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Kap121p-Mediated Nuclear Import Is Required for Mating and Cellular Differentiation in Yeast

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To further our understanding of how the nucleocytoplasmic transport machinery interfaces with its cargoes and how this affects cellular physiology, we investigated the molecular mechanisms of phenotypes associated with mutations in karyopherin Kap121p. Two previously unreported phenotypes of *kap121* cells were observed: defects in mating and in the transition from the normal yeast form to the pseudohyphal, invasive form. In parallel, we searched for Kap121p cargoes by using Kap121p as a probe in overlay assays of yeast nuclear proteins. One of the major interacting proteins identified by this procedure was Ste12p, a transcription factor central to both the mating response and the pseudohyphal transition. We therefore investigated whether defects in these differentiation processes were due to an inability to import Ste12p. Both immunopurification and in vitro binding studies demonstrated that Ste12p interacted specifically with Kap121p in a Ran-GTP-sensitive manner and that Ste12p was mislocalized to the cytoplasm by inactivation of Kap121p in a temperature-sensitive mutant. The Kap121p-specific nuclear localization signal (NLS) of Ste12p was determined to reside within a C-terminal region of Ste12p. Furthermore, by overexpression of *STE12* or expression of a *STE12*-cNLS fusion in *kap121* cells, the invasive-growth defect and the mating defect were both suppressed. Together these data demonstrate that Ste12p is imported into nuclei by Kap121p and that mating and differentiation defects associated with *kap121* mutants are primarily attributable to the mislocalization of Ste12p.

When cells respond to extracellular cues, the signals received at the plasma membrane are often transduced to the nucleus, where gene expression is altered. The transduction of these signals can be accomplished by changing the phosphorylation state and/or the localization of transcription factors or accessory proteins, which in turn coordinately activate the transcription of a set of specific genes (27, 30). The nuclear envelope functions as a barrier to this signaling by physically separating the nuclear DNA from the cytoplasm. The movement of macromolecules between the nucleus and cytoplasm occurs solely through circular pores in the nuclear envelope joining these two compartments (reviewed in references 54 and 67). Large octagonally symmetric protein complexes termed nuclear pore complexes (NPCs) reside within these pores and govern the bidirectional movement of macromolecules between the two compartments. In yeast, NPCs comprise ~30 proteins, or nucleoporins, which are present in multiple copies to yield an ~50-MDa complex. The numbers of nucleoporins in humans and other vertebrate species are not known, but in these cells the NPCs appear more elaborate and have estimated masses of over 100 MDa. The structural organization of NPCs, and indeed many nucleoporins, are highly conserved from yeast to mammals (54, 67). In all species examined, nucleoporins can be divided into two broad categories: those that contain degenerate repeats of dipeptide FG and those that do not.

These FG repeat-containing nucleoporins have been shown to interact with a class of soluble factors termed karyopherins or kaps. Proteins and other macromolecules are directed across the nuclear envelope by the presence of specific nuclear localization signals (NLSs) (11) or nuclear export signals (NESs) (16, 19); karyopherins (kaps) bind to the NLSs and NESs of proteins and RNAs in the cytoplasm or nucleoplasm and mediate their translocation across the NPC. Karyopherins fall into two structurally related families: the β-karyopherin and the α-karyopherin protein families (reviewed in references 21, 41, 47, and 69). There are 14 β-kaps and only one α-kap represented in yeast, but in higher eukaryotes the families appear to be larger. To date, the general rule for kap cargo function suggests that β-kaps interact directly with their cognate cargoes and escort them across the NPC. The exception to this comes from the first import pathway characterized. In this case, classical NLS (cNLS)-containing substrates are recognized by kap α (Kap60p/Srp1p in yeast), which in turn is bound to kap β1 (Kap95p in yeast), and this complex mediates nuclear import. Thus it appears that kap α functions as an adapter for kap β1 (15, 20, 47, 49). In yeast, the β-karyopherin family includes 11 kaps that function as import receptors, 3 export kaps, and 1 kap that functions as both an import and export receptor (70). Surprisingly, only four of these karyopherins are essential, despite the fact that nonessential karyopherins transport cargoes essential for cell viability, suggesting

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considerable cross talk between the various transport pathways.

The soluble factor Ran is a small GTPase, believed to be a key regulator that provides directionality to nucleocytoplasmic transport, in part by regulating the formation of kap/cargo complexes (reviewed in references 6, 21, 42, 54, and 67). Two forms of Ran exist in the cell, a GTP-bound form and a GDP-bound form. The cytoplasmic localization of the Ran GTPase-activating protein (RanGAP) and the nuclear localization of the guanine nucleotide exchange factor (RanGEF) are believed to create a Ran-GDP/Ran-GTP gradient across the nuclear envelope, which appears to control the association and dissociation of kap/cargo complexes. For example, import kap/cargo complexes form in the cytoplasm. Once they traverse the NPC and encounter the high concentration of Ran-GTP in the nuclear environment, Ran-GTP binds the kap, stimulating complex dissociation and cargo release (51). In contrast, export complexes comprising the kap, its cargo, and Ran-GTP are formed cooperatively in the nucleus (18, 33), and these complexes are disrupted once they reach the cytoplasm, where RanGAP induces GTP hydrolysis (17, 31, 33). Other soluble factors such as Yrb1p (58), Yrb2p (64), and Nup2p (9, 10) appear to act as coactivators of the Ran cycle and aid in controlling the interaction of Ran with karyopherins and the NPC.

Ste12p is a transcription factor that plays a central role in a signaling pathway from the plasma membrane to the nucleus, which promotes yeast either to mate or to become filamentous, change their budding pattern, and invade solid substrates such as agar. Although the outcome depends on the cell type and the stimuli they receive, in either case, some of the same signaling elements are involved (Ste20p, Ste11p, and Ste7p), and both signal transduction pathways terminate in the phosphorylation and activation of Ste12p (13, 14, 22, 35, 46, 52). In the mating response, Ste12p forms a homomultimer, which binds to pheromone response elements (PREs) found upstream of genes required during mating (23, 26), inducing their transcription and triggering a number of cellular responses, which include polarized growth (or shmooing), cell cycle arrest in G_1, and increased expression of proteins required for cell and nuclear fusion. In contrast, in the filamentous or pseudohyphal and invasive-growth transitions, activated Ste12p forms a heterodimer with a second transcription factor, Tec1p (39, 44). This protein complex then interacts with promoters containing PREs in close proximity to Tec1p binding sites (collectively termed filamentation/invasion response elements), and this induces the transcription of genes required for filamentous and invasive growth.

In this study we identify Ste12p as a nuclear import cargo for Kap121p. Kap121p is an essential member of the yeast β-karyopherin family. We show that *KAP121* mutants are defective for mating responses and cellular differentiation. Furthermore, we use new temperature-sensitive alleles of *kap121*, as well as overlay assays, in vitro binding assays, and in vivo fluorescence localization studies, to characterize the role of Kap121p in Ste12p import into the nucleus.

MATERIALS AND METHODS

Plasmids and strains. Yeast strains were derived from DF5 (*mat***a**/*matα ura3-52*/*ura3-52 his3Δ200*/*his3Δ200 trp1-1*/*trp1-1 leu2-3,112*/*leu2-3,112 lys2-801*/*lys2-801*). *KAP121* strains are derivatives of DF5 and were produced as follows. The entire *KAP121* open reading frame (ORF) was replaced on one chromosome in DF5 diploid cells by direct integration of a PCR product containing the *URA3* gene. Correct integration was confirmed by PCR (2). For selection purposes, *URA3* was then replaced with *HIS3* (7). The *KAP121* gene including 1,000 nucleotides upstream and downstream of the ORF was cloned into pRS316 and pRS314 to yield *KAP121-URA3* and *KAP121-TRP1*, respectively. p*KAP121-URA3* was transformed into diploid DF5 cells carrying one disrupted copy of *KAP121* (*kap121*::*ura3*::*HIS3*). The diploids were sporulated, tetrads were dissected, and *kap121*::*ura3*::*HIS3*/p*KAP121-URA3* haploids were selected and termed *kap121*Δ/p*KAP121-URA3*.

The *STE12* ORF was amplified by PCR from yeast genomic DNA and cloned into the *Eco*RI and *Hin*dIII sites of pYX242 (53), and the resulting recombinant plasmid was termed pSte12-GFP. *STE12* gene fragments (nucleotides 1 to 759, 760 to 1485, and 1486 to 2064) were produced by PCR using oligonucleotides containing either a single *Eco*RI restriction site or both *Eco*RI and *Hin*dIII restriction sites at their 5′ ends. These PCR fragments were cloned into the *Eco*RI site of pGEX-4T-3 (Amersham Pharmacia Biotech), and the resulting recombinants were termed pGST-Ste12p (aa1-252), pGST-Ste12p (aa253-493), and pGST-Ste12p (aa494-688), respectively. The same PCR fragments of the *STE12* gene were also cloned into the *Eco*RI and *Hin*dIII sites of p12-GFP2-NLS (pKW431; a gift from K. Weis, University of California, San Francisco) (62). In each case, this resulted in the removal of the cNLS encoded by the p12-GFP-NLS plasmid, replacing it with the *STE12* coding sequence in frame with a mutant (p12) NES followed by two green fluorescent protein (GFP) coding sequences. The resulting recombinant plasmids were termed pSte12p (aa1-252)-GFP, pSte12p (aa253-493)-GFP, and pSte12p (aa494-688)-GFP. Multicopy plasmid pRS426-*STE12* was constructed by PCR amplification of the *STE12* gene, including 500 nucleotides upstream and downstream of the ORF, from yeast genomic DNA and ligation of the fragment into the *Hin*dIII and *Xho*I sites of pRS426. The *STE12*-cNLS fusion was produced by PCR amplification of the *STE12* gene, including 500 nucleotides upstream of the ORF, and an in frame fusion on the simian virus 40 (SV40) large T antigen NLS coding sequence (29) at its 3′ end. This PCR fragment was cloned into the *Hin*dIII and *Eco*RI sites of pRS316.

Construction of the temperature-sensitive alleles. *kap121* mutants were made by the gapped plasmid repair method (45). Briefly, the *KAP121* gene was amplified under mutagenic-PCR conditions (100 μM dGTP, 100 μM dCTP, 100 μM dTTP, 5 μM dATP) and cotransformed in a 10:1 ratio with the p*KAP121-TRP1* plasmid (which had been digested with *Bal*I and *Nde*I and gel purified to remove nucleotides 1 to 2392 of the *KAP121* ORF) into *kap121*Δ/p*KAP121-URA3* cells. The transformants were plated on SC-Ura-Trp (synthetic complete medium minus uracil and tryptophan) and incubated at 23°C. Colonies were replica plated to 5-fluoroorotic acid-containing medium (3) and incubated at 23 and 37°C to select cells lacking p*KAP121-URA3*. Mutants that grew at 23°C but not at 37°C were characterized further. Temperature-sensitive colonies were replica plated to YEPD (1% yeast extract, 2% peptone, 2% dextrose) and incubated at 23 and 37°C to confirm that the temperature-sensitive phenotype was not the result of nutritional requirements. Finally, mutants were tested for their ability to be complemented with a wild-type copy of *KAP121*. Colonies that fulfilled all of these requirements were classified as *kap121* temperature-sensitive strains, and the plasmids containing the temperature-sensitive *kap121* alleles were isolated and termed p*kap121-18*, p*kap121-26*, p*kap121-34*, and p*kap121-41*.

The *kap95-14* temperature-sensitive strain is a derivative of DF5 and was produced as previously described (1).

Overlay blot assay. Yeast nuclei were prepared as previously described (56). The proteins were denatured in sodium dodecyl sulfate (SDS) and fractionated by hydroxyapatite (HA) high-pressure liquid chromatography (HPLC). Pooled column fractions were further separated by standard formic acid HPLC separation (56, 68). The proteins in each of the resulting column fractions were concentrated by trichloroacetic acid precipitation. Aliquots were then separated by SDS-polyacrylamide gel electrophoresis (PAGE) and transferred to nitrocellulose membranes. The membranes were blocked and probed with cytosolic fractions isolated from yeast cells expressing protein A (pA)-tagged Kap121p and Kap123p as previously described (1, 57). Karyopherin-interacting proteins were identified by mass spectrometry as described previously (48, 55)

Recombinant-protein preparation. Glutathione *S*-transferase (GST) fusion proteins were expressed separately in *Escherichia coli* BL21(DE3)(pLYS-S) (Novagen, Madison, Wis.). Overnight cultures were diluted and grown for 30 min at 37°C. The cells were induced to express the GST fusions by incubation at 37°C in 2 mM IPTG (isopropyl-1-thio-β-D-galactopyranoside) for an additional 5 h. Cells were harvested, washed with water, and flash frozen in a dry ice-ethanol bath. The cell pellets were resuspended in STE (100 mM NaCl, 10 mM Tris-HCl

[pH 7.5], 1 mM EDTA)–1% Triton X-100 containing 1/100 solution P (0.4 mg of pepstatin A/ml, 18 mg of phenylmethylsulfonyl fluoride/ml [1]) and lysed by sonication. The lysates were cleared by centrifugation at 17,500 × *g* for 15 min at 4°C and stored at −80°C in 1-ml aliquots.

Ran preparation. Yeast Ran (Gsp1p) was expressed as a GST fusion in *E. coli* as described above. A 1-ml aliquot of the resulting protein lysate was thawed on ice and incubated with 200 μl of glutathione (GT) resin (Amersham Pharmacia Biotech) for 1 h at 4°C with rotation. Beads were pelleted by centrifugation and washed four times with binding buffer (20 mM HEPES-KOH [pH 6.8], 2 mM magnesium acetate, 150 mM potassium acetate [KOAc]). Ran was cleaved from the column with 0.3 U of thrombin (Sigma) at room temperature for 30 min with rotation, and 1 U of hirudin (Sigma) was then added to inhibit the thrombin. The cleaved Ran was collected through a microcentrifuge spin column, cleared of any residual GST proteins via incubation with 20 μl of fresh GT resin at 4°C for 30 min, and collected a second time through a fresh column. Purified Ran was then loaded with either GTP or GDP as described previously (51) and stored at −80°C.

In vitro binding assays. (i) Protein purification and binding. Protein lysates, produced as described above, were thawed, adsorbed to GT resin as described above, washed three times with transport buffer (20 mM HEPES-KOH [pH 7.4], 110 mM KOAc, 2 mM $MgCl_2$, 1 μM $CaCl_2$, 1 μM $ZnCl_2$, 1 mM EDTA, 1 mM dithiothreitol, 0.1% Tween 20), once with transport buffer plus 500 mM NaCl, and three times with transport buffer. Kap121p was removed from the GT resin via incubation with 0.1 U of thrombin (Sigma) overnight at room temperature, and 1 U of hirudin (Sigma) was then added to inhibit the thrombin. Cleaved Kap121p was collected through a microcentrifuge spin column and cleared of any remaining GST-containing proteins as described above.

For in vitro binding reactions involving two proteins, the initial binding and purification steps were carried out as described above. Purified Kap121p was incubated with immobilized GST-Ste12p fusion proteins for 1 h at 4°C with rotation. Samples were centrifuged, and the unbound protein fractions were prepared for SDS-PAGE. The bound protein complexes were washed as described above and prepared for SDS-PAGE. SDS-PAGE and Coomassie blue staining were then used to analyze equal amounts of both the unbound and bound fractions.

(ii) Ran release. Protein complexes were formed as described above and separated into aliquots. Ran-GTP, Ran-GDP, or GTP-loading buffer alone was added, and complexes were incubated with rotation at room temperature for 30 min. Unbound protein fractions were collected and prepared for SDS-PAGE. The bound protein complexes were washed three times with transport buffer and released from the beads by incubation with SDS-PAGE buffer. Proteins in the bound and unbound fractions were separated by SDS-PAGE and visualized by Coomassie blue staining. Kap121p present in the gel was quantified with ImageQuant as previously described (34). The amounts of Kap121p present in both the unbound and bound fractions were determined, and the Kap121p released from the complex was represented graphically as a percentage of total Kap121p (released Kap121p/[released Kap121p + bound Kap121p]) × 100%).

Microscopy. Wild-type and mutant cells expressing GFP-tagged proteins were grown in selection media, and the GFP chimeras were visualized directly by fluorescence microscopy with a Zeiss Axioskop 2. Images were captured with a Spot camera (Diagnostic Instruments Inc., Sterling Heights, Mich.). Where temperature shifts were required, cultures were grown to mid-logarithmic phase at 23°C and then shifted to a 30 or 37°C water bath for 3 h and maintained at this temperature on a heated microscope stage during examination.

Immunopurification studies and immunoblotting procedures. Aliquots of the GST-Ste12p (aa1-252), GST-Ste12p (aa253-493), and GST-Ste12p (aa494-688) bacterial lysates were thawed on ice and incubated with 200 μl of GT-Sepharose resin (Amersham Pharmacia Biotech) for 1 h at 4°C with rotation. The beads were washed three times with transport buffer (20 mM HEPES-KOH [pH 7.4], 110 mM KOAc, 2 mM $MgCl_2$, 1 μM $CaCl_2$, 1 μM $ZnCl_2$, 1 mM EDTA, 1 mM dithiothreitol, 0.1% Tween 20, 1/1,000 solution P [1]), once with transport buffer plus 500 mM NaCl, and three times with transport buffer. Cleared whole-cell lysates from yeast strains expressing Kap95-pA (1, 57), Kap104-pA (1), Kap121-pA (57), and Kap123-pA (57), prepared as described previously (40), were incubated with the immobilized GST chimeras overnight at 4°C with rotation. The immobilized protein complexes were washed extensively with wash buffer (150 mM NaCl, 0.1 mM $MgCl_2$, 0.1% Tween 20, 50 mM Tris-HCl [pH 7.5], 1:1,000 solution P) and successively with wash buffer containing 500 mM, 1 M, and 2 M $MgCl_2$ and finally with 0.5 M acetic acid. The proteins in each fraction were then precipitated with trichloroacetic acid and prepared for SDS-PAGE. Protein samples were separated by SDS-PAGE and stained with Coomassie blue or transferred to nitrocellulose and blocked with 5% skim milk (1). pA-tagged fusions were detected with rabbit immunoglobulin B (IgG; Cappel) and visualized with horseradish peroxidase-conjugated secondary antibodies and enhanced chemiluminescence.

fus1-LACZ* assays.** DF5 *mata** and *kap121-34 mat***a** cells were transformed with pSB234 (66). These cells were grown to an optical density at 600 nm (OD_{600}) of 0.200, and the cultures were separated into aliquots, treated with 5 μM α-factor, and incubated at 23, 30, or 37°C. Samples were taken at various intervals after pheromone treatment, divided in half, and prepared for Western blot analysis or β-galactosidase activity assays. For Western blot analysis whole-cell protein lysates were separated by SDS-PAGE, transferred to nitrocellulose membranes, and blocked with 5% skim milk (1). The β-galactosidase fusion and Gsp1p were detected with monoclonal anti-β-galactosidase (57) and anti-Gsp1p, respectively, and visualized as described above. The β-galactosidase activity assays were preformed as described in the Matchmaker 2-Hybrid manual (Clontech Laboratories, Inc.). Briefly, the OD_{600} of each culture was determined, and the cells were harvested, washed with Z buffer (60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, 1 mM $MgSO_4$) (Clontech Laboratories, Inc.), and resuspended in 100 μl of Z buffer. The cells were lysed by flash freezing in liquid nitrogen and quick thawing in a 37°C water bath three times. Then 700 μl of 0.27% β-mercaptoethanol and 0.56 mg of o-nitrophenyl-β-D-galactopyranoside were added to each sample. The reaction mixtures were incubated at 30°C for 3 min, reactions were stopped by the addition of 400 μl of Na_2CO_3, mixtures were centrifuged, and the OD_{420} of each sample was determined. Units of β-galactosidase activity were calculated with the following formula: 1,000 × OD_{420} of centrifuged reaction mixture/(OD_{600} of culture × volume of culture × minutes of assay) (66).

Quantitative mating assay. Quantitative mating assays were performed as previously described (61). Briefly, mid-logarithmic cultures of the strains to be tested and wild-type tester strains were grown at 23°C in selective or rich media. Culture concentrations were determined, and 2 × 10^6 cells of the strain to be tested were mixed with 10^7 cells of the wild-type tester strain. These mixtures were incubated at 30°C for 5 h and plated in a 1:1,000 dilution onto selective media to select for diploids. To determine the number of viable haploid cells of the strain being tested, the mating mixtures were also plated in a 1:1,000 dilution onto selective media to select for these haploids. The resulting haploid and diploid colonies were counted. Mating efficiency was expressed as a percentage of the input haploids of the strain being tested that formed diploid colonies (32).

Invasion assay. To obtain invasion-competent strains with which to assay the role of *KAP121 kap121-ts*/p*KAP121-URA3*, haploids were crossed with SK1 (*matα*) (37), an invasion-competent *Saccharomyces cerevisiae* strain. The resulting diploids were sporulated, and tetrads were dissected. The segregants were patched on SC-His-Ura, SC-His-Trp, and SC-His-Ura-Trp plates to identify strains containing p*KAP121-URA3* as the only copy of *KAP121*. To determine which of these haploid strains were invasion competent, the cells were patched to YEPD plates with a toothpick and incubated at 30°C for 2 days and then the plates were washed with a gentle stream of deionized water to rinse cells from the agar surface, revealing cells that had invaded the agar (52). Four haploid strains, termed *121INV1-4*, were identified. *121INV1* cells were then transformed with *kap121* temperature-sensitive alleles on *TRP1*-based plasmids p*kap121-18*, p*kap121-34*, and p*kap121-41*. These transformants were then replica plated to 5-fluoroorotic acid-containing media to select for cells that had lost the wild-type *KAP121*-containing plasmid (*pKAP121-URA3*), yielding (otherwise) isogenic strains containing the temperature-sensitive allele of *kap121* as the only copy of *KAP121* (*121INV1-kap121-18*, *121INV1-kap121-34*, and *121INV1-kap121-41*). *121INV1-kap121-34* cells were also transformed with pRS426-*STE12* and pRS316-*STE12*cNLS.

RESULTS

Temperature-sensitive *KAP121* mutants. Previous studies have demonstrated that *KAP121* is an essential gene (60). To further understand the essential role Kap121p plays and to identify specific cargoes and other interactions, we used a random PCR mutagenesis approach (45) to isolate temperature-sensitive mutant alleles of *KAP121*. Four temperature-sensitive clones were isolated based on the ability of strains carrying them to grow at 23°C but not at 37°C (Fig. 1). Immunoblotting with anti-Kap121p antibodies whole-cell lysates obtained from both the wild-type and temperature-sensitive strains during a temperature shift time course demonstrated that each allele produced proteins with the same molecular mass as wild-type Kap121p (data not shown), indicating that the protein was

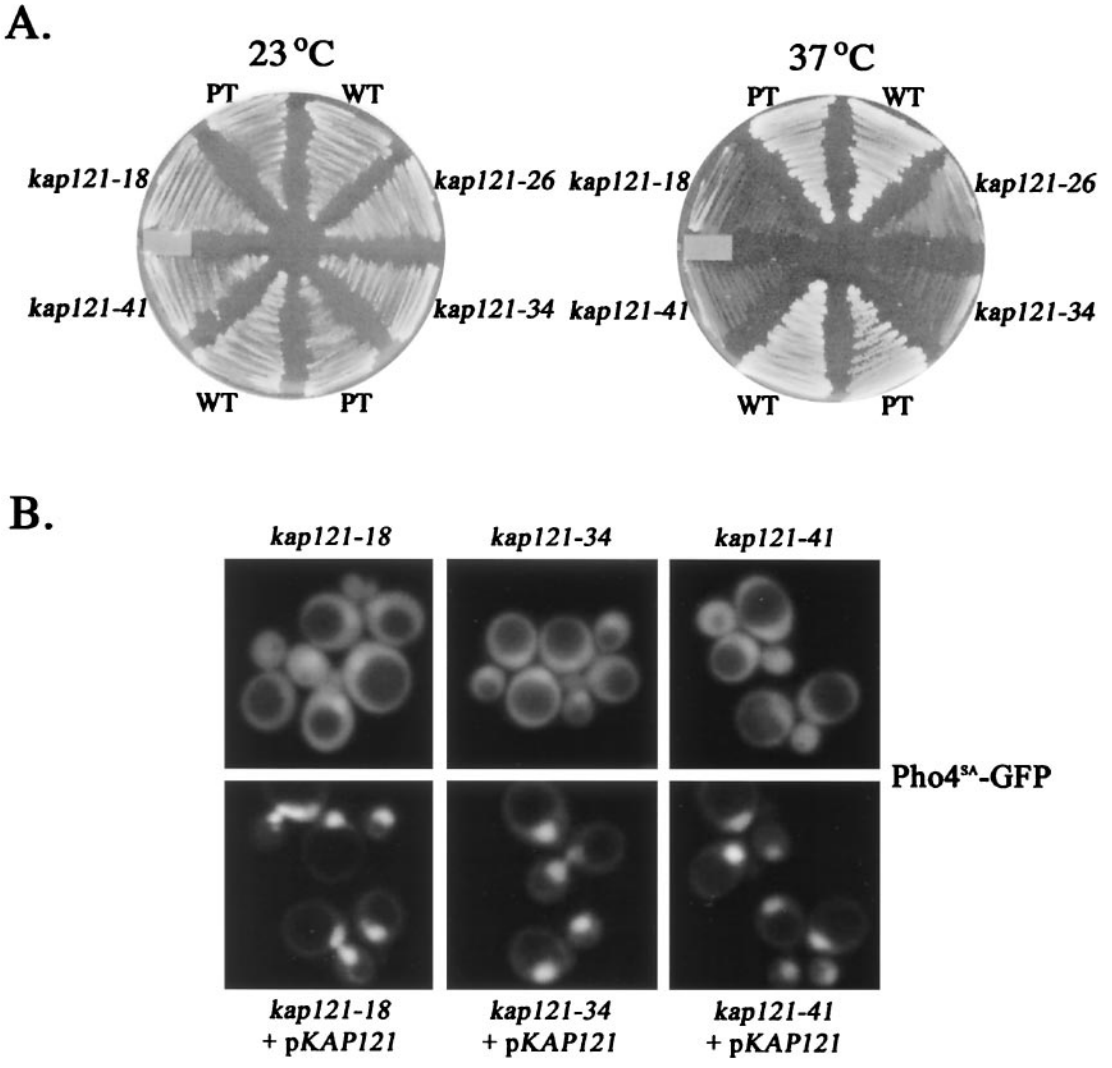

FIG. 1. Growth of parental, wild-type, and *kap121* temperature-sensitive strains. (A) Parental (PT) DF5 cells, *kap121Δ/KAP121* (wild-type [WT]), and the *kap121* temperature-sensitive yeast strains were streaked on YEPD and incubated for 2 days at 23 or 37°C. When incubated at 23°C, all strains grew, but growth of the temperature-sensitive strains (*kap121-18*, *kap121-26*, *kap121-34*, and *kap121-41* strains) was dramatically inhibited at 37°C. (B) The Pho4^{SA}-GFP fusion (28) was expressed in *kap121-18*, *kap121-34*, and *kap121-41* haploid cells (top) and in the same strains also expressing a wild-type *KAP121* allele (bottom). The cultures were grown to mid-log phase at 23°C, and GFP was detected by fluorescence microscopy. Note that, when expressed in the *kap121* temperature-sensitive mutants, the GFP fusion was predominantly localized to the cytoplasm (also see reference 40a.

present but not functional at the nonpermissive temperature (37°C). To determine if the temperature-sensitive alleles isolated here contained mutations that impaired Kap121p-mediated nuclear import, the cellular localization of a Pho4^{SA}-GFP fusion containing a constitutively active form of the Pho4p NLS (28) was determined in the *kap121-18*, *kap121-34*, and *kap121-41* strains. As previously observed with the *pse1-1* mutant (28), this chimera was predominantly mislocalized to the cytoplasm of each strain at the permissive temperature (Fig. 1) and completely mislocalized at 37°C (data not shown). When a wild-type allele of *KAP121* was added back to each strain, Pho4^{SA}-GFP relocalized to the nucleus, indicating that these *kap121* temperature-sensitive alleles specifically impaired the Kap121p transport pathway in the same manner as the *pse1-1* allele (Fig. 1). As we could not detect obvious differences between the cargoes that are imported by the three *kap121* strains examined (see below), we focused our attention on one strain, the *kap121-34* strain. DNA sequence analysis of the *kap121-34* strain identified 20 point mutations, which result in 13 amino acid residue changes throughout the length of the protein (Table 1). Initial analysis of *kap121-34* cells demonstrated that this strain grew slower than the parental strain at all temperatures tested, but we also noted that, when crossed with other strains, the *kap121-34* strain did not mate as well as other laboratory strains (see Fig. 8). To date no Kap121p cargoes that specifically relate to the mating process have been identified; therefore we sought to identify novel Kap121p cargoes, some of which may be implicated in this phenotype.

Identification of Ste12p as a Kap121p cargo. A pA-tagged Kap121p chimera (Kap121-pA) was used in an overlay assay to

TABLE 1. *kap121-34* mutations[a]

Nucleotide change	Position	Amino acid change	Position
A→C	305	I→T	102
A→G	977	D→G	326
T→C	1087	Y→H	363
A→G	1274	Q→R	425
A→T	1336	T→S	446
A→G	1630	K→E	544
T→G	2042	L→R	681
A→G	2105	E→G	702
T→C	2317	S→P	773
A→T	2566	N→Y	856
C→T	2732	S→F	911
T→C	3068	V→A	1023
A→G	3109	I→V	1037

[a] p*kap121-34-TRP1* was isolated from *kap121-34* cells, and the *kap121-34* DNA sequence was determined by automated dideoxynucleotide chain termination sequencing. Presented are the nucleotide substitutions that result in predicted amino acid changes in Kap121p.

detect nuclear proteins that interact with Kap121p. Yeast nuclei were purified, the proteins were fractionated by HA HPLC, separated by SDS-PAGE, and transferred to nitrocellulose membranes (Fig. 2A). The membranes were incubated with cytosols from yeast cells expressing Kap121-pA, and the bound chimera was detected with rabbit IgG and enhanced chemiluminescence. Because previous studies have shown that the Kap121p and Kap123p transport pathways overlap (57), the Kap121-pA profiles were compared with profiles obtained from experiments conducted using Kap123-pA as a probe. Fractions enriched with Kap121p-interacting proteins were pooled and further fractionated by reverse-phase HPLC and SDS-PAGE and transferred to nitrocellulose membranes (Fig. 2B). These nitrocellulose membranes were again separately probed with Kap121-pA and Kap123-pA cytosols (Fig. 2C), and the karyopherin-interacting proteins were identified by mass-spectrometric analysis of the same bands in Coomassie blue-stained SDS-polyacrylamide gels. A number of nuclear proteins that interacted with Kap121p were identified; among these was Ste12p, a transcription factor that regulates mating responses and cellular differentiation. Because of the central role Ste12p plays in regulating mating responses, we chose to further characterize the Kap121p/Ste12p interaction (22, 35, 37, 52).

Ste12p is mislocalized in *kap121* cells. To determine if Ste12p is a cargo of the Kap121p-mediated transport pathway, Ste12p was C-terminally tagged with GFP and its cellular localization in *kap121* cells was examined. At 23°C, Ste12p-GFP was predominantly nuclear but was also detectable in the cytoplasm of *kap121* cells. After 3 h at 30°C the cytoplasmic pool of Ste12p-GFP increased, and at 37°C Ste12p appeared almost completely mislocalized to the cytoplasm, with little or no defined nuclear signal (Fig. 3). When a functional copy of *KAP121* was added back to *kap121-34* cells, Ste12p-GFP remained nuclear at all temperatures. Similar observations were made for *kap121-18* and *kap121-41* cells (Fig. 3). These data demonstrated that partial or catastrophic loss of Kap121p function caused the redistribution of Ste12p to the cytoplasm, while the presence of a functional copy prevented this mislocalization. As a control, we monitored the localization of the cNLS of SV40 large T antigen and Rpl25NLS (data not shown)

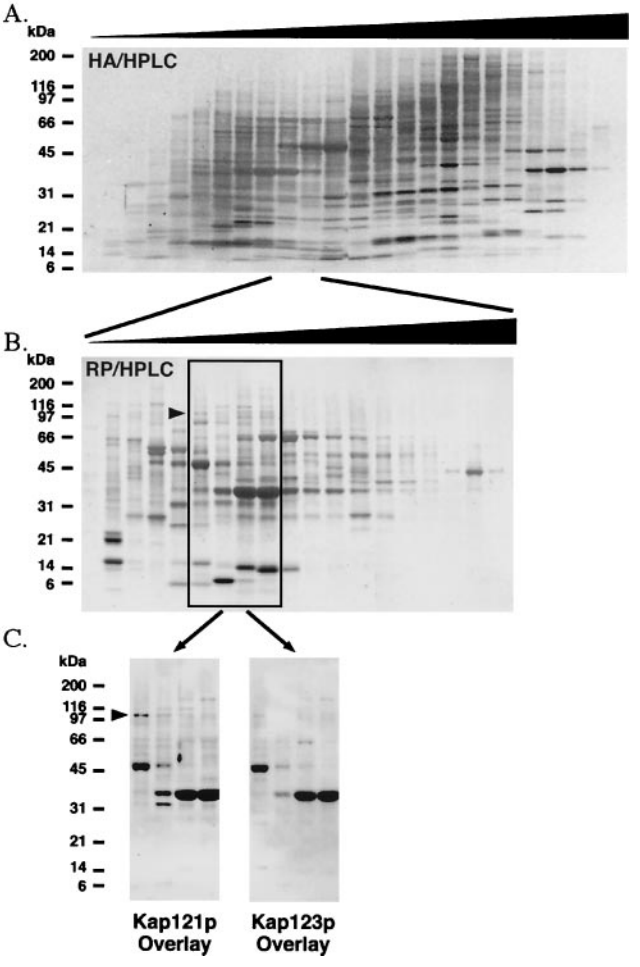

FIG. 2. Kap121-pA interacts with Ste12p. (A) Yeast nuclear proteins were purified, fractionated by HA-HPLC, separated by SDS-PAGE, transferred to nitrocellulose membranes, and detected by amido black staining. These fractions were then probed with cytosols from cells expressing either Kap121-pA or Kap123-pA. (B) Fractions enriched with Kap121p-interacting proteins were pooled and further fractionated by reverse-phase (RP) HPLC, separated by SDS-PAGE, transferred to nitrocellulose membranes, and detected by amido black staining. (C) Membranes were separately probed with cytosols from cells expressing either Kap121-pA or Kap123-pA, and bound chimeras were detected with horseradish peroxidase-conjugated anti-rabbit IgG and ECL. Karyopherin-interacting proteins were identified by mass spectrometry of protein bands in an identical gel.

fused to GFP, whose import into the nucleus is mediated by Kap95p/Kap60p and Kap123p, respectively, in the *kap121-34* strain. We found that both the cNLS-GFP and Rpl25NLS-GFP chimeras were nuclear at both the permissive and nonpermissive temperatures, suggesting that the mislocalization of Ste12p was specific to the Kap121p transport pathway.

The C-terminal portion of Ste12p interacts specifically with Kap121p and contains a functional NLS. Both the overlay assay and the in vivo import data suggested that Kap121p interacts with Ste12p and mediates its nuclear import. To test whether Kap121p could bind directly to Ste12p, we attempted to reconstitute this interaction in vitro by using recombinant proteins. A GST fusion protein containing full-length Ste12p was constructed and expressed in *E. coli* to analyze this inter-

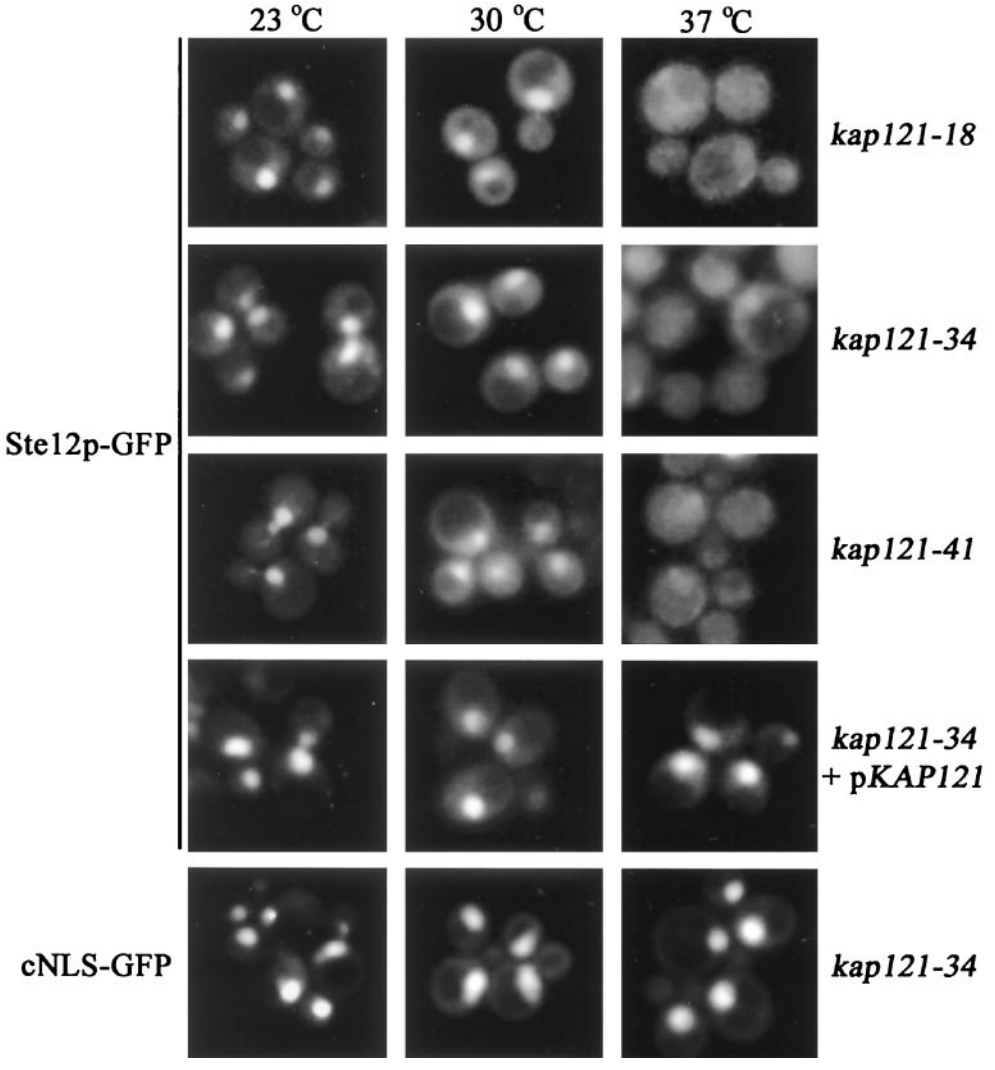

FIG. 3. Ste12p mislocalizes in *kap121* temperature-sensitive strains. Ste12p-GFP was expressed in the indicated *kap121* temperature-sensitive haploid cells. As controls Ste12p-GFP was also expressed in *kap121* cells containing a wild-type copy of *KAP121* (*kap121-34* + p*KAP121*), and a cNLS-GFP chimera was expressed in *kap121* cells (*kap121-34*). Cultures grown at 23°C or shifted to 30 or 37°C for 3 h were observed by fluorescence microscopy. Note that in *kap121* cells Ste12p-GFP shifted to the cytoplasm at higher temperatures.

action; however this chimera was highly susceptible to proteolysis (data not shown). Deletion constructs were therefore produced to further characterize the Kap121p/Ste12p interaction and concomitantly identify the region of Ste12p that contains the NLS. GST chimeras containing amino acid residues 1 to 252, 253 to 493, and 494 to 688 of Ste12p [GST-Ste12p (aa1-252), GST-Ste12p (aa253-493) and GST-Ste12p (aa494-688), respectively] and GST alone were expressed in *E. coli*, purified, immobilized on GT-Sepharose, and incubated with recombinant Kap121p. Kap121p bound directly to the C-terminal fragment of Ste12p (amino acids 494 to 688) but failed to bind to either the N-terminal (amino acids 1 to 252) or central portion (amino acids 253 to 493) of the protein or GST alone (Fig. 4A).

To evaluate the potential of each fragment to be imported into nuclei in vivo, each of the Ste12p protein fragments was C-terminally fused to a tandem repeat of two GFPs, expressed under the control of the *ADH1* promoter, and visualized directly by fluorescence microscopy of wild-type (DF5) yeast cells (Fig. 4B). In agreement with the in vitro binding studies, only the C-terminal, Kap121p-interacting fragment of Ste12p (amino acids 494 to 688) was targeted to the nucleus, while the other fragments were diffusely localized throughout the cell. Furthermore, the import of the Ste12p (aa494-688)-GFP chimera was dependent on functional Kap121p. In *kap121-34* cells, Ste12p (aa494-688)-GFP was localized predominantly to nuclei at 23°C but was mislocalized to the cytoplasm at 37°C (Fig. 4C), and, moreover, the nuclear import of this GFP chimera was rescued by introducing a wild-type copy of *KAP121* into the *kap121-34* strain. As a control, we also monitored the localization of Ste12p (aa494-688)-GFP in a *kap95* temperature-sensitive strain (*kap95-14* strain). In this strain, the prototypical yeast β-karyopherin (Kap95p) is mutated, and consequently cNLS-containing reporters are mislocalized to the cytoplasm at restrictive temperatures (data not shown). We found that, in these cells, the Ste12p (aa494-688)-GFP chimera

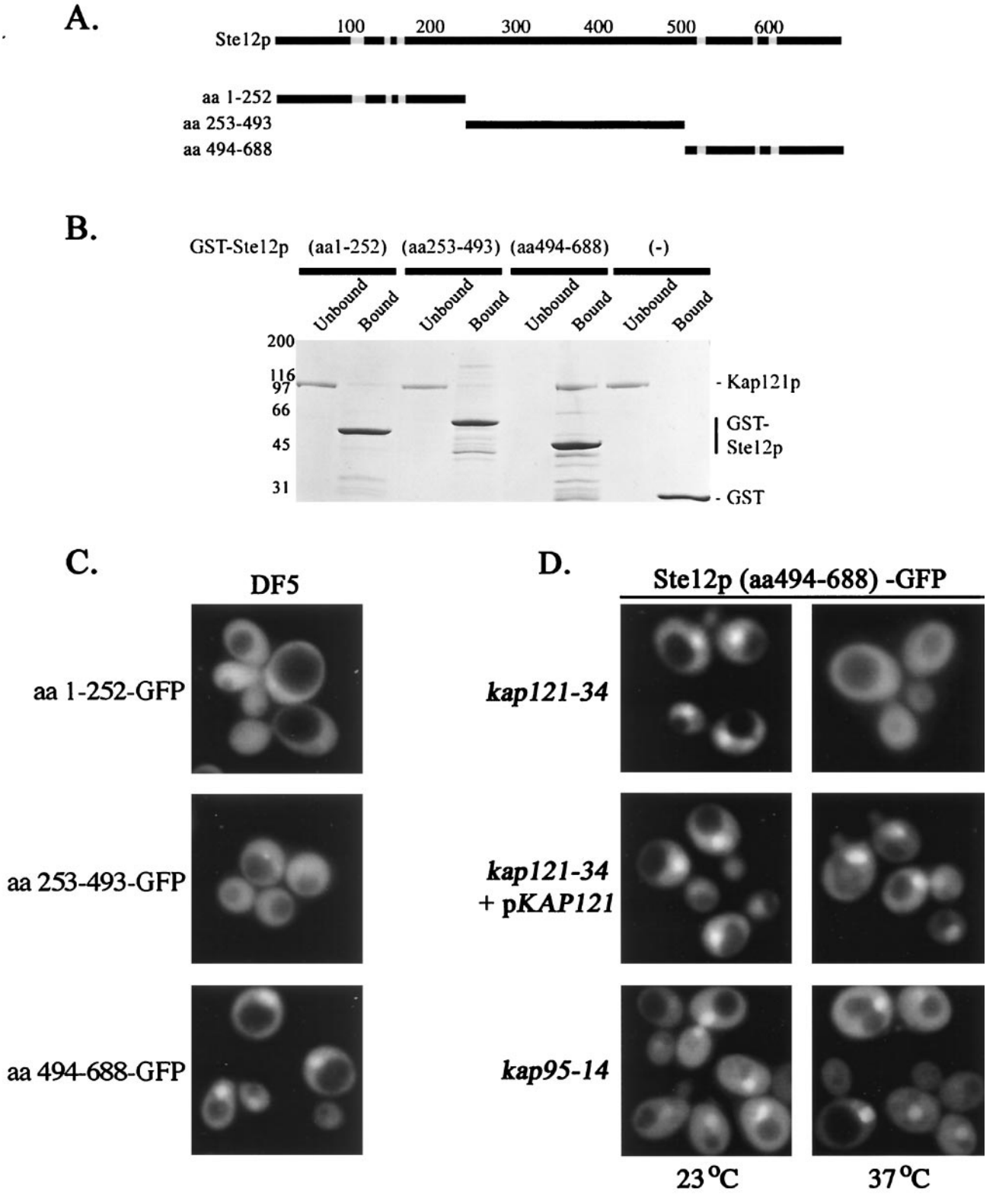

FIG. 4. The C-terminal portion of Ste12p interacts directly with Kap121p and contains a functional NLS. (A) Schematic diagram showing the fragments of Ste12p expressed as GST fusions. Gray segments are basic regions considered potential NLS sequences. (B) GST alone and the fragments of Ste12p were expressed as GST fusion proteins in *E. coli* and ~400 ng of each was immobilized on GT-Sepharose 4B beads. The immobilized fusion proteins were incubated with recombinant Kap121p (~200 ng). After extensive washing, bound protein fractions were released from the GT resin by incubation with SDS-PAGE buffer. Equal amounts of both the bound and unbound protein fractions were analyzed by SDS-PAGE and Coomassie blue staining. (C) Ste12p deletion constructs were expressed as GFP fusions in haploid DF5 cells. Cultures were grown to mid-log phase at 23°C, and GFP chimeras were observed by fluorescence microscopy. Note the nuclear localization of the C-terminal portion of Ste12p. (D) The C-terminal GFP fusion [Ste12p (aa494-688)-GFP] was expressed in *kap121-34*, *kap121-34*/p*KAP121*, and *kap95-14* cells. Cultures were grown to mid-log phase at 23°C; GFP chimeras were detected by fluorescence microscopy either at this temperature or after cultures had been shifted to 37°C for 3 h. Note the cytoplasmic accumulation of Ste12p (aa494-688)-GFP in *kap121-34* cells after the temperature shift.

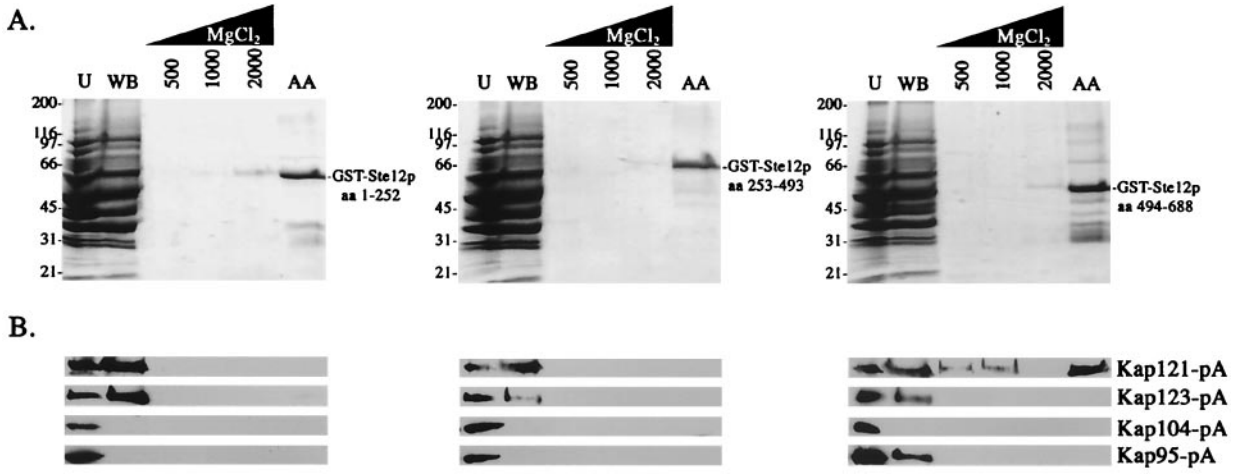

FIG. 5. The C terminus of Ste12p specifically enriches for Kap121-pA from whole-cell lysates. Recombinant GST-Ste12p (aa1-252), GST-Ste12p (aa253-493), and GST-Ste12p (aa494-688) fusion proteins were immobilized on GT-Sepharose 4B beads and incubated with whole-cell lysates from strains expressing pA chimeras of kaps. Bound protein complexes were washed with binding buffer (WB), followed by a $MgCl_2$ gradient ($MgCl_2$ concentrations are in millimolar) and finally 0.5 M acetic acid (AA). Proteins from each fraction were separated by SDS-PAGE. U, unbound lysate. (A) Coomassie blue-stained gel resulting from lysates derived from cells expressing Kap121-pA. (B) Western blots for detecting the chimeras within bound and eluted fractions from cells expressing Kap95-pA, Kap104-pA, Kap121-pA, or Kap123-pA. Note that the only detected interaction was between Kap121p and the C-terminal portion of Ste12p.

remained nuclear at both the permissive and nonpermissive temperatures (Fig. 4C). Taken together, these data demonstrated that Kap121p mediates the nuclear import of Ste12p through a direct interaction with an as yet undefined NLS present in the C terminus of Ste12p.

The C-terminal fragment of Ste12p interacts specifically with Kap121p. Previous studies suggest that the Kap121p and Kap123p transport pathways overlap and are capable of mediating the nuclear import of some common substrates (24, 57). To investigate the specific role that Kap121p plays in the nuclear import of Ste12p, immunopurification studies were used to determine whether recombinant, *E. coli*-expressed, GST-Ste12p fusion proteins could interact with other β-karyopherins. Each GST-Ste12p chimera was immobilized on GT-Sepharose and incubated with lysates derived from yeast strains expressing pA-tagged copies of Kap95p, Kap104p, Kap121p, and Kap123p. Bound proteins were eluted from the column with increasing concentrations of $MgCl_2$ and then with 0.5 M acetic acid. Coomassie blue staining and immunoblotting (Fig. 5) demonstrated that of the four karyopherins tested, the C terminus of Ste12p was enriched only with Kap121p. Together, the immunopurification data and the overlay blot analysis provided complementary evidence that Kap121p interacts specifically with Ste12p through the NLS-containing C-terminal fragment of the protein.

Ran-GTP dissociates the Kap121p-Ste12p complex. Ran-GTP is a key regulator and a driving force of nucleocytoplasmic transport (5, 6, 38, 42, 63). Previous studies have shown that the binding of Ran-GTP to import-bound β-karyopherins, including Kap95p (51), Kap104p (34), Kap123p (58), and Kap121p (28, 58), contributes, to various degrees, to the displacement of import substrates. To test if Ran-GTP had a

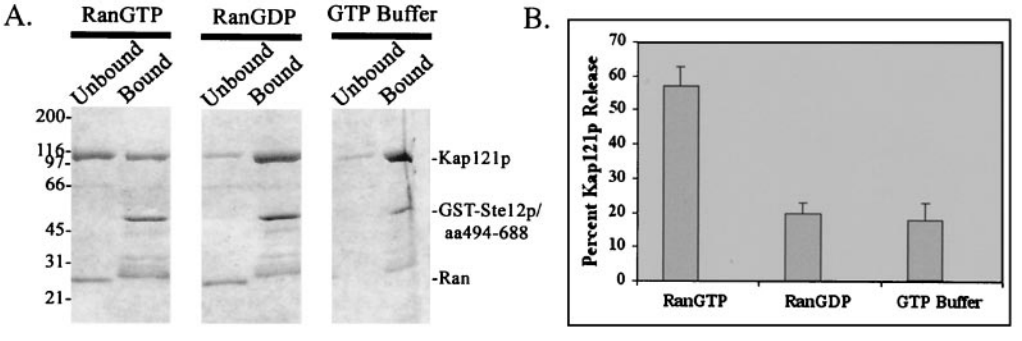

FIG. 6. Ran-GTP dissociates Kap121p-Ste12p (aa494-688) protein complexes. (A) Complexes containing recombinant Kap121p bound to GST-Ste12p (aa494-688) and immobilized on GT-Sepharose were incubated with Ran-GTP (~120 ng), Ran-GDP (~120 ng), or GTP-loading buffer, as indicated, for 30 min at room temperature. Proteins remaining bound to the resin were released by incubation in SDS-PAGE buffer. The bound and released proteins were analyzed by SDS-PAGE and Coomassie blue staining. (B) The results of three separate experiments were quantified as described in Materials and Methods. Data are percentages of Kap121p released by each treatment. Note that Kap121p was preferentially released when the protein complex was incubated with Ran-GTP.

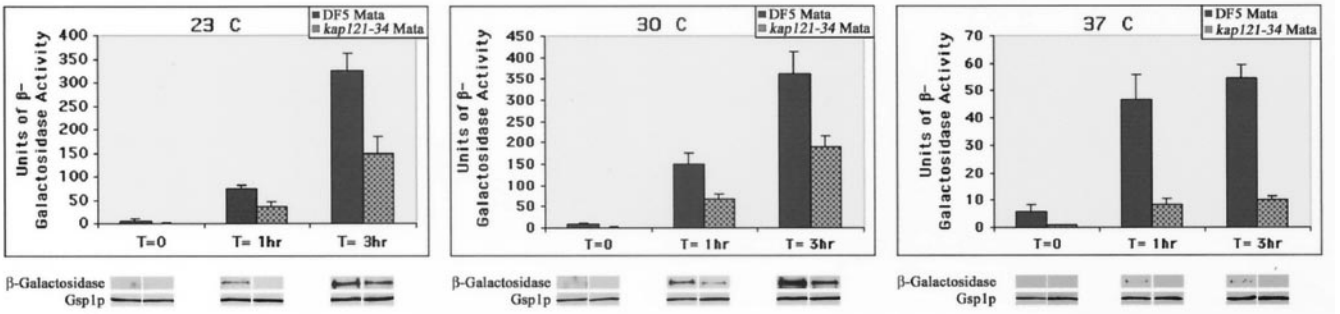

FIG. 7. Ste12p-induced transcription is compromised in *kap121-34* cells. *kap121-34 mat***a** and wild-type *mat***a** cells containing a *fus1-LACZ* fusion were induced with 5 μM α-factor (Sigma) and incubated at 23, 30, or 37°C. Samples were taken at the indicated intervals after pheromone treatment and prepared for Western blot analysis or β-galactosidase activity assays. Units of β-galactosidase activity were determined as described in Materials and Methods. The standard errors of the means were calculated from three separate experiments. Western blots detecting the *fus1-LACZ* chimera and Gsp1p (loading control) were from whole-cell lysates obtained from the same experiment represented graphically. Note the β-galactosidase activity and the β-galactosidase fusion protein produced by the *kap121-34* cells were significantly less than those produced by wild-type cells at all temperatures.

similar effect on the Kap121p-Ste12p (aa494-688) import complex, this complex was immobilized on GT-Sepharose and then incubated with either Ran-GTP, Ran-GDP, or GTP-loading buffer. The unbound and bound protein fractions were analyzed by SDS-PAGE and visualized by Coomassie blue staining (Fig. 6A). The addition of Ran-GTP to the complex resulted in the release of Kap121p into the unbound protein fraction. By comparison, when the complex was challenged with Ran-GDP or GTP-loading buffer, Kap121p remained predominantly in the bound fraction. Quantification of Kap121p release in three separate experiments revealed that Ran-GTP stimulated approximately threefold more Kap121p release from Ste12p than did either Ran-GDP or buffer alone (Fig. 6B). The specific dissociation of the Kap121p-Ste12p (aa494-688) protein complex by Ran-GTP is similar to other nuclear import carrier/cargo interactions and is thought to mimic the conditions that these complexes encounter as they enter the nucleus in vivo (24, 28, 34, 51, 57, 58).

Ste12p-induced transcription is severely compromised in *kap121-34* cells. The data presented above demonstrate that Kap121p mediates the nuclear import of Ste12p. Therefore, we hypothesized that Ste12p-induced transcription would also be affected in *kap121* temperature-sensitive strains. To address this, we used a well-characterized Ste12p-dependent β-galactosidase reporter, *fus1-LACZ* (66), to monitor Ste12p-induced transcription in response to mating pheromone α-factor in *kap121-34 mat***a** and wild-type *mat***a** cells. In both strains, β-galactosidase activity peaked at 3 h after pheromone treatment, but at all temperatures tested *kap121-34* cells displayed significantly less activity than wild-type cells and this was more pronounced at 37°C (Fig. 7). It should also be noted that the overall enzymatic activity was decreased in both wild-type and mutant cells at 37°C. The reasons for this are unclear, but this may partially explain a poor mating efficiency of even wild-type cells at 37°C (50) (data not shown).

***kap121-34* cells do not mate efficiently or grow invasively.** The compromised activity of Ste12p revealed above, combined with its coincident partial mislocalization (Fig. 3) in *kap121* cells at all temperatures, provided an opportunity to assay the functional relevance of Kap121p-mediated import under permissive growth conditions. Because both mating and invasive growth require Ste12p-dependent transcriptional activity, quantitative mating and invasive growth assays were performed with the *kap121* temperature-sensitive mutants at 30°C. The mating efficiency of *kap121-34* cells (and that of other *kap121* strains [data not shown]) was at least threefold less than that observed for wild-type cells (Fig. 8A). Similarly *kap121* cells were unable to grow invasively, whereas otherwise isogenic cells containing a wild-type copy of *KAP121* were capable of undergoing the physiological changes required to invade the agar (Fig. 8B). Taken together, these data further supported a role for Kap121p-mediated import of Ste12p, which thereby affects the differentiation reactions requiring nuclear Ste12p. However, because Kap121p likely imports many cargoes, we wished to evaluate if these phenotypes were specifically related to inefficient Ste12p import. We therefore transformed *kap121-34* cells with a multicopy plasmid containing *STE12* and assayed both mating and invasive growth. Indeed, under these conditions, both defects were suppressed (Fig. 8). These data suggest that, in *kap121* cells at permissive temperatures, Ste12p import (and thus activity) is not completely inhibited. Thus, overexpressing *STE12* likely increases the Ste12p concentrations in both the cytoplasm and nucleus sufficiently to suppress the observed phenotypes resulting from compromised import. However, other studies have demonstrated that overexpression of *STE12* can suppress many different mutants with mating defects (12, 59) and stimulate pseudohyphal and invasive growth (36, 65). Thus, here overexpression could potentially mask the loss of components, other than Ste12p, required for these processes. To address this, we determined if bypassing the import defect in *kap121* cells could restore Ste12p-dependent activities. A cNLS from SV40 large T antigen (29) was fused to the 3′ end of *STE12*, allowing the expressed chimera to be imported by the Kap95p/Kap60p import pathway. Synthesis of Ste12p with the appended cNLS in *kap121-34* cells restored both mating and invasion activities (Fig. 8), thereby establishing that the observed transcriptional defect and subsequent inability of *kap121-34* cells to mate or grow invasively was directly related to their inability to efficiently import Ste12p.

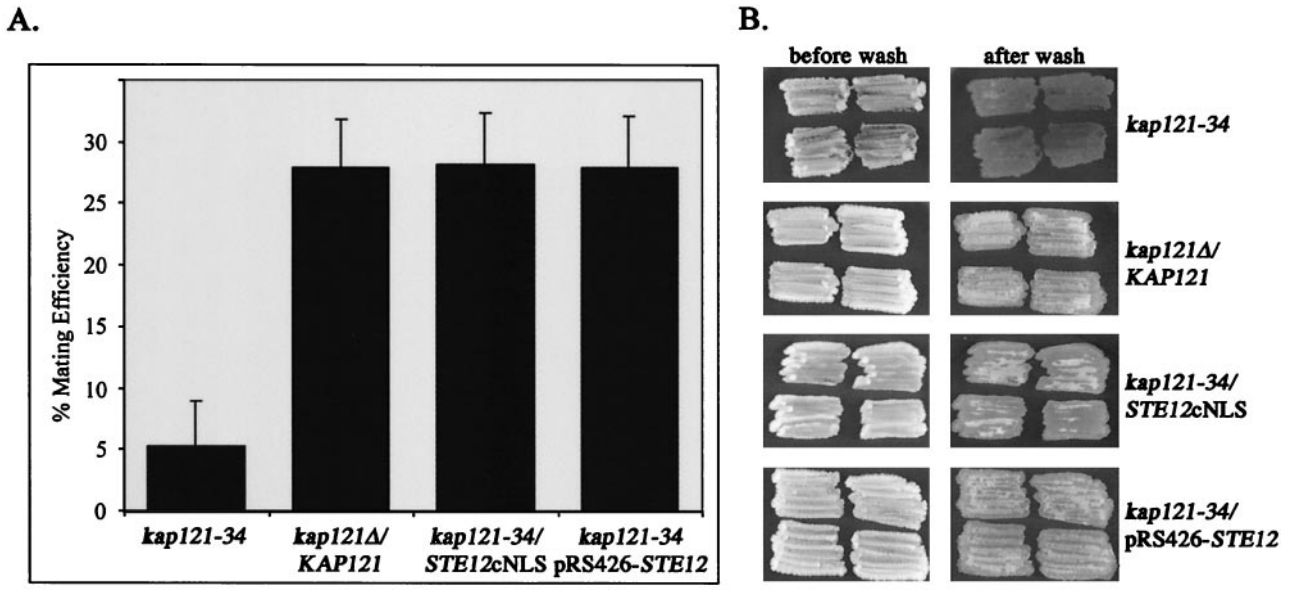

FIG. 8. *kap121-34* cells are unable to mate or grow invasively. (A) After pregrowth at 23°C, *kap121-34*, *kap121*Δ/*KAP121*, *kap121-34*/pRS426-*STE12*, and *kap121-34*/pRS316-*STE12*cNLS strains were mixed with the tester strain and incubated at 30°C for 5 h. The mating mixtures were plated on the appropriate dropout media to select for diploids and haploids. The resulting colonies were counted. Mating efficiency is expressed as a percentage of the input haploids (of the strain being tested) that formed diploid colonies. (B) *kap121-34* cells (*121INV1-kap121-34*), *kap121*Δ/*KAP121* cells (*121INV1*), and *kap121-34* cells (*121INV1-kap121-34*) containing either wild-type *STE12* on a multicopy plasmid (pRS426-*STE12*) or the *STE12*-cNLS fusion were patched onto YEPD media and incubated at 30°C for 2 days. The plates were then washed with a gentle stream of deionized water to reveal invasive growth (52). Note that *kap121-34* cells were unable to grow invasively but that *kap121-34* cells expressing *KAP121*, multiple copies of *STE12*, or the *STE12*-cNLS fusion were able to invade the agar.

DISCUSSION

One of the important goals of understanding nuclear transport is to identify the networks of nuclear transport pathways that allow cells to maintain their homeostasis, control their growth, and govern their responses to external stimuli. We and others have begun to identify connections within these networks, but it is a very complex system; there are 15 members of the yeast karyopherin family, which together mediate the nuclear transport of approximately 2,000 proteins, as well as numerous RNAs and ribonuclear protein particles. Furthermore, considering that many cargoes are transported by each karyopherin, that the mislocalization of each cargo has the potential to cause a phenotype, and that many cargoes can be transported (to a greater or lesser extent) by multiple karyopherins, understanding the links between a mutation in a karyopherin and the consequential phenotypes is a daunting systems biology problem.

Here, we have focused on beginning to disentangle the web of interactions for a typical karyopherin, Kap121p. The transport pathway mediated by Kap121p heavily overlaps with that mediated by its closest relative, Kap123p. Both proteins import ribosomal proteins into the nucleus (57). However each also appears to have its own set of cargoes. For example, Kap121p appears to specifically import Pho4p (28), Pdr1p (8), and Spo12p (4), transcription factors that regulate phosphate homeostasis, multidrug resistance, and sporulation, respectively.

We sought to further characterize Kap121p by making new *kap121* temperature-sensitive mutants and characterizing the phenotypes associated with them and, in parallel, attempting to comprehensively identify many potential Kap121p cargoes by overlay assays of nuclear proteins. One particularly interesting set of phenotypes associated with *kap121* cells that we observed were defects in both the mating response and the transition to the pseudohyphal invasive form. While these two, apparently distinct, differentiation programs arise from different external stimuli and utilize different mitogen-activated protein (MAP) kinase pathways to convey their signals to the nucleus, the pathways share some signaling elements and converge on a single nuclear transcription factor, Ste12p (22, 35, 52), a protein also identified as specifically interacting with Kap121p in our overlay assays. Depending on the MAP kinase pathway activated, the differential phosphorylation of Ste12p, and its interaction with partners, different transcriptional programs are activated, inducing either the mating response or pseudohyphal differentiation and invasive growth. Indeed, on the basis of our current knowledge, Ste12p is unique among known nuclear proteins in that its mislocalization could specifically disable both of these differentiation programs.

We thus tested if the nuclear import of Ste12p was dependent on Kap121p. Analysis of Ste12p-GFP chimeras demonstrated that Ste12p was not efficiently imported into nuclei of *kap121* cells and remained cytoplasmic at the nonpermissive temperature. However, when a functional copy of *KAP121* was added back to these cells, apparently normal Ste12p import was restored. Other transport pathways in these cells were not directly affected, establishing that the Kap121p transport pathway specifically affected the mislocalization of Ste12p. Furthermore, although Kap121p function has previously been shown to overlap with that of Kap123p, in agreement with our overlay assays, immunopurification studies suggest that Ste12p does

not interact with Kap123p or other β-karyopherins tested in this study.

Using in vitro binding studies we demonstrated that, like most other β-karyopherins, Kap121p interacts directly with its cargo, Ste12p. In vivo fluorescence localization studies combined with in vitro binding studies determined that the C-terminal amino acids 494 to 688 of Ste12p contain this Kap121p-specific NLS. The previously identified NLS sequences of Kap121p cargoes are basic in nature but fail to bear any other notable similarities (4, 8, 25, 28, 43, 57). Furthermore, some of these experiments have found exceptionally large karyopherin-interacting NLS domains, suggesting that the similarity in NLSs recognized by Kap121p lies, in part, in the secondary structure. Although we have not fully defined which residues are necessary and sufficient for Ste12p NLS function, amino acid sequence alignments between the previously characterized Kap121p-interacting NLS of Pho4p and full-length Ste12p identified two short sequences within this C-terminal region of Ste12p and a larger sequence containing a gap that show similarity to the Pho4p NLS. However, only mutational and structural analysis of Ste12p will fully illustrate which residues in this C-terminal fragment constitute the minimal NLS and are required for Kap121p interaction. Furthermore, these results do not rule out the possibility that additional amino acid residues, perhaps outside of the C-terminal fragment, contribute to efficient Ste12p nuclear import and/or retention.

A role for Kap121p in Ste12p import suggests that an inability to efficiently import Ste12p in *kap121* cells is the primary cause of the observed mating and invasive-growth defects. Indeed, expression of a Ste12p-dependent reporter in *kap121* cells was compromised. Moreover, expression of a *STE12*-cNLS fusion or overexpression of *STE12* in the *kap121-34* strain suppressed both phenotypic defects. Together, these data suggest that, although other Kap121p cargoes are mislocalized in *kap121-34* cells, the phenotypes we observed under these conditions are linked directly to Ste12p.

Here we have presented data demonstrating that Kap121p imports Ste12p into the nucleus and that *kap121* mutants disrupt Ste12p function, thereby blocking the Ste12p-induced transcription and the differentiation pathways mediated by Ste12p. This work underscores the validity of our parallel approaches and provides a framework for future studies aimed at linking phenotypes associated with *kap* mutants to their transport cargoes. However, it is also apparent from our results that our ability to unravel the complexities of nuclear transport requires a comprehensive inventory of kap-cargo interactions. We are currently exploring methods to define these interactions.

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