Dissection of the Karyopherin α Nuclear Localization Signal (NLS)-binding Groove

FUNCTIONAL REQUIREMENTS FOR NLS BINDING*-

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Classical protein import, mediated by the binding of a classical nuclear localization signal (NLS) to the NLS receptor, karyopherin/importin α, is the most well studied nuclear transport process. Classical NLSs are either monopartite sequences that contain a single cluster of basic amino acids (Lys/Arg) or bipartite sequences that contain two clusters of basic residues separated by an unconserved linker region. We have created mutations in conserved residues in each of the three NLS-binding sites/regions in Saccharomyces cerevisiae karyopherin α (SRP1). For each mutant we have analyzed binding to both a monopartite and a bipartite NLS cargo in vitro. We have also expressed each karyopherin α mutant in vivo as the only cellular copy of the NLS receptor and examined the impact on cell growth and import of both monopartite and bipartite NLS-containing cargoes. Our results reveal the functional significance of specific residues within karyopherin α for NLS cargo binding. A karyopherin α variant with a mutation in the major NLS-binding site exhibits decreased binding to both monopartite and bipartite NLS cargoes, and this protein is not functional in vivo. However, we also find that a karyopherin α variant with a mutation in the minor NLS-binding site, which shows decreased binding only to bipartite NLS-containing cargoes, is also not functional in vivo. This suggests that the cell is dependent on the function of at least one bipartite NLS cargo that is imported into the nucleus by karyopherin α. Our experiments also reveal functional importance for the linker-binding region. This study provides insight into how changes in binding to cellular NLS sequences could impact cellular function. In addition, this work has led to the creation of conditional alleles of karyopherin α with well characterized defects in NLS binding that will be useful for identifying and characterizing novel NLS cargoes.

In eukaryotic cells, cellular responses to extracellular signals often involve the transport of macromolecules between the nucleus and the cytoplasm. This transport occurs through nuclear pore complexes embedded within the nuclear envelope (1–5). The exchange of information entails both the import of signaling factors into the nucleus and the export of response molecules to the cytoplasm, both of which are highly regulated and selective processes (4). The transport process is mediated by soluble receptors termed karyopherins, also known as importins and exportins, which carry cargoes into and out of the nucleus (5–7). General transport via karyopherin receptors requires direct binding of cargo to the karyopherin receptor. In the case of classical nuclear protein import, a nuclear localization signal (NLS) within the cargo protein is recognized by an adaptor protein, karyopherin/importin α, which mediates binding to the transport receptor, karyopherin/importin β (8, 9).

Classical NLSs are categorized as either monopartite, containing a single cluster of basic amino acid residues (K/R), or bipartite, containing two clusters of basic amino acid residues separated by a linker of 10–12 unconserved amino acids. The prototypical monopartite NLS is the SV40 large T antigen NLS (PKKKRKV), and the prototypical bipartite NLS is that of nucleoplasmin (KRPAATKKAGQAKKKKL) (10–12). Several recent studies have examined the structural and energetic contributions of specific residues within the NLS to binding to karyopherin α (13–16). These studies provide insight into which residues within the NLS are critical for NLS function. For example, the most critical lysine (K) defined in the monopartite NLS consensus (K/K/R/X/K/K/R) where X is any amino acid is stabilized by three amino acid residues (Gly161, Thr166, and Asp203) in Saccharomyces cerevisiae karyopherin α (see Fig. 1A) (17).

Karyopherin α is composed of three domains, an N-terminal domain that mediates binding to karyopherin/importin β known as the importin β binding (IBB) domain, a large central NLS-binding domain that is composed of 10 armadillo (ARM) repeats, and a C-terminal domain that interacts with the nuclear export factor CAS/Cse1p (18–23). Recent studies have provided a great deal of information about the molecular interactions between karyopherin α and NLS cargoes (13, 14, 16, 17). The resolution of the crystal structure of truncated S. cerevisiae karyopherin α with an NLS peptide revealed how the flexible ARM repeats create a binding groove for binding of both monopartite and bipartite NLSs (17). Subsequent studies with the full-length mouse karyopherin α revealed that the structure and NLS binding mode are similar for vertebrate karyopherin α (14, 24).

The NLS-binding groove is lined by a regular array of con-
served asparagine and tryptophan residues that is proposed to spatially orient and accommodate the lysine and arginine residues in an NLS (see Fig. 1A) (17). In addition, the positively charged lysine and arginine residues of NLSs are stabilized by amino acids that line the binding pockets for each NLS residue (14, 17). The regular array of asparagine and tryptophan residues is interrupted by a conserved tyrosine and a conserved charged lysine and arginine residues of NLSs are stabilized by residues in an NLS (see Fig. 1A).

The structural studies predict that three regions of the karyopherin α central ARM domain are critical for the direct interaction with classical NLS cargoes (see Fig. 1A) (14, 17). The major NLS-binding site, composed of ARM repeats 2–4, interacts with both monopartite NLSs and with the larger cluster of basic residues within bipartite NLSs. The minor binding site, composed of ARM repeats 7 and 8, interacts with the second smaller cluster of basic residues in bipartite NLSs. Finally, the two NLS-binding sites are linked by a less defined region, the linker-binding region, that is predicted to interact with the peptide backbone of the linker within a bipartite NLS (14, 16, 17). More recent structural studies of mouse karyopherin α in complex with bipartite NLSs (of human retinoblastoma and *Xenopus* NIN2) suggest that the linker-binding region interacts with not only the backbone chain of the bipartite NLS linker but also with side chains of amino acid residues; however, the latter interactions appear to be specific to the individual NLS (16).

In the present study, we dissect the contributions of the different regions of the NLS-binding groove of karyopherin α to NLS binding using a combination of *in vitro* quantitative binding assays and *in vivo* functional tests in *S. cerevisiae*. For this study we have designed a series of NLS cargos that can be used to compare quantitative binding to karyopherin α in vitro and NLS cargo targeting to the nucleus *in vivo*. Both the *in vitro* and *in vivo* NLS cargoes consist of the NLS sequence fused N-terminal to GFP such that the NLS is in the same context in both cargos. This experimental design facilitates the direct comparison of the *in vitro* and *in vivo* data.

We have created mutations in each of the three NLS-binding sites/regions in *S. cerevisiae* karyopherin α (SRP1). For each mutant we have analyzed binding to both a monopartite and a bipartite NLS cargo *in vitro*. We have also expressed each karyopherin α mutant *in vivo* as the only copy of the NLS receptor in the cell and examined the impact on cell growth and import of both monopartite and bipartite NLS containing cargos. Our results reveal the importance of various residues within karyopherin α for binding to different NLS cargos. A variant of karyopherin α with a mutation in the major NLS-binding site exhibits decreased binding to both monopartite and bipartite NLS cargoes, and this protein is not functional *in vivo*. However, we also find that a karyopherin α variant with a mutation in the minor NLS-binding site, which shows decreased binding only to bipartite NLS-containing cargos, is also not functional *in vivo*. This suggests that the cell is dependent on the function of at least one bipartite NLS cargo that must be imported into the nucleus by karyopherin α. Our experiments also reveal functional importance for the linker-binding region. Finally, these studies have led to the creation of conditional alleles of karyopherin α with well characterized defects in NLS binding that will be useful for identifying and characterizing NLS cargos.

### Experimental Procedures

**Yeast Strains and Plasmids**—Yeast transformations and genetic manipulations were carried out by standard methods (25). Yeast strains and DNA plasmids used in this study are listed in Table I. Plasmids used for *in vitro* bacterial expression of truncated yeast karyopherin α, AIRB-Srp1p, were generated by subcloning AIRB-SRP1 variants (lacking residues 1–88) into the pProEX bacterial expression vector (17). Plasmids used for *in vitro* expression of NLS-GFP (in pET28a) were previously described (15, 26). Plasmids used for *in vivo* expression of Srp1p in yeast cells were created by subcloning PCR-amplified fragments, which contain the SRP1 coding region and its respective 5′ and 3′ genomic flanking regions, into the yeast vector pRS315 (57). Plasmids used for *in vivo* expression of NLS-GFP-GFP in yeast were made by subcloning PCR-amplified NLS-GFP fragments (from *in vitro* plasmids) in-frame using XbaI and Clal restriction enzyme sites, into pAC825, which contains an additional copy of the C-terminal GFP (28). In creating the *in vitro* NLS-GFP-GFP fusion protein, the His tag and 10 amino acids upstream of each NLS were removed; however, each NLS remains in the same context as the corresponding *in vitro* NLS-GFP fusion protein used for quantitative binding experiments.

**Generation of Mutations in Yeast Karyopherin α (SRP1)—** Mutations in the SRP1 gene were generated using PCR-directed mutagenesis by

### Table I: Yeast strains and plasmids

| Strains       | Plasmids                                              |
|---------------|-------------------------------------------------------|
| ACY192        | MATa leu2Δ1 trp1 ura3-52 (33)                         |
| ACY324        | MATa his3Δ200 leu2Δ1 lys2 ura3-52 ∆SRP1::His3::SRP1::URA3 (29) |
| pRS315        | LEU2 CEN AMPR (27)                                    |
| pAC493        | ΔIBB-SRP1 AMPR pProEX-HTB bacterial expression vector (17) |
| pAC825        | ΔIBB-Srp1 AMPR pProEX-HTB bacterial expression vector (17) |
| pAC876        | SRP1 URA3 CEN AMPR (29)                               |
| pAC1008       | srp1-E402R LEU2 CEN AMPR                             |
| pAC1009       | srp1-D203K LEU2 CEN AMPR                             |
| pAC1059       | pMET25-BPSV40T3 NLS-GFP-GFP URA3 CEN KAN             |
| pAC1065       | pMET25-SV40 NLS-GFP-GFP URA3 CEN KAN                 |
| pAC1104       | SRP1 LEU2 CEN AMPR                                    |
| pAC1105       | srp1-Y283A/R321A LEU2 CEN AMPR                        |
| pAC1107       | srp1-D203N LEU2 CEN AMPR                              |
| pAC1207       | SV40 NLS-GFP KANR pET28a bacterial expression vector (26) |
| pAC1296       | srp1-E402Q LEU2 CEN AMPR                              |
| pAC1416       | ∆IBB-srp1-E402R AMPR pProEX-HTB bacterial expression vector |
| pAC1417       | ∆IBB-srp1-D203K AMPR pProEX-HTB bacterial expression vector |
| pAC1418       | ∆IBB-srp1-E402Q AMPR pProEX-HTB bacterial expression vector |
| pAC1419       | ∆IBB-srp1-D203N AMPR pProEX-HTB bacterial expression vector |
| pAC1420       | ∆IBB-srp1-Y283A/R321A AMPR pProEX-HTB bacterial expression vector |
| pAC1421       | pMET25-BPSV40A4 NLS-GFP-GFP URA3 CEN AMPR            |
| pAC1481       | BPSV40T3 NLS-GFP KANR pET28a bacterial expression vector (15) |
| pAC1478       | BPSV40A4 NLS-GFP KANR pET28a bacterial expression vector (15) |
using mutation-containing primers for PCR amplification of the SRP1 gene. PCR fragments were subsequently subcloned into either the bacterial or yeast expression vectors (Table I). Each resulting plasmid was sequenced to verify the presence of the desired mutation and the absence of any other mutations.

**Protein Expression and Purification**—Purified proteins (ΔIBB-Srp1p and NLS-GFP) used in this study were expressed in *Escherichia coli* BL21 (DE3) cells and purified by nickel affinity chromatography as described previously (7, 15, 26). The monopartite NLS cargo used was QMGRGSEFESPPKKRKVE containing the monopartite SV40 NLS sequence and the two bipartite NLSs were KRTADGSFESEPRTKKRKVE (BPSV40T3) and KRTADGSFESEPRTKKRKVE (BPSV40A4), which are engineered bipartite variants of the SV40 NLS (15).

**In Vitro Karyopherin α (ΔIBB-Srp1p) Binding Assay**—To measure the binding of various NLS cargoes to the karyopherin α variants, we utilized a solution binding fluorescence depolarization assay in which all anisotropy measurements were taken using an ICS PC1 fluorimeter (15, 26). For all in vitro NLS binding experiments, we utilized yeast karyopherin α (Srp1p) that does not contain the karyopherin β-binding domain (ΔIBB-Srp1p), which binds NLSs with a similar affinity to full-length Srp1p in the presence of karyopherin β (26). The dissociation constants (Kd values) for the binding of monopartite and bipartite NLS-GFPs to ΔIBB-Srp1p were measured and calculated as previously described (15, 26). Briefly, for each binding assay, a constant amount of the NLS-GFP cargo (~20 nM) is titrated with increasing amounts of wild type or mutant ΔIBB-Srp1p protein. The change in anisotropy is then used to calculate the binding isotherm for each binding reaction and subsequently fitted by nonlinear regression to determine the dissociation constant (Kd) (15, 26). For some weak binding variants (I203K and E402R), binding constants could only be estimated because the binding curves did not show an obvious inflection point. These binding constants were estimated based on the assumption that the binding isotherm would saturate at approximately the same anisotropy value as measured for other tighter binding variants. Binding reactions were maintained at 25 °C for most experiments, but some were also carried out at 37 °C as indicated. All of the binding constants were measured at least two times for each karyopherin α protein variant.

For this study, we used three NLS-GFPs (SV40, BPSV40T3, and BPSV40A4), all of which bind with similar affinity to ΔIBB-Srp1p (see Fig. 1B) (15). We employed SV40 to examine monopartite NLS binding in all experiments. To assay bipartite NLS binding to the mutant Glu283 and Tyr320/Arg321 ΔIBB-Srp1p proteins, we utilized the engineered BPSV40T3 NLS, which contains the classical threonine mutation at the critical lysine within the monopartite SV40 sequence (10). Because residue Asp203 is predicted to stabilize this critical lysine, to assess the binding of the mutant Asp203 protein we used BPSV40A4 (alanine mutation at the second lysine residue within the NLS) instead of BPSV40T2 (15).

**In Vivo Functional Assay**—A plasmid shuffle assay was used to test the function of the various mutant Srp1 proteins. We used *S. cerevisiae* cells deleted for the endogenous wild type copy of SRP1 and maintained by a URA3 plasmid that contains an exogenous copy of SRP1 expressed from its own promoter (ACT324) (29). To create and test the viability of cells containing only the full-length mutant Srp1 protein, ΔSrp1 cells were transformed with a LEU2 plasmid containing the mutant srp1p. Immunoblot analysis of lysates of cells expressing each of the Srplp variants demonstrated that all are expressed at approximately the same level as wild type Srplp (data not shown). For growth analysis, cells were grown to saturation (~2 × 10^6 cells/ml), serially diluted, and spotted onto selective medium or 5-fluoroorotic acid (5-FOA) plates, which selects against the wild type SRP1-URA3 maintenance plasmid (30). Under these conditions, the cells on 5-FOA plates contain only the LEU2 test plasmid encoding the Srp1p variant.

**In Vivo Localization Assay**—The localization of NLS-GFP-GFP in yeast cells was examined by direct fluorescence microscopy. SRP1-, srp1p-ΔD63N-, srp1p-ΔE402Q-, and srp1p-T283AΔR321A-expressing cells were grown to saturation, and lysed cells were used for fluorescence microscopy. NLS-GFP-GFP fusion proteins expressed from the inducible methionine promoter, pMET25 (Table I) (28). The cells were cultured in methionine-deficient medium (4–6 h) to induce the expression of the NLS-GFP-GFP fusion protein prior to visualization. Immunoblots demonstrated that NLS-GFP-GFP cargo were expressed at approximately the same level in all of the cells examined (data not shown). For temperature-sensitive Srp1p yeast transformants, the cells were grown to log phase at the permissive temperature (25 °C) and then shifted to the nonpermissive temperature (37 °C) for 2–4 h before viewing the cells. DNA was stained with 4,6-diamidino-2-phenylindole (1 µg/ml) to confirm the position of the nucleus (data not shown). Direct fluorescence images were captured using an Olympus BX60 epifluorescence microscope equipped with a GFP-optimized filter and a Photometrics Quantix digital camera.

**RESULTS**

To assess the functional consequences of mutations within the NLS-binding groove, we generated several variants of *S. cerevisiae* karyopherin α (Srp1p) and characterized them through a combination of *in vitro* binding assays and *in vivo* functional assays. For our mutational analysis, we have focused on four conserved residues within karyopherin α that represent different binding sites/regions within the NLS-binding groove: Asp203 of the major NLS-binding site, Glu283 of the minor NLS-binding site, and Tyr320 and Arg321 of the bipartite NLS linker-binding region (Fig. 1A). To define the function of each binding site/region, we analyzed these Srp1p variants by measuring binding to a monopartite NLS (SV40) and a bipartite NLS (BPSV40T3 or BPSV40A4), all of which bind to wild type Srp1p with comparable Kd values (Fig. 1B and Table I).
Dissection of the Karyopherin α NLS-binding Groove

All in vitro NLS-GFP binding experiments were carried out using a truncated form of yeast karyopherin α, ΔIBB-Srp1p (15, 26). Monopartite (MP) and bipartite (BP) NLS-GFP cargo binding to purified Srp1p protein variants was measured using fluorescence anisotropy (see "Experimental Procedures"). The binding assays were carried out at 25 or 37 °C as indicated. The binding affinities are expressed as dissociation constants (Kd values), which are averaged values based on two or more independent binding experiments. The standard errors in the data are indicated.

| ΔIBB-Srp1p | NLS | MP/BP | Temperature °C | Kd (nM) |
|------------|-----|-------|----------------|--------|
| Wild type  | SV40 | MP    | 25             | 10 ± 4 |
|            | SV40 | MP    | 37             | 21 ± 7 |
|            | BPSV40T3 | BP  | 25             | 19 ± 5 |
|            | BPSV40T3 | BP  | 37             | 32 ± 7 |
|            | BPSV40A4 | BP  | 25             | 5 ± 4  |
| D203K      | SV40 | MP    | 25             | ≥3000  |
|            | BPSV40A4 | BP  | 25             | ≥1900  |
| D203N      | SV40 | MP    | 25             | 66 ± 8 |
|            | BPSV40A4 | BP  | 25             | 13 ± 4 |
| E402R      | SV40 | MP    | 25             | 50 ± 8 |
|            | BPSV40T3 | BP  | 25             | 335 ± 35 |
| E402Q      | SV40 | MP    | 25             | 22 ± 6 |
|            | SV40 | MP    | 37             | 31 ± 11 |
|            | BPSV40T3 | BP  | 25             | 33 ± 16 |
|            | BPSV40T3 | BP  | 37             | 165 ± 75 |
| Y283A/R321A | SV40 | MP    | 25             | 25 ± 7 |
|            | BPSV40T3 | BP  | 25             | 195 ± 78 |

In addition, as a functional in vivo test, we assessed the ability of the various srp1 mutants to replace the essential SRP1 gene (31) in S. cerevisiae. For srp1 mutants that could support cell growth, we then examined the steady state localization of both monopartite and bipartite NLS cargoes in cells expressing the mutant Srp1 protein as the only cellular NLS receptor.

Functional Analysis of the Major Binding Site—For our analyses of the major NLS-binding site, we focused on a residue, Asp203, which is one of the three residues that stabilize the critical lysine within an NLS (Fig. 1A) (17). We changed Asp203 to lysine (D203K) to reverse the negative charge to positive) or to asparagine (to remove the charge). To assess the effect that mutations at Asp203 have on monopartite and bipartite NLS binding, we analyzed the binding of Srp1-D203N to both monopartite (SV40-GFP) and bipartite (BPSV40A4-GFP) NLSs using fluorescence anisotropy. Typical curves for binding of SV40 NLS-GFP to wild type, D203K, and D203N karyopherin α are shown Fig. 2A. As described under "Experimental Procedures," these curves are used to calculate the binding constants (Kd values) for the interaction between NLS cargo and karyopherin α. The Kd values for wild type, D203K, and D203N are shown for both the monopartite and bipartite NLS cargoes (Fig. 2B and Table II). When Asp203 is mutated to lysine (D203K), binding to both monopartite and bipartite NLSs is greatly reduced as compared with wild type Srp1p. Binding is decreased ~300-fold for the monopartite NLS and ~400-fold for the bipartite NLS (Fig. 2B and Table II). When we examined in vitro NLS binding of the more conservative Srp1p-D203N variant, the effect is more subtle as indicated by a ~7-fold decrease in monopartite NLS binding and a ~2-fold decrease in bipartite NLS binding compared with wild type Srp1p (Fig. 2B and Table II).

Our in vitro binding data show that the reverse charge D203K mutation in the major NLS-binding site severely decreases binding to both monopartite and bipartite NLSs. To examine the consequences of these NLS binding defects in vivo, we expressed either the D203K or D203N mutant protein in S. cerevisiae as the only copy of Srp1p in the cell. Under
conditions where only the Srp1p-D203K mutant protein is expressed (5-FOA), the cells are not viable (Fig. 2C), suggesting that the D203K protein is not functional. As controls, the cells that contain wild type SRP1 (WT on 5-FOA) grow, whereas cells with no SRP1 (VEC on 5-FOA) show no growth. The D203N protein appears to be functional because cells expressing this Srp1p variant show growth that is indistinguishable from the wild type Srp1p control (Fig. 2C, compare D203N and WT on 5-FOA). Immunoblotting demonstrates that both Asp203Srp1p variants are expressed at approximately the same level as wild type Srp1p (data not shown). To further assess the function of the D203N mutant Srp1 protein, we examined protein import using a GFP reporter in cells expressing the D203N protein variant as the only copy of Srp1p. We analyzed the steady state localization of cargoes (NLS-GFP-GFP) that contain the same NLSs in the same spatial context as those analyzed in our in vitro assays. For D203N, we saw no change in the localization of either the monopartite (MP-GFP) or the bipartite (BP-GFP) NLS cargo (Fig. 2D) as compared with wild type cells. This assay could not be carried out for the D203K mutant because these cells are not viable.

Functional Analysis of the Minor Binding Site—To characterize the minor binding site of karyopherin α, we focused on residue Glu402 in Srp1p. This amino acid stabilizes the positive charge of one of the basic amino acids in the minor cluster of a bipartite NLS (Fig. 1A) (17). Similar to our approach for studying the major binding site, we either reversed the charge (negative to positive) of the residue by changing it to arginine or neutralized the charge by changing it to glutamine. Based on the co-crystal structure, changing Glu402 of the minor NLS-binding site is predicted to impact bipartite NLS but not monopartite NLS binding (Fig. 1A) (14, 17). We used fluorescence anisotropy to examine the binding of these minor binding site mutations to both monopartite and bipartite NLS cargoes. As indicated by the calculated dissociation constants (Table II), binding is decreased ~18-fold for bipartite NLS (BP) with the reversed charge Srp1p mutant protein, E402R (Fig. 3A and Table II). There is also a slight ~5-fold decrease in binding to monopartite (MP) NLS. Interestingly, growth analysis of S. cerevisiae cells that express only the E402R mutant of Srp1p reveals that the E402R mutant cannot function in vivo (Fig. 3B, compare WT and E402R on 5-FOA). We also analyzed the function of the neutral charge mutant, E402Q. We found no significant difference in NLS binding to the E402Q protein as compared with wild type Srp1p protein (Fig. 3A and Table II). However, when we performed our in vitro functional assay, we noticed that cells expressing the E402Q mutant protein as the only copy of Srp1p grow more slowly than cells expressing wild type Srp1p (Fig. 3B, compare WT and E402Q on 5-FOA). This slow growth defect is greatly enhanced when cells are incubated at a higher temperature (37 °C), indicating that the srp1-E402Q mutation within the minor NLS-binding site confers a temperature-sensitive growth phenotype.

The above in vivo data suggest that the function of the E402Q protein may be temperature dependent. Because our initial binding experiments, which showed no significant decrease in NLS binding, were carried out at 28 °C, we investigated the in vitro binding activity of the E402Q protein and a wild type control protein at higher temperature (37 °C) (Fig. 4A). No change in the binding of wild type karyopherin α to NLS was observed at 37 °C; however, our experiments demonstrate that the E402Q mutant protein binds more weakly to a bipartite NLS at 37 °C than at 25 °C. Importantly, there is no significant effect on monopartite (MP) NLS binding to Srp1p-E402Q at either temperature when compared with wild type (Fig. 4A and Table II). We took advantage of the conditional growth phenotype of E402Q cells to assess whether the meningitis in vitro binding analysis, the minor NLS-binding site analysis of Srp1p and mutant Srp1p-E402Q proteins (E402Q and E402R) is shown. The dissociation constants (Kd) are plotted on a logarithmic scale. Each value results from an average of at least two independent experiments, and the standard errors in the data are indicated. B, yeast cells with Srp1p deleted that are maintained by a wild type Srp1p gene on a URA3 plasmid were transformed with wild type, E402R, or E402Q Srp1. The cultures were grown to saturation, serially diluted, and spotted onto minimal (URA) or 5-FOA plates. On the URA plates the wild type Srp1 maintenance plasmid is present. On the 5-FOA plates, this Srp1 plasmid is lost, and cells express only the karyopherin α variant indicated on the left. Cells that express either wild type karyopherin α (WT) or a vector control (VEC) are shown as positive and negative controls, respectively. To assess the growth of cells expressing the different Srp1p mutants, the plates were incubated at 30 °C for 2–4 days or 37 °C for 1–2 days.

Functional Analysis of the Linker-binding Region—As described in the Introduction, the regular array of asparagine and tryptophan residues within the central ARM domain of karyopherin α is interrupted by two evolutionarily conserved residues, Tyr283 and Arg321 in S. cerevisiae karyopherin α, which are found between the major and minor NLS-binding sites (17). Here, we refer to the region containing these residues as the linker-binding region, in reference to their potential interaction with the linker sequence that connects the two basic clusters in a bipartite NLS (14, 17). To examine the contribution of this linker-binding region, we have mutated the two conserved residues to alanine (YR→AA). Results from our in vitro NLS fluorescence anisotropy assay indicate that the mutant Srp1p-AA protein has reduced (~10-fold) binding to bipartite (BP) NLS when compared with wild type Srp1p and only a slight decrease (~2.5-fold) in monopartite (MP) NLS binding (Fig. 5A and Table II). When we express the linker-binding mutant Srp1p-AA as the only copy of Srp1p in cells, they grow slowly (Fig. 5B, compare WT and AA on 5-FOA). When we examined the localization of NLS cargoes in cells expressing Srp1p-AA as the only copy of Srp1p, we observed a more cyto-
plasmic localization of bipartite NLS-GFP cargo than observed for cells expressing wild type Srp1p (Fig. 5C, BP-GFP). As predicted from the in vitro binding data, there was no apparent difference in monopartite NLS-GFP localization in cells expressing Srp1- AA protein as compared with wild type cells (Fig. 5C).

**DISCUSSION**

Studies of the co-crystal structures of NLS peptides with yeast and mouse karyopherin α suggest three distinct functional regions within the NLS-binding groove: the major NLS-binding site, the minor NLS-binding site, and the (bipartite) NLS linker-binding region (14, 16, 17, 24). On the basis of these structural studies, we generated and examined three Srp1p variants to dissect the functional contribution of each individual proposed region to NLS binding. Consistent with the co-crystal structural information, our mutational analyses of the stabilizing residue Asp\(^{203}\) (D203K) confirm the function of the major NLS-binding site in mediating both monopartite and bipartite NLS binding to karyopherin α (Fig. 2B) (14, 17). Similarly, our analyses of the residues Glu\(^{402}\) and Tyr\(^{203}/\)Arg\(^{211}\) support the predicted functions of the minor NLS-binding site and the NLS linker-binding region in bipartite NLS binding to karyopherin α (Figs. 3A and 5A).

Taking advantage of molecular genetics in *S. cerevisiae*, we were able to examine the in vivo consequences of impaired NLS binding by the various karyopherin α mutants. To correlate defects observed in vitro with in vivo phenotypes, we used the same monopartite and bipartite NLS cargos for our binding assays and our steady state NLS cargo localization experiments. This approach allows for direct comparison of the binding constants measured in vitro and the capacity for nuclear targeting in vivo.

**Fig. 4. In vitro and in vivo temperature-sensitive phenotypes of *srp1-402Q*. A, in vitro binding affinities (K\(_d\)) of monopartite (○) and bipartite (gray box) NLS-GFP binding to wild type (WT) Srp1p and mutant Srp1p-E402Q protein were determined from binding assays carried out at both 25 and 37 °C. The dissociation constants (K\(_d\) values) are plotted on a logarithmic scale. Each value results from an average of at least two independent experiments, and the standard errors in the data are indicated. B, to determine the in vivo localization of monopartite and bipartite NLS cargoes, plasmids encoding monopartite or bipartite NLS-GFP-GFP were transformed into cells expressing either wild type or E402Q Srp1p. The intracellular localization of the NLS cargoes was visualized by direct fluorescence microscopy. MP-GFP and BP-GFP denote monopartite SV40- and bipartite BPSV40T3- NLS-GFP-GFP cargoes, respectively.

**Fig. 5. In vitro and in vivo analyses of mutations within the NLS linker-binding region of Srp1p. A, in vitro binding of monopartite (○) and bipartite (gray box) NLS-GFP binding to wild type (WT) Srp1p and mutant Srp1p-AA protein (Y283A/R321A) was measured using fluorescence anisotropy, and the binding affinities were calculated. The dissociation constants (K\(_d\) values) are plotted on a logarithmic scale. Each value results from an average of at least two independent experiments, and the standard errors in the data are indicated. B and C are functional analyses of the Srp1p-AA mutant in *S. cerevisiae*. B, yeast cells with Srp1p deleted and maintained by a wild type Srp1p gene on a URA3 plasmid were transformed with either wild type Srp1p or srp1- AA. The cultures were grown to saturation, serially diluted, and spotted onto minimal (URA) or 5-FOA plates. On the URA plates the wild type Srp1 maintenance plasmid is present. On the 5-FOA plates, this Srp1 plasmid is lost, and cells express only the karyopherin α variant indicated on the left. Cells that express either wild type karyopherin α (WT) or a vector control (VEC) are shown as positive and negative controls, respectively. To assess the growth of cells expressing the srp1- AA mutant, the plates were incubated at 30 °C for 2–4 days. C, in vivo localization of monopartite and bipartite NLS cargoes were carried out by expressing plasmids containing monopartite or bipartite NLS-GFP-GFP in either wild type or srp1- AA mutant cells. The intracellular localization of NLS cargos was visualized by direct fluorescence microscopy. MP-GFP and BP-GFP denote monopartite SV40- and bipartite BPSV40T3-NLS-GFP-GFP cargoes, respectively.

Mutations that reversed the charge of residues Asp\(^{203}\) and Glu\(^{402}\) both created karyopherin α proteins that did not function in vivo; however, conservative mutations at these sites were far less detrimental. We hypothesize that the substitution of the positively charged residue for the normally negatively charged amino acid repels the positive basic residues of the NLSs and in turn disrupts the interactions with the binding sites. Asp\(^{203}\) is part of the pocket that stabilizes and accommodates the critical lysine within the major basic amino acid cluster that contributes the most energy to NLS binding to karyopherin α (15, 17). Glu\(^{402}\) stabilizes the arginine residue that contributes the most binding energy to the minor binding site for bipartite NLSs (15, 16). Hence, our data are consistent with the prediction that repelling either of the two critical NLS residues from the respective binding pockets significantly disrupts the NLS-karyopherin α interaction. Previous work showed that simultaneously reversing the charge at both of
these residues (Asp$^{203}$ and Glu$^{402}$ in S. cerevisiae and Asp$^{189}$ and Glu$^{286}$ in Xenopus) alleviated binding to an NLS cargo (29, 32). However, this is the first time that the individual contribution of each residue and hence each binding site has been examined.

The more conservative major binding site mutant, D203N, which has little or no impact on NLS binding, has no apparent effect on cell viability or in vitro NLS cargo import or interaction (Fig. 2, C and D). Although this negatively charged residue (Asp$^{203}$) normally stabilizes the essential lysine within an NLS (15, 17), the lack of any profound defect observed for the D203N mutation may be attributed to the contribution of the two additional residues, glycine 161 and threonine 166, which are also predicted to interact with and stabilize this critical lysine.

Interestingly, our in vivo analyses of the conservative E402Q mutant of the minor NLS-binding site identified a conditional allele of yeast karyopherin α. Furthermore, this in vivo temperature-sensitive growth phenotype is mimicked in vitro where binding of an NLS cargo to Srp1p-E402Q is also temperature-sensitive. This correlation between the in vivo and in vitro phenotypes strongly suggests that the growth defect observed in vivo is a direct result of decreased binding affinity of this Srp1p variant for bipartite NLS containing cargoes in the cell.

Our mutational analysis of the linker-binding region indicates the importance of this region in interacting with bipartite NLS sequences. Even though both of the NLS-binding sites are intact in the Srp1p-AAA (Tyr$^{283}$/Arg$^{321}$) mutant, we still see decreased binding to a bipartite NLS cargo in vitro. In addition, cells that express this mutant form of karyopherin α as the only copy in the cell have a slow growth phenotype. The co-crystal structure of nucleoplasmic bipartite NLS and mouse karyopherin α revealed that residues Tyr$^{277}$ and Arg$^{215}$ (mouse counterparts of S. cerevisiae Tyr$^{283}$ and Arg$^{221}$ residues) are involved in interactions with the peptide backbone of the linker sequence within a bipartite NLS (14). However, more recent analysis of the co-crystal structure of mouse karyopherin α bound to either the human retinoblastoma bipartite NLS or the Xenopus NIN2 bipartite NLS showed that these evolutionarily conserved residues also interact extensively with side chains within the linker region of these particular bipartite NLSs (16). Moreover, their interaction with the linker peptide is specific to the particular bipartite NLS sequence (16). This structural finding is consistent with our observation that these two conserved residues in the linker-binding region are important for the interaction of a bipartite NLS with karyopherin α. One possibility is that these interactions may be fine-tuned for the particular bipartite NLS sequence within a bipartite NLS (14). However, mutations in the minor binding site (E402R and E402Q) and linker-binding region (AA) decrease binding to bipartite NLS cargo without profoundly impacting binding to monopartite NLS cargo. The E402R variant, which shows a severe but specific decrease in binding to bipartite NLS cargo, is not functional in vivo. Interestingly, a triple mutant that combines the conservative major pocket mutant, D203N, with the linker-binding region mutations (Y283A/R321A) also yields karyopherin α nonfunctional in vivo (data not shown), presumably because of weakened interactions of the bipartite NLS with the major binding site and the linker-binding region. Altogether, the in vivo phenotype of karyopherin α mutants that are defective in bipartite NLS binding in vitro suggests that for cells to function properly, they require the import of at least one essential bipartite NLS cargo.

As a result of mutational analyses, we have generated a panel of mutant alleles of karyopherin α that will serve as useful tools for both identifying and characterizing potential NLS cargoes. We have identified two conditional alleles of karyopherin α, srp1-E402Q and srp1-Y283A/R321A, that could be used for identifying essential bipartite cargoes in yeast. In addition, the mutants of the different binding sites/regions may be useful in isolating monopartite or bipartite NLS cargoes via biochemical means. Furthermore, the combination of our in vivo and in vitro assays will be useful in characterizing the import mechanism of novel NLS cargoes.

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