In *Saccharomyces cerevisiae*, Spo12p is involved in mitosis and is essential for meiosis. We found that Spo12p is imported into the nucleus by the karyopherin Kap121p. A complex containing Spo12p and Kap121p was isolated from cytosol and was also reconstituted with recombinant proteins, indicating that this interaction is direct. Spo12p was mislocalized to the cytosol in *pse1–1*, a temperature-sensitive strain harboring a mutation of Kap121p, at the permissive temperature, confirming an essential role for Kap121p in Spo12p import. Spo12p was also mislocalized in a *pse1–1/pse1–1* homozygous strain, suggesting it is imported via the same pathway in diploid cells. Furthermore, we found that *pse1–1/pse1–1* shows a sporulation defect similar to that of *spo12Δ/spo12Δ*. In addition, we have characterized the Spo12p nuclear localization signal, mapped it to residues 76–130, and identified residues within this region that are important for nuclear localization signal function.

Bidirectional exchange of macromolecules between the nucleus and the cytoplasm occurs through a large complex embedded in the nuclear envelope, the nuclear pore complex (NPC).¹ In *Saccharomyces cerevisiae* (herein referred to as yeast), the NPC is composed of about 30 proteins (nucleoporins), which provide docking sites for a family of soluble and structurally related transport factors, karyopherins (also known as importins/exportins/transportins). In addition to docking at the NPC, karyopherins bind cargo to be transported into and out of the nucleus (see Refs. 1 and 2 for a review). They recognize specific sequence elements in their cognate transport substrates that constitute the signal for import (nuclear localization sequence, or NLS) or export (nuclear export sequence, or NES). Kap11 needs an adapter (Kapo) in order to bind to most substrates. Kapo recognizes proteins containing a classical NLS (cNLS), which consists of one or two stretches of basic amino acid residues (3, 4). In yeast, there is one Kapo and 14 Kapfs (herein referred to as kaps). The kaps share a low but significant identity (about 20%), most striking at the amino terminus (1, 2, 5). To date, all yeast kaps except Kap120p have been shown to function in import or in export. The involvement of a kap in both processes has recently been discovered (6), and it remains to be elucidated whether more share the same ability. There are hundreds of yeast nuclear proteins or proteins with a nuclear life style, but only 14 kaps. This necessarily means that some kaps will be responsible for the transport of a large number of cargoes. Sequences of the NLSs that have been mapped so far do not resemble the cNLS or each other. Hence, the basis for kap/substrate specificity and the overall mechanism of substrate recognition by karyopherins remain to be further elucidated.

Meiosis is one of the few differentiation pathways in yeast, and its regulation necessarily depends on the expression control of subsets of genes. Regulation of nuclear transport has been increasingly recognized as an important mechanism for the control of gene expression (see Ref. 7 for a review). It is not known how most of the proteins involved in meiosis are transported into the nucleus to participate in its regulation. Spo12p was first identified as a protein whose absence causes sporulating diploid cells to skip one meiotic division, leading to the formation of two diploid spores (8). More recently, its overexpression has been found to suppress a number of late mitotic mutants (9, 10). Consistent with a role in exit from mitosis, cells in G2/M are over-represented in a *spo12Δ* population (11). Spo12p is synthetically lethal with Dbf2p, a kinase required for events in anaphase/telophase and has been suggested by genetic studies to be an activator of Dbf2p and its relative Dbf20p (12). Its biological function remains unknown, and its functional differences in mitosis and meiosis await further clarification.

Spo12p has been shown to be nuclear in both haploid and diploid cells (13). Here we report that in haploid cells Spo12p is imported into the nucleus by Kap121p. Kap121p is a previously characterized karyopherin, first implicated in ribosomal protein import (14) and mRNA export (15) and later shown to be responsible for import of the transcription factors Pho4p and Pdr1p (16, 17) (see “Discussion”). Evidence for import of Spo12p by Kap121p includes binding to Kap121p both in vitro and in vivo and mislocalization of Spo12p to the cytosol in *pse1–1*, a temperature-sensitive strain of Kap121p. We have also characterized the Spo12p NLS and identified residues within this region that are important for NLS function. Since Spo12p has a role in both mitosis and meiosis, we sought to determine whether the import pathway differs in diploid cells. In this case, we found that Spo12p was mislocalized in a *pse1–1/pse1–1* homozygous strain, indicating that it is imported using the same pathway in both haploid and diploid cells.

**EXPERIMENTAL PROCEDURES**

**Yeast Strains and Plasmids**—The procedures for yeast manipulation were as described (18). A diploid strain expressing Spo12p-Protein A (PrA) was obtained by integrative transformation of DF5 (19) with the coding sequence of four and half IgG binding repeats of *Staphylococcus aureus* protein A immediately upstream of the *SPO12* stop codon as described (20). Haploid strains were obtained by sporulation and tetrad

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¹ The abbreviations used are: NPC, nuclear pore complex; kap, karyopherin; NLS, nuclear localization signal; NES, nuclear export sequence; cNLS, classical NLS; PrA, Protein A from *S. aureus*; MS, matrix-assisted laser desorption ionization/time of flight mass spectrometry; MALDI-TOF, matrix-assisted laser desorption ionization/time of flight; PAGE, polyacrylamide gel electrophoresis; GST, glutathione S-transferase; GFP, green fluorescent protein; PCR, polymerase chain reaction; TB, transport buffer; DIC, differential interference contrast.
dissection. Proper integration was assessed by PCR and Western blot analysis. kapi2Δ (6), kapi2Δ (14), pse1–1, PSY580, and PSY581 (15), kapi2Δ (20), kapi114Δ (21), kapi119Δ (22), kapi104A (23), kapi111Δ (24), and kapi108A (25) have been described previously. A pse1–1/kapi121 heterozygous strain was generated by mating PSY581 with pse1–1. After sporulation and tetradsdissection of pse1–1/kapi121, pse1–1 (matting type α) was obtained. A pse1–1/pse1–1 homozygous diploid strain was generated by mating pse1–1 with pse1–1 (α).

The entire coding region of SPO12 was PCR-amplified from S. cerevisiae genomic DNA and inserted into pXY242-GFP (26) using primer-encoded EcoRI and HindIII sites to generate an in-frame fusion with the green fluorescent protein (GFP), pSpo12-GFP, pSpo12-GFP derivatives pSpo12-76–130-GFP, pSpo12-76–105-GFP, pSpo12-76–173-GFP, pSpo12-91–173-GFP, pSpo12-116–173-GFP, pSpo12-76–143-GFP, pSpo12-76–130-GFP, pSpo12-76–153-GFP, and pSpo12-116–153-GFP were generated similarly. A PCR fragment containing a deletion of amino acids 76–130 of Spo12p (76–130) was created by the overlap extension method and cloned into pXY242-GFP as described above to generate pSpo12-76–130-GFP. Alanine-scanning mutants in the region 76–130 in pSpo12-GFP were generated by the same method. Oligonucleotides that sequentially introduced five alanine codons in place of wild-type sequences were used. Detailed information on plasmid construction is available upon request. Yeast strains were transformed with plasmids via the lithium acetate method or electroporation (18).

Spo12p fragments 1–173, 76–130, and 76–130 were PCR-amplified from pSpo12-GFP, pSpo12-76–130-GFP, and pSpo12-76–130-GFP, and ligated into the bacterial expression vector pGEX4T4v (27) using primer-encoded EcoRI and SalI sites, resulting in N-terminal fusions with GST. All constructs were verified by sequencing analysis. hLa-GFP and Lhp1-GFP (26), GST-Kap114p (21), and pGEX-Kap121p (28) were described previously. hLa-GFP and Lhp1-GFP, in the 2.5–4.5 M MgCl2 fractions.

Cells were harvested and resuspended in NETN buffer (100 mM NaCl, 110 mM KOAc, 2 mM MgCl2, 0.1% Tween 20), proteins were washed with NETN and proteins eluted with a step gradient of MgCl2. Proteins of collected fractions, as well as the final NETN wash, were separated by SDS-PAGE and stained with Coomassie Blue. The band representing Kap121p is indicated. The predicted molecular size of Spo12p-PrA is 20 kDa. The antibody was detected with a Western blot with anti-GFP antibody (Cell Signaling). The band size of Spo12p-PrA was determined by mass spectrometry analysis.

Results

Spo12p Binds to Kap121p in Vivo—Spo12p was previously localized to the nucleus (13). Kap proteins involved in import form stable complexes with their substrates in the cytosol, prior to their import into the nucleus. Once inside the nucleus, specific factors disrupt the complex, leading to the deposition of the cargo and the recycling of the empty kap to the cytosol for another round of import. To determine which karyopherins play a role in Spo12p import, we isolated proteins interacting with the cytosolic pool of Spol12p. To do so, we first genomically tagged the Spol12p C terminus in frame with the IgG binding domain of S. aureus PrA. This resulted in the endogenous expression of Spol12p-PrA from the SPO12 promoter. We then

FIG. 1. Cytosolic Spo12p interacts with Kap121p. A, cytosol from cells expressing Spo12p-PrA was incubated with IgG-Sepharose. Bound proteins were washed with TB and eluted with a step gradient of MgCl2. Proteins of collected fractions, as well as the final TB wash, were separated by SDS-PAGE and stained with Coomassie Blue. The band representing Kap121p is indicated. The predicted molecular size of Spo12p-PrA is 20 kDa. The antibody was detected with a Western blot with anti-GFP antibody (Cell Signaling). The band size of Spo12p-PrA was determined by mass spectrometry analysis.

FIG. 2. Spo12p and Kap121p interact directly. Purified recombinant GST or GST-Spo12p were immobilized on glutathione-Sepharose and incubated with Kap121p or Kap114p, as a control. After washing with TB, bound proteins were eluted with sample buffer, separated by SDS-PAGE, and visualized with Coomassie Blue staining.
prepared a post-nuclear, post-ribosomal cytosolic fraction of
this strain. After incubation with IgG-Sepharose, bound pro-
teins were eluted with a step gradient of MgCl₂. The eluted
fractions were analyzed by SDS-PAGE and Coomassie Blue
staining. The results of a typical experiment are shown in Fig.
1A. Spo12p-PrA, by virtue of the high affinity of PrA to IgG, is
expected to elute only with high salt concentrations. Although
a band of the expected size of Spo12-PrA is evident throughout
the elution (indicated by an asterisk in Fig. 1A), immunoblot
analysis indicated that Spo12-PrA eluted only in the expected
fractions (Fig. 1B). A fortuitously concomitant band eluted at
lower MgCl₂ concentrations. A protein with an apparent mo-
lecular mass of ~116 kDa consistently eluted in the 0.1–1 M
fractions, the typical range of MgCl₂ concentrations needed to
disrupt the kap/cargo complex (Fig. 1A). The band was excised,
digested with trypsin, and resulting peptides submitted to
MALDI-TOF MS analysis. 27 peaks of a total of 29 were con-
sistent with the mass of peptides resulting from the theoretical
digest of Kap121p. The amount of isolated Kap121p appears to
be in excess of Spo12p-PrA. However, since this is a semiquan-
titative assay, we cannot comment on the complex stoichiome-
try. Some proteins have decreased solubility in high concentra-
tions of MgCl₂, which results in a lack of correlation between
the amount of isolated proteins and the amount visible on the
gel.

We also sought to determine what fraction of cytosolic
Kap121p was associated with Spo12p. After isolation of
Spo12p-PrA in the manner described above, fractions were run
on an SDS-PAGE gel and transferred to nitrocellulose. Western
blot analysis with a Kap121p antibody show that the majority of
Kap121p present in the cytosol remains in the unbound
fraction and is not, therefore, associated with Spo12p (Fig. 1C).

**Spo12p Binds to Kap121p Directly**—Since the Spo12p/
Kap121p complex was isolated from cytosol, it is possible that
this interaction is bridged by another protein. Therefore, we
investigated whether the interaction between Spo12p and
Kap121p is direct. IgG-Sepharose-bound Spo12p-PrA purified
from yeast cytosol was incubated with purified recombinant
GST-Kap121p, washed, and bound proteins eluted with sample
buffer. After SDS-PAGE and Coomassie Blue staining, we de-
tected binding of GST-Kap121p in addition to the endogenous
Kap121p (data not shown). We next immobilized bacterially
expressed GST and GST-Spo12p on glutathione-Sepharose and
then incubated with recombinant Kap121p or Kap114p, which
was used as a control. The resin was washed, bound proteins
were eluted with sample buffer and analyzed by SDS-PAGE
and Coomassie Blue staining. As seen in Fig. 2, we found that
Kap121p bound to GST-Spo12p. We did not detect either kap
bound to GST alone, while only a small quantity of Kap114p
bound to GST-Spo12p relative to Kap121p. We conclude that
Spo12p and Kap121p interact directly.

**Spo12p Is Mislocalized in a Kap121p Mutant Strain**—To
determine the role of Kap121p in Spo12p nuclear import, we
examined the localization of Spo12p fused to GFP in vivo. In an
initial attempt, the fusion was expressed under the control of
the SPO12 natural promoter from a low copy number (CEN)
plasmid, but no fluorescence was observed (data not shown).
We then constructed a plasmid to overexpress Spo12p fused to
GFP from a high copy number (2µ) plasmid, and this construct
was expressed under the control of the natural SPO12 promoter.
Spo12p-GFP localization was monitored in diploid wild-type (PSY580/PSY581),
spo12Δ homozygous (spo12Δ/spo12Δ) strains, grown at room temperature. Co-
incident Nomarski (DIC) images are also shown (lower panels).

![Figure 4](image)

**Figure 4.** Spo12p is mislocalized in *pse1–1* homozygous diploid cells. Spo12p-GFP localization was monitored in diploid wild-type (PSY580/PSY581), *pse1–1* homozygous (*pse1–1/KAP121*), and *pse1–1* homozygous (*pse1–1/pse1–1*) strains, grown at room temperature. Coincident Nomarski (DIC) images are also shown (lower panels).

**Table 1**

| Colony | Wild-type | pse1–1/pse1–1 |
|--------|-----------|--------------|
| A      | 10.4      | 32.4         |
| B      | 5         | 30           |
| C      | 8.6       | 42.2         |
| D      | 9.5       | 26           |
| Average| 9.5 ± 0.9 | 29.5 ± 3.2   |

Approximately 100 asci in each of four different colonies were counted for each strain. The outstanding values (underscored) were ignored and the remaining three averaged.
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FIG. 5. Identification of the Spo12p NLS. A, deletion mutants of Spo12p were cloned in frame with GFP. Localization of Spo12p proteins was assessed in living cells by virtue of the fluorescent GFP. Localization of Spo12p proteins was assessed in living cells (Fig. 5A). We found the region containing amino acids 76–130 to be both necessary (Spo12p76–130-GFP) and sufficient (Spo12p76–130-GFP) for import into the nucleus (Fig. 5B). However, we detected some mislocalization of Spo12p76–130-GFP to the cytoplasm. This was decreased or abolished when larger constructs were used (Fig. 5A), suggesting that larger domains might stabilize the kap/substrate interaction, leading to higher transport efficiency.

The fusion Spo12p76–130-GFP is mislocalized to the cytosol in pse1–1(Fig. 5B), indicating that it is targeted to the nucleus in a Kap121p-dependent manner. To further address this point, we determined whether this region was also necessary and sufficient for direct interaction with Kap121p. Purified recombinant GST, GST-Spo12p, GST-Spo12p76–130, and GST-Spo12p76–130 were immobilized on glutathione-Sepharose and incubated with purified recombinant Kap121p. After washing, bound proteins were eluted with sample buffer and analyzed by SDS-PAGE and Coomassie Blue staining. This seems to suggest a connection between the amount of Kap121p and the extent of Spo12p nuclear accumulation, consistent with this hypothesis. There is also the possibility that another karyopherin imports Spo12p in this strain. It has been suggested that some karyopherins can compensate for the...

FIG. 6. Kap121p interacts directly with the Spo12p NLS. Purified recombinant GST, GST-Spo12p, GST-Spo12p76–130, and GST-Spo12p76–130 were immobilized on glutathione-Sepharose and incubated with purified recombinant Kap121p. After washing, bound proteins were eluted with sample buffer, separated by SDS-PAGE, and visualized by Coomassie Blue staining.

not shown), suggesting that this protein contains a protease sensitive site. Note that there was binding of Kap121p observed to this fusion and not to GST-Spo12p76–130, even though there was more GST-Spo12p76–130 than GST-Spo12p76–130 or even GST-Spo12p.

In order to more clearly define which residues of Spo12p are necessary for import of Spo12p. Kap121p was first described as a karyopherin whose overexpression was able to substitute for Kap123p in the import of the ribosomal protein L25 NLS (14). Later, also suggesting an overlapping pathway with Kap123p, mRNA export was shown to be defective in a pse1–1/kap123Δ double mutant but not kap123Δ or pse1–1 (15). However, it should be noted that the observed defect may be indirect. It may be that deficient import of a protein involved in mRNA export in pse1–1/kap123Δ would result in the observed phenotype. More recently, Kap121p was shown to be required for transcription factor Pho4p import under phosphate starvation conditions. This role was not dependent on Kap123p, since Pho4p did not interact with Kap123p nor was it mislocalized in kap123Δ (16). This was the first time Kap121p and Kap123p were attributed separate functions. While this report was under revision, Kap121p was shown to be responsible for import of transcription factor Pdr1p (17).

We have shown that Spo12p is imported by Kap121p. We have communomioslated the two proteins from yeast cytosol and reconstituted this complex with recombinant proteins, which indicates this interaction is direct. Spo12p is mislocalized in pse1–1, but not in any of the other kap mutant strains tested (including kap123Δ) supporting a primary role for Kap121p in Spo12p import. The fact that some nuclear accumulation of Spo12p was still observed in pse1–1 remains to be clarified. One hypothesis is that since almost 50% of Kap121p remains in this strain at the permissive temperature (15), the Kap121p still present is sufficient for import of some of the Spo12p-GFP fusion. In fact, when we studied Spo12p localization in diploid cells, a slight accumulation of the fusion in the cytosol of pse1–1/PSY581 was observed, whereas there was none in wild-type cells and comparable levels in pse1–1/ pse1–1 and haploid pse1–1. This seems to suggest a connection between the amount of Kap121p and the extent of Spo12p nuclear accumulation, consistent with this hypothesis. There is also the possibility that another karyopherin imports Spo12p in this strain. It has been suggested that some karyopherins can compensate for the...
absence of others, suggesting they can function in overlapping pathways and that family members have redundant functions (14, 21). Since Kap121p is essential, a complex of Spo12p with another kap in the absence of Kap121p could not be identified in vivo.

Spo12p absence in sporulating diploid cells results in the formation of dyads (a rather unique characteristic). We found that diminished activity of Kap121p in sporulating diploid cells leads to an increased number of asci containing two spores. This observation is consistent with deficient import of Spo12p in pse1–1/pse1–1; nonetheless, we cannot claim it is directly related. Kap121p associates with a large number of proteins in the cytosol (data not shown). These proteins might constitute additional substrates. Identification of these substrates should provide more insight into Kap121p’s distinctive cellular roles and why it is essential for viability. Kap121p might be responsible for import of essential substrates or constitute a backup pathway in the import of a large number of proteins, and thus be vital in compensating for the loss or malfunction of other kaps (for example, see Ref. 21).

We have characterized the Spo12p NLS and determined that the region containing residues 76–130 is both necessary and sufficient for import and for direct binding to Kap121p. When we attempted to determine which residues were important for NLS function by alanine scan mutagenesis, we only identified 1 group (out of 11 generated) of five residues, which, if mutated, led to mislocalization of Spo12p to the cytoplasm (30). One explanation for this observation is that many residues throughout the entire NLS interact with Kap121p. Since the mapped NLS is large, it could be that mutation of five residues to alanine was not sufficient in order to observe a phenotype. On the other hand, the fact that we could not obtain a shorter deletion mutant with nuclear accumulation could mean that a large domain of the protein is needed in order to provide the proper structure necessary for interaction with Kap121p. This study emphasizes the caution one must use while performing NLS analysis. The need for a large domain to confer complete nuclear accumulation has been reported previously. Furthermore, the region containing residues 112–234 of Lhp1p confers Kap108-dependent import. However, some cytoplasmic signal is observed, in contrast with the full-length protein (26). Similarly, residues 283–414 of Npl3p are necessary and sufficient for import and binding to its import receptor, Kap111p.

Again, more accumulation in the cytoplasm is observed in comparison with the full-length protein (30). The Spo12p NLS is located between residues 76 and 130, but only a structural analysis will fully elucidate which residues are required for interaction with Kap121p and thus constitute the actual/minimal NLS. It is, nonetheless, clear that it is different from the cNLS. It is larger, and even though there are some stretches of basic residues in the mapped NLS, these are not sufficient for nuclear accumulation, nor does mutation of these residues lead to mislocalization. Although a large number of proteins have predicted cNLSs, it should not be assumed that they will be imported via the Kapα/β heterodimer.

The mapped Spo12p NLS and the Pho4p NLS do not bear any noticeable resemblance, so a pattern of substrate recognition by Kap121p could not be derived. The Rpl25p and the Pdr1p NLSs were not shown to bind Kap121p directly; nonetheless, they also do not reveal any sequence similarities. NLS mapping of additional substrates might reveal a pattern of recognition by Kap121p. As is the case for Kap121p, other kaps likely recognize numerous dissimilar NLSs. It is, however, probable that only after structures of a large number of kap/substrate complexes are known might we start to better understand nature’s solution to this daunting molecular recognition problem.

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