We have developed a quantitative in vitro steady-state fluorescence depolarization assay to measure the interaction of a nuclear localization signal (NLS) substrate with its receptors. This assay relies on the change in fluorescence depolarization of an NLS fused to the green fluorescent protein upon binding to receptor. No binding is observed in the absence of a functional NLS, and binding affinities measured correlate with previous in vivo studies of NLS function. We have used this assay to test an auto-inhibitory model for the interaction of an NLS with the NLS receptor complex. This model suggests that NLS binding to importin α is modulated by an auto-inhibitory sequence within the N terminus of importin α, which is displaced by importin β binding. Consistent with this model, NLS substrates bind tightly to an N-terminally truncated importin α lacking the auto-inhibitory domain (Kd ~10 nM), but measurable binding to full-length importin α is only observed upon addition of importin β. Our quantitative results support the auto-inhibitory model and suggest a mechanism for a switch between a cytoplasmic, high affinity and a nuclear, low affinity NLS receptor. This predicted mode of interaction would facilitate binding of substrate in the cytoplasm and its subsequent release into the nucleus.

In eukaryotic cells, selective transport of proteins into the nucleus is mediated by short amino acid sequences that are referred to as nuclear localization signals (NLSs). The “classical” NLS motif contains one (monopartite) or two (bipartite) clusters of basic amino acids (1). The monopartite NLS is exemplified by the NLS of SV40 large T-antigen (K26P2KKRK23V), whereas the bipartite, consisting of two small clusters of basic residues separated by a linker sequence, is found in the NLS of nucleoplasmin (K256KPAATKKAGQAKKKK270) (2, 3).

A heterodimeric receptor for the classical nuclear protein import pathway has been identified. This receptor consists of two proteins referred to as importin α and importin β (4). The molecular and biochemical characterization of importin α shows that it recognizes and binds directly to the NLS peptide (5–8). Importin α acts as an adapter in the formation of a trimeric import complex containing the NLS-bearing cargo, importin α, and the import receptor importin β. Importin β interacts with components of the nuclear pore complex known as nucleoporins (9–11). As a result of these interactions the import complex is targeted to the nuclear pore complex and then translocated into the nucleus (9, 12) via a process that requires the activity of the small GTPase Ran (4, 13).

The mechanism for the recognition of NLS peptides by importin α has been enigmatic because of the diversity in the amino sequences of experimentally defined NLSs. The structural basis for recognition of at least one type of NLS by Saccharomyces cerevisiae importin α is now known from a crystallographic analysis of the NLS binding domain of the 50-kDa yeast importin α fragment bound to an SV40 NLS peptide (8). This 50-kDa importin α fragment lacks the N-terminal importin β-binding (IBB) domain and consists of 10 helical repeats known as armadillo (ARM) motifs. Each ARM motif is characterized by three α helices whose arrangement results in an array of binding sites for SV40-NLS recognition. Biochemical evidence supports the structural model and suggests that organization of the repeats determines the specificity of importin α for the NLS-containing cargo (7, 14).

The recently reported crystal structure of full-length mouse importin α in the absence of NLS substrate revealed the presence of a proposed internal NLS within the N-terminal IBB domain (15). This internal NLS may serve as an auto-inhibitory domain that regulates NLS binding. The structure of importin β bound to the IBB domain of importin α also indicates that the IBB domain changes conformation upon complex formation (16). Therefore, the interaction of importin α with importin β would be expected to displace the auto-inhibitory sequence of the IBB from the NLS-binding site and release the inhibition of the NLS binding to importin α. Consistent with the hypothesis that the IBB competes with the NLS for binding to importin α, a previous study demonstrated that overexpression of the IBB domain (α1–61) is sufficient to inhibit nuclear import of an NLS-containing substrate in vivo (17). Thus, the crystallographic data in combination with the in vivo experiment support a model where there is a regulatory switch between the cytoplasmic form of importin α, which has high affinity for NLS substrate and the nuclear form, which has a low affinity for the NLS.

Although NLS receptors from different species share structural and functional homology, the mechanism that regulates the recognition of an NLS by importin subunits and the importance of this regulation in the nuclear import process have not yet been investigated quantitatively. Since modulation of the affinity of importin α for the NLS-cargo may contribute to the regulation of NLS-dependent nuclear import, it is necessary to...
characterize the binding energies for each step involved in the NLS-importin α interaction. Therefore, we have used a method based on steady-state fluorescence depolarization to examine quantitatively the regulatory mechanism of NLS recognition by the NLS-binding protein, importin α.

The depolarization of fluorescence emission from a solution excited by a polarized light source yields a measure of the rotational diffusion of a fluorophore. This parameter, when expressed as fluorescence anisotropy, provides a useful method for monitoring protein-protein interactions (18). When small fluorophores are excited with polarized light, they tumble rapidly in solution relative to the fluorescence lifetime of the fluorophore. Thus, the light emitted from these fluorophores is significantly depolarized resulting in a low anisotropy value. If the fluorophore binds to a large macromolecule, the tumbling rate decreases. The reduction in the tumbling rate diminishes the depolarization of the emitted light yielding a relatively high anisotropy value. This relationship of size to anisotropy can provide information about the binding of a small rapidly tumbling fluorescent ligand to a large slowly rotating receptor.

We have applied this method to the study of protein-protein interactions by monitoring the change in fluorescence depolarization of the green fluorescent protein tagged with a nuclear localization signal (NLS-GFP) as the NLS substrate binds to the large import receptor, importin α. We first show the utility of this method by measuring the binding equilibrium between the NLS-GFP substrate and a fragment of importin α lacking the putative auto-inhibitory IBB domain (ΔIBB importin α). The binding of GFP to the importin α fragment is absolutely dependent on the fused NLS sequence. We then demonstrate that the relative affinity between an amino acid sequence and importin α measured in vitro correlates with the ability of that sequence to act as a nuclear localization signal in vivo. Next we provide experimental support for a mechanism of unidirectional transport where the affinity of importin α for an NLS cargo is modulated by interactions with importin β. The quantitative analysis of these interactions will provide a detailed thermodynamic model for the mechanism of protein import into the nucleus.

EXPERIMENTAL PROCEDURES

Expression and Protein Purification—The SV40 NLS-GFP was cloned into a pET-28a expression vector (Novagen) as an N-terminal His6 tag followed by the SV40 NLS sequence (SPKKKRKVEAS), a 10-residue linker, and finally GFP with a second C-terminal His6 tag. The SV40 NLS-GFP was overexpressed at 30 °C in the Escherichia coli strain BL21 (DE3). The E. coli cells were grown in LB medium containing 30 μg/ml kanamycin, and expression was induced, at an optical density of 0.6 (600 nm), by addition of 0.2 mM isopropyl-1-thio-β-D-galactopyranoside for 5 h. Bacterial cultures were harvested by centrifugation and resuspended in 20 mM Tris-HCl, pH 8.0, 100 mM NaCl, 1 mM EDTA, 2 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, and 2 μg/ml each of leupeptin, pepstatin A, aprotinin, and chymostatin. Cells were lysed using a French pressure cell, and cell debris was harvested by high speed centrifugation. Importin β was subsequently purified to homogeneity by size exclusion, hydrophobic interaction, and anion exchange chromatography (Amersham Pharmacia Biotech). The protein was then concentrated to 2 mg/ml in 50 mM Tris-HCl, pH 7.8, buffer containing 10% glycerol and stored at –80 °C.

Fluorescence Depolarization Assays—Fluorescence anisotropy measurements were carried out using an ISS PC1 fluorimeter fitted with polarization filters. The SV40 NLS-GFP was diluted into the desired concentration using PBS 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 importin α, or ΔIBB importin α were successively added to the assay volume. The sample was excited with a polarized beam with a wavelength of 492 nm. The emitted light was collected using a high-pass filter with a cut-off wavelength of 510 nm.

The binding assay is based on the change in the depolarization of fluorescent emissions from an NLS-GFP-containing protein upon binding to import receptors. The rapid rotational motion of the free small NLS-GFP protein in solution, relative to its fluorescence lifetime, results in the depolarization of emitted fluorescent light. The binding of importin α to NLS-GFP causes changes in the depolarization of the fluorescent light because the formation of a larger protein complex reduces the rotational motion of the GFP fluorophore. Thus, the binding of importin α to the NLS-GFP is monitored by measuring changes in the fluorescence anisotropy. The anisotropy (A) is defined as the difference between the parallel and perpendicular emitted light intensity (I∥ and I⊥) with respect to the total intensity when parallel polarized excitation is used (see Equation 1).

\[
A = \frac{I_{\parallel} - I_{\perp}}{I_{\parallel} + 2I_{\perp}} \quad (Eq. 1)
\]

The fluorescence anisotropy is related to the correlation time (τc) of the fluorophore through the Perrin Equation (2), (20)

\[
\frac{A_0}{A} - 1 = \frac{\tau}{\tau_c} \quad (Eq. 2)
\]

with A0 being the limiting anisotropy of the fluorophore, a known constant, and τc the fluorophore lifetime. Therefore, the fluorescence anisotropy value as a function of the concentration of a macromolecule provides a measure of the extent of its binding to the fluorophore.

Solid Phase Binding Assay—Either importin β or myoglobin was covalently coupled to epoxy-activated Sepharose (Amersham Pharmacia Biotech) at a concentration of 1 mg/ml gel. Approximately 0.2 mg of purified full-length importin α and ΔIBB importin α were incubated with 0.5 ml of importin β or myoglobin-coupled gel in binding buffer (50 mM Tris-HCl, pH 7.8, 100 mM NaCl, 20 mM dithiothreitol) for 3 h at 4 °C. The resins were then washed five times with 1 ml of binding buffer, and fractions were eluted with 50 mM Tris-HCl, pH 7.8, 1 M NaCl. Bound and unbound fractions were analyzed on a 10% SDS gel and visualized by Coomassie Brilliant Blue staining.

RESULTS

A Fluorescence Anisotropy-based Assay to Study Protein-Protein Interactions—We used fluorescence depolarization to develop an in vitro assay to measure the binding affinity of the protein import receptor complex for an NLS peptide. Initial experiments were designed to determine the utility of our proposed assay in providing a quantitative measurement of the affinity between importin α and the NLS sequence. The in vitro assay was performed using the SV40 NLS sequence fused to the green fluorescent protein (GFP) at the DNA level. A fragment of importin α spanning residues 88–530 was assayed for its affinity for the NLS-GFP fusion. This fragment, denoted ΔIBB importin α, contains the NLS binding ARM domain yet lacks the N-terminal putative auto-inhibitory importin β-binding (IBB) domain. The atomic structure of ΔIBB importin α...
bound to an SV40 NLS peptide, determined through crystallographic analysis, allows a direct comparison of quantitative measurements of affinity with the structure of the complex (8).

We determined the binding affinity between ΔIBB importin α and the SV40 NLS-GFP by measuring changes in fluorescence depolarization of the GFP fluorophore as a function of the concentration of the ΔIBB importin α fragment (Fig. 1). The depolarization of GFP fluorescence, plotted as fluorescence anisotropy, sharply increases from a value of ~0.31 for the NLS-GFP alone to a value of ~0.335 upon its binding to the ΔIBB importin α fragment. The resulting data were fit to a simple equilibrium equation yielding a binding constant \( K_D \) of 10 nM. The specificity of binding is indicated by the fact that GFP without the SV40 NLS shows no detectable binding to the importin α fragment (Fig. 1). These results demonstrate that the fluorescence anisotropy-based assay allows a quantitative characterization of the interactions involved in the NLS-importin α binding.

**Correlation between in Vivo Function and in Vitro Binding Affinity**—To test the specificity of the observed importin α-SV40 NLS-GFP interaction we compared the binding affinities between ΔIBB importin α and variants of the SV40 NLS. The NLS variants described in Table I were constructed as fusions to GFP. The SV40M mutant contains a threonine (Thr) instead of a lysine (Lys), which is known to abolish the nuclear import of the NLS-cargo in vivo (2). In the SV40A variant an arginine (Arg) was substituted with an alanine (Ala) to generate a milder variation of the wild type sequence. The anisotropy profiles observed with the SV40M and SV40A probes clearly showed that the binding of the NLS variants to importin α was severely reduced (Fig. 2). In comparison to the wild type SV40 NLS, the SV40A mutant showed a weaker interaction with the importin α fragment with a \( K_D \) of 37 nM, whereas the SV40M mutant showed an even greater decrease in the binding affinity for the importin α fragment. Although the binding was not saturated due to the limited solubility of the importin α fragment, we determined the binding constant of the SV40M mutant assuming that there should be an anisotropy change similar to that observed for the wild type SV40. Based on this assumption, we calculated the binding constant of the SV40M mutant to be approximately 3 \( \mu \)M. These results establish a quantitative correlation between the strength of an NLS-importin α interaction and the ability of the NLS to function in vivo.

**The IBB Domain Affects the Binding Affinity of Importin α**

For the NLS-cargo—Crystallographic evidence suggests that the IBB domain of importin α functions as an auto-inhibitory domain, modulating the affinity of importin α for the NLS-containing cargo through intramolecular competitive inhibition (15). Further structural evidence intimates that the binding of importin β to importin α displaces the auto-inhibitory IBB domain from the NLS-binding site (16). To examine these hypotheses, we compared the binding of SV40 NLS-GFP to purified full-length importin α versus the ΔIBB importin α fragment. The binding profile showed that the full-length importin α had no measurable binding to the NLS-GFP as compared with that of the ΔIBB importin α fragment (Fig. 3A). These observations are consistent with the hypothesis that the IBB domain modulates NLS binding to importin α.

To test further whether the IBB domain and an NLS peptide compete for binding to the ARM domain of importin α, we examined binding of an IBB-GFP fusion protein to the ΔIBB importin α fragment. As shown in Fig. 3B, the IBB-GFP bound to the ΔIBB importin α fragment in trans. Furthermore, this binding was competed by an NLS peptide but not by an unrelated control peptide (Fig. 3B). These data are consistent with a model for direct competitive inhibition between the IBB domain and an NLS peptide.

**Importin β Affects the Binding Affinity of Importin α for the NLS-cargo**—The crystal structure of an IBB domain-importin β complex suggests that the interaction of importin α with importin β may displace the auto-inhibitory sequences of the IBB from the NLS-binding site and release the inhibition of NLS binding to importin α (16). To ensure that the bacterially expressed full-length importin α can bind to importin β via the IBB domain, we examined the binding of purified yeast importin β to purified yeast full-length importin α or ΔIBB importin α using a solid phase binding assay. An importin β affinity gel was incubated with full-length importin α or ΔIBB importin α. The bound fractions were eluted from gel after thorough washing and were analyzed by SDSPolyacrylamide gel electrophoresis followed by Coomassie staining. As seen in Fig. 4, full-length importin α binds efficiently to importin β, but dele-
After extensive washing, bound proteins were eluted with 1M NaCl. No binding of either full-length or SDS-polyacrylamide gel electrophoresis and Coomassie Blue staining. Myoglobin (lanes 5–8; 0.5 mg) was observed. DsGFP as compared with that of the IBB importin α. As a control for specific binding, neither full-length nor lane 4 VADELNKMLL) with unrelated amino acid sequence. No competition was observed upon addition of a control peptide (CINE-

The IBB domain modulates NLS binding to importin α. A, fluorescence anisotropy measurements of full-length importin α (●) and ΔIBB importin α binding to the SV40 NLS-GFP ( ●) were performed as described under “Experimental Procedures.” Curves show that full-length importin α has no measurable binding to the NLS-SV40 GFP as compared with that of the ΔIBB importin α. B, competition assays were performed using an IBB-GFP fusion protein and NLS peptide. IBB-GFP (30 nm) was incubated with increasing concentrations of ΔIBB importin α. Before the binding was saturated (see arrow), increasing amounts of peptide were sequentially added. The binding profile indicates that the SV40 NLS peptide (SPKKHKKVEAS) efficiently competes with IBB-GFP for binding to the ΔIBB importin α (○). No competition was observed upon addition of a control peptide (CINE-VADELNKMLL) with unrelated amino acid sequence ( ●).

Analysis of NLS-Importin α Interaction

Importin β regulates the affinity of importin α for the SV40 NLS-GFP. A, addition of a stoichiometric amount of importin β (15 μM) to the in vitro assay increases the affinity of full-length importin α for the SV40 NLS-GFP (Kd ~33 nM, ○) as compared with full-length importin α alone (●). Results shown are from two independent experiments. The mean anisotropy values and standard deviations are indicated. B, addition of a stoichiometric amount of importin β (15 μM) to the in vitro assay does not change the affinity of ΔIBB importin α for the SV40 NLS-GFP (○) as compared with the ΔIBB importin α alone ( ●). C, SV40 NLS-GFP (30 nm) shows no detectable binding to importin β in the absence of full-length importin α (○). GFP lacking an NLS (30 nm) also shows no measurable binding to full-length-importin α in the presence of 15 μM importin β (●).

Importin β (15 μM) was incubated with increasing concentrations of ΔIBB importin α, fluorescence anisotropy measurements of full-length importin α (●) and ΔIBB importin α binding to the SV40 NLS-GFP ( ●) were performed as described under “Experimental Procedures.” Curves show that full-length importin α has no measurable binding to the NLS-SV40 GFP as compared with that of the ΔIBB importin α. As a control for specific binding, neither full-length nor lane 4 VADELNKMLL) with unrelated amino acid sequence. No competition was observed upon addition of a control peptide (CINE-

As a direct test of our hypothesis that the IBB domain competes with NLS substrate for binding to the ARM domain of importin α in a manner that is regulated by importin β binding, we examined the binding of full-length importin α to the SV40 NLS-GFP when a stoichiometric amount of importin β was added to the assay. The binding profiles show that the presence of importin β increases the affinity of full-length importin α for the NLS-GFP (Kd ~33 nM) when compared with the binding of full-length importin α alone (Kd >10 μM) (Fig. 5A). In contrast, the addition of a stoichiometric amount of importin β did not change the affinity of ΔIBB importin α for the NLS-GFP (Fig. 5B). In control experiments, GFP without the SV40 NLS showed no detectable binding to the importin α-β complex and the SV40 NLS-GFP had no measurable binding to importin β alone (Fig. 5C). These data, along with the solid phase binding assay results, suggest that importin β binds to the IBB domain and thereupon releases the inhibition of NLS binding mediated by the IBB sequence.
This study presents quantitative in vitro measurements for the binding affinity of the import receptor complex to the monopartite SV40 NLS sequence. Although detailed structural and some functional analyses of the interactions between importin α, importin β, and an NLS peptide have already been reported (8, 15, 16), few quantitative studies of these interactions have been performed (21). Comprehensive understanding of the mechanisms of signal-dependent nuclear import requires rigorous quantitative analysis of the thermodynamic events, and this study can be seen as a step in this direction.

We developed an in vitro fluorescence anisotropy assay to generate a quantitative model for the unidirectional transport of proteins into the nucleus. Fluorescence anisotropy, as a solution-based methodology, has been shown to be useful in the study of protein-nucleic acid and protein-protein interactions (18). Furthermore, this method is performed at equilibrium and does not require separation of free and bound species (18, 22, 23). For this reason we used fluorescence anisotropy to examine quantitatively the binding specificity and affinities between an NLS ligand and the receptor importin α.

One important aspect of this study is that we are able to correlate an interaction energy measured in vitro (the binding of a nuclear localization signal to importin α) with the functional consequences of this interaction in vivo (transport of the NLS-cargo to the nucleus). By utilizing the NLS-binding domain of importin α (ΔIBB importin α), we measured the interaction energy between the import receptor and three variants of the SV40 nuclear localization signal fused to GFP. The binding constant for the native SV40 NLS, which can functionally act as a nuclear localization signal in vitro, was measured to be 10 nM. The SV40M variant of this NLS is not capable of functioning as a nuclear localization signal in vivo (2). The binding of this variant NLS to ΔIBB importin α was observable but with a dissociation constant 300-fold weaker than that of the functional NLS sequence. Thus, as one might expect, there is a functional correlation between the affinity of importin α for an NLS and the ability of that NLS to function in vivo. In future experiments, the combination of the in vitro assay along with in vivo functional assays can thus provide a model for the relative partitioning of an NLS-containing protein between the nucleus and the cytoplasm as a function of the interaction energy with importin α.

Productive nuclear import requires importin α to bind tightly to the NLS-cargo and to enter the nucleus while bound to importin β. The binding of importin β has been shown to increase the affinity of importin α for its cargo (6, 9, 11, 12). Two possible mechanisms can be considered to explain the effect of importin β on the binding between importin α and the NLS-cargo. First, importin β could stabilize the interaction between importin α and NLS-cargo through direct contacts with the cargo or with the ARM domain of importin α. Second, as proposed by Kobe (15), the importin β binding domain (IBB) of importin α could bind to the NLS-binding site of importin α, thereby obstructing the binding site for the NLS-containing cargo. To determine which of the two possibilities is true, we first compared the affinity of full-length importin α for NLS to that of ΔIBB importin α which lacks the putative inhibitory domain. Although the NLS-GFP fusion bound tightly to ΔIBB importin α, there was no observable binding of the NLS substrate to full-length importin α. If the proposed auto-inhibitory mechanism regulates the interaction between importin α and the NLS, we predict that the IBB domain would bind importin α in trans. Therefore, we examined the binding of an IBB-GFP to ΔIBB importin α. We found that the IBB domain can indeed bind to ΔIBB importin α in trans. Furthermore, this binding is specifically competed by an NLS peptide. All these data support the model that the IBB domain regulates the interaction between importin α and the NLS-containing cargo.

The most critical test of our hypothesis is that binding of importin β to importin α should displace the auto-inhibitory IBB domain from the NLS-binding site of importin α and increase the affinity of full-length importin α for NLS substrate. Thus, we measured the binding of the full-length importin α to SV40 NLS in the presence of importin β. We found that binding of full-length importin α-β complex to SV40 NLS was nearly as efficient as that of ΔIBB importin α. In addition, we excluded the possibility that importin β increases the affinity of full-length importin α for the NLS-GFP through direct contacts with the ARM domain by measuring the binding of ΔIBB importin α to NLS-GFP in the presence of importin β. We found that the addition of importin β did not increase the affinity of ΔIBB importin α for the NLS-GFP. Therefore, our in vitro results support the auto-inhibitory model that identifies importin β as a regulator of importin α affinity for the NLS-cargo through the interaction between importin β and the IBB domain of importin α.

Recently, an ELISA-based binding assay was used to examine the interactions of both yeast and mouse importin α with various NLS peptides (21). Although ELISAs can produce quantitative results, this assay is not performed under equilibrium conditions. Thus, this methodology is limited in the range of binding energies that can be accurately measured. Hu and Jana (21) observed tight binding between full-length importin α and the SV40 NLS. In addition, this tight interaction was not affected by the presence of importin β (21). We found that the interaction between full-length importin α and the SV40 NLS was absolutely dependent on importin β. This disparity could be due to the inaccuracies inherent in measuring equilibrium binding constants through solid phase ELISA. The dependence of the importin α-NLS interaction on importin β observed in the current report is consistent with structural analyses of both an importin β-importin α complex (15, 16) and an importin α-NLS peptide complex (8).

The present study uses a quantitative approach to examine interactions that are essential for the initial recognition of NLS substrate in the cytoplasm. In the future, this analysis could be extended by the addition of other components required to complete a full cycle of nuclear transport. For example, addition of Ran-GTP to the system would mimic the scenario that may occur in the nucleus where levels of Ran-GTP are elevated. A thorough examination of the changes in the importin-substrate complex that occur upon addition of Ran-GTP could yield a quantitative model for the release of the cargo into the nucleus. Conversely, export of substrates could be examined by measuring the assembly of export complexes in the presence of Ran-GTP. Thus, the experimental approach detailed in this study presents an opportunity for quantitative analysis of multiple steps that occur in the course of transporting a substrate from one side of the nuclear envelope to the other.

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Quantitative Analysis of Nuclear Localization Signal (NLS)-Importin α Interaction through Fluorescence Depolarization: EVIDENCE FOR AUTO-INHIBITORY REGULATION OF NLS BINDING

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