS-GFP and ΔIBB-α or
NLS-cargo binding to the variant importin α proteins when that residue of either the NLS (SV40 ■ and Myc □) or importin α (■) is replaced with an alanine is plotted above each residue letter.

FIG. 3. **Functional analysis of the importin α mutants in vivo.** A, The SRP1 deletion strain (ACY324) maintained by a plasmid encoding wild-type importin α and expressing either wild-type or mutant importin α protein was spotted onto minimal media (control) or 5-FOA plates as described in Experimental Procedures. All plates were incubated at the indicated temperature for 3-5 days. B, Levels of importin α expressed in wild-type cells (ACY192) were examined by immunoblotting with an anti-myc antibody. Ten micrograms of total yeast protein was loaded in each lane. C, The integrated importin α-GFP proteins were viewed in wild-type (ACY192) cells by direct fluorescence (panels A-E). All cultures were grown to log phase at 30°C. Corresponding DIC images are shown (panels F-J).

FIG. 4. **In vivo analysis of R54-importin α.** A, The SRP1 deletion strain (ACY324) maintained by a plasmid encoding wild-type importin α and expressing either wild-type or mutant importin α protein was spotted onto minimal media (control) or 5-FOA plates as described in Experimental Procedures. All plates were incubated at the indicated temperature for 3-5 days. B, The integrated importin α-GFP proteins were viewed in wild-type (ACY192) cells by direct fluorescence microscopy (panels A-C). All cultures were grown to log phase at 30°C. Corresponding DIC images are shown (panels D-F).
FIG. 5. **In trans localization of the auto-inhibitory sequence.** *A*, Wild-type cells (ACY192) expressing GFP-GFP (*panel A*), SV40-NLS-GFP-GFP (*panel B*) or the auto-inhibitory sequence (importin α residues 49-60) fused to GFP-GFP (*panel C*) were grown at 30°C to log phase and viewed by direct fluorescence microscopy. Corresponding DIC images are shown (*panels D-F*).

FIG. 6. **Genetic analysis of NLS release factors mutants.** *A*, Double and triple mutants of *srp1-55, cse1-1* and Δnup2 were generated as described in Experimental Procedures. These cells were grown to saturation and spotted onto minimal media (*control*, which contains a *SRP1* maintenance plasmid) or 5-FOA plates (no maintenance plasmid). All plates were incubated at the indicated temperature for 3-5 days. *B*, High copy suppression analysis of *srp1-55* by NLS release factors. Suppression of the *srp1-55* cold-sensitive phenotype by importin β, Cse1p and Nup2p overexpression was assessed by growth analysis. Negative (vector) and positive (importin α) controls are shown. Cells were grown to saturation and spotted onto minimal media (*control*, which contains a *SRP1* maintenance plasmid) or 5-FOA plates (no maintenance plasmid). All plates were incubated for 3-5 days.

FIG. 7. **A55-importin α cells accumulate in G2/M at 18°C.** *A*, Wild-type (ACY192) and *srp1-55* cells expressing integrated *TUB1*-GFP were grown to log phase at 30°C or 18°C and viewed by direct fluorescence microscopy (*panels A-D*). Corresponding DIC images are shown (*panels E-H*). *B*, *srp1-55* cells (transformed with either vector, *SRP1* or *CSE1*) were grown to log phase at 30°C and 18°C before being prepared for FACS analysis (Experimental Procedures). Histograms of fluorescence intensity versus cell number are shown. 1N, unreplicated DNA; 2N, replicated DNA.
| Strain/plasmid          | Description                                                                 |
|------------------------|-----------------------------------------------------------------------------|
| ACY192 (wild-type)     | MATα ura3-52 leu2Δ1 trp1 (43)                                               |
| ACY247 (wild-type)     | MATα/α ura3-52/ura3-52 leu2Δ1/ leu2Δ1 his3Δ200/his3Δ200 ade2/ADE2 ade3/ADE3 lys2/LYS2 trp1/TRP1 (44) |
| ACY324 (ΔSRP1)         | MATα ura3-52 leu2Δ1 lys2 his3Δ200 SRP1::HIS3 (SRP1-CEN-URA3) (23)          |
| ACY642 (srp1-55)       | MATα ura3-52 his3Δ200 leu2Δ1 trp1 srp1-55::LEU2 (SRP1-CEN-URA3) (44)      |
| ACY687 (srp1-55)       | MATα ura3-52 his3Δ200 leu2Δ1 srp1-55::LEU2 (SRP1-CEN-URA3) (23)          |
| ACY690 (Δnup2)         | MATα ura3-52 his3Δ200 leu2Δ1 NUP2::KAN (SRP1-CEN-URA3) (23)               |
| ACY692 (srp1-55 Δnup2) | MATα ura3-52 his3Δ200 leu2Δ1 srp1-55::LEU2 NUP2::KAN (SRP1-CEN-URA3) (23) |
| ACY694 (cse1-1)        | MATα ura3-52 his3Δ200 leu2Δ1 cse1-1::HIS (SRP1-CEN-URA3) (23)             |
| ACY696 (srp1-55 cse1-1)| MATα ura3-52 his3Δ200 leu2Δ1 srp1-55::LEU2 cse1-1::HIS (SRP1-CEN-URA3) (23) |
| ACY698 (srp1-55 cse1-1)| MATα ura3-52 his3Δ200 leu2Δ1 srp1-55::LEU2 NUP2::KAN cse1-1::HIS (SRP1-CEN-URA3) (23) |
| pAC3 (pRS315)          | CEN, LEU2, AMP^R (34)                                                      |
| pAC6 (pRS426)          | 2µ, TRP1, AMP^R (34)                                                       |
| pAC8 (pRS426)          | 2µ, URA3, AMP^R (34)                                                      |
| pAC9 (pRS304)          | LEU2 integrating, AMP^R (34)                                               |
| pAC369                 | RSL1^a, AMP^R, pET based importin β bacterial expression vector (29)        |
| pAC492                 | SRP1^a, AMP^R, pProEX-HTB importin α bacterial expression vector (24)      |
| pAC592                 | RSL1, 2µ, TRP1, AMP^R                                                      |
| pAC855                 | A3 (^54KRR^56→AAA)-SRP1, CEN, LEU2, AMP^R (23)                             |
| pAC856                 | SRP1, CEN, LEU2, AMP^R (23)                                               |
| pAC876                 | SRP1, CEN, URA3, AMP^R (23)                                               |
| pAC   | Description                                                                 |
|-------|----------------------------------------------------------------------------|
| pAC857 | **A54-SRP1, CEN, LEU2, AMP^R**                                           |
| pAC858 | **A55-SRP1, CEN, LEU2, AMP^R**                                           |
| pAC859 | **A56-SRP1, CEN, LEU2, AMP^R**                                           |
| pAC963 | **SRP1-c-myc (3X), CEN, TRP1, AMP^R**                                    |
| pAC982 | **R54-SRP1, CEN, LEU2, AMP^R**                                           |
| pAC1021| **pGAL1-10-GFP-GFP, 2µ, URA3**                                           |
| pAC1022| **pGAL1-10-SV49-NLS-GFP-GFP, 2µ, URA3**                                  |
| pAC1128| **Srp1-55, integration, LEU2, AMP^R**                                    |
| pAC1330| **pGAL1-10-49DEALAKRRNFIP^60 SRP1-GFP-GFP, 2µ, URA3**                    |
| pAC1156| **SRP1-GFP, integration, URA3, AMP^R (23)**                              |
| pAC1157| **A3-SRP1-GFP, integration, URA3, AMP^R (23)**                            |
| pAC1207| **SV40-NLS-GFP, KAN^R, pET-28a bacterial expression vector (29)**       |
| pAC1303| **CSE1, 2µ, TRP1, AMP^R**                                                |
| pAC1321| **A54-SRP1-GFP, integration, URA3, AMP^R**                               |
| pAC1322| **R54-SRP1-GFP, integration, URA3, AMP^R**                               |
| pAC1323| **A56-SRP1-GFP, integration, URA3, AMP^R**                               |
| pAC1324| **A55-SRP1-GFP, integration, URA3, AMP^R**                               |
| pAC1331| **A3-SRP1-c-myc (3X), CEN, TRP1, AMP^R**                                 |
| pAC1332| **A54-SRP1-c-myc (3X), CEN, TRP1, AMP^R**                                |
| pAC1333| **R54-SRP1-c-myc (3X), CEN, TRP1, AMP^R**                                |
| pAC1334| **A55-SRP1-c-myc (3X), CEN, TRP1, AMP^R**                                |
| pAC1335| **A56-SRP1-c-myc (3X), CEN, TRP1, AMP^R**                                |
| pAC1344| **TUB1-GFP, integration, URA3, AMP^R (33)**                             |
pAC1354  \textit{SRP1 CEN, TRP1, AMP^R} \\
pAC1385  \textit{NUP2, 2\mu, TRP1, AMP^R} \\
pAC1386  \textit{A54-SRP1, AMP^R, pProEX-HTB bacterial expression vector} \\
pAC1387  \textit{A55-SRP1, AMP^R, pProEX-HTB bacterial expression vector} \\
pAC1388  \textit{A56-SRP1, AMP^R, pProEX-HTB bacterial expression vector} \\
pAC1389  \textit{R54-SRP1, AMP^R, pProEX-HTB bacterial expression vector} \\
pAC1390  \textit{Myc-NLS-GFP, KAN^R, pET-28a bacterial expression vector (26)}

* The \textit{S. cerevisiae} proteins Srp1p (importin \(\alpha\)) and Rsl1p (importin \(\beta\)) were used for all experiments.
TABLE II. Binding of importin α proteins to NLS-cargo

| Importin α | K\textsubscript{d} (nM) \((-\text{ Importin }\beta)\) | K\textsubscript{d} (nM) \((+\text{ Importin }\beta)\) |
|------------|---------------------------------|---------------------------------|
| A3         | 73 ± 5                           | 20 ± 5                           |
| A54        | 83 ± 20                          | 23 ± 5                           |
| R54        | 90 ± 20                          | 20 ± 5                           |
| A55        | 240 ± 35                         | 25 ± 6                           |
| A56        | 1300 ± 900                       | 18 ± 10                          |
| wild-type  | 500 ± 5                          | 18 ± 4                           |
Figure 3
Figure 4

A

vector
WT
A54
R54

control
30°C
30°C
16°C
5-FOA
5-FOA

B

WT
A54
R54

GFP

DIC

A
B
C

D
E
F
Figure 5

SV40

GFP

IBB 49-60

-GFP

DIC

AB C

DE F
Figure 6

A

|        | control             | FOA-25°C   | FOA-16°C   |
|--------|---------------------|------------|------------|
| WT     | ![Image](image1)    | ![Image](image2) | ![Image](image3) |
| srp1-55| ![Image](image4)    | ![Image](image5) | ![Image](image6) |
| cse1-1 | ![Image](image7)    | ![Image](image8) | ![Image](image9) |
| Δnup2  | ![Image](image10)   | ![Image](image11) | ![Image](image12) |
| srp1-55 cse1-1 | ![Image](image13) | ![Image](image14) | ![Image](image15) |
| srp1-55 Δnup2 | ![Image](image16) | ![Image](image17) | ![Image](image18) |
| cse1-1 Δnup2 | ![Image](image19) | ![Image](image20) | ![Image](image21) |
| srp1-55 cse1-1 Δnup2 | ![Image](image22) | ![Image](image23) | ![Image](image24) |

B

|        | control             | FOA-16°C   |
|--------|---------------------|------------|
| vector | ![Image](image25)   | ![Image](image26) |
| Importin α | ![Image](image27) | ![Image](image28) |
| Importin β | ![Image](image29) | ![Image](image30) |
| Cse1p   | ![Image](image31)   | ![Image](image32) |
| Nup2p   | ![Image](image33)   | ![Image](image34) |
Figure 7

A

|       | 30°C | 18°C |
|-------|------|------|
| WT    | A    | E    |
| srp1-55 | B   | F    |
|       | C    | G    |
|       | D    | H    |

Tub1-GFP

DIC

B

- Tub1-GFP
- DIC

|       | cell number |
|-------|-------------|
| 1N2N  | 80          |
| 1N    | 80          |
| 2N    | 80          |

- srp1-55 + vec
- srp1-55 + vec
- srp1-55 + SRP1
- srp1-55 + CSE1

30°C

18°C

18°C
Characterization of the auto-inhibitory sequence within the N-terminal domain of importin alpha
Michelle T. Harreman, Pamela E. Cohen, Mary R. Hodel, Glyn J. Truscott, Anita H. Corbett and Alec E. Hodel

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