Protein transport between the nucleus and cytoplasm requires interactions between nuclear pore complex proteins (nucleoporins) and soluble nuclear transport factors (karyopherins, importins, and exportins). Exactly how these interactions contribute to the nucleocytoplasmic transport of substrates remains unclear. Using a synthetic lethal screen with the nucleoporin NUP1, we have identified a conditional allele of NUP82, encoding an essential nuclear pore complex protein in Saccharomyces cerevisiae. This nup82Δ-3 allele also exhibits synthetic genetic interactions with mutants of the karyopherin MSN5. nup82Δ-3 mutants accumulate the Msn5 export substrate Pho4 within the nucleus at non-permissive temperatures. The nuclear import of the RPA complex subunit Rfa2 is impaired in nup82Δ-3 and in mutants of the karyopherin KAP95, but is not affected by the loss of MSN5. Interestingly, deletion of MSN5 results in retention of Rfa2-GFP within the nucleus under conditions in which it normally diffuse out. These data provide evidence that Nup82 is important for Msn5-mediated nuclear protein export and Kap95-mediated protein import. In addition, Msn5 may play a role independent of import in the localization of Rfa2.

The nuclear and cytoplasmic compartments of eukaryotic cells are separated by the concentric lipid bilayers that constitute the nuclear envelope (NE). The regulated movement of polypeptides across the NE generates distinct populations of proteins in the nucleus and cytoplasm, allowing each compartment to carry out distinct activities. This regulated transport between the nuclear and cytoplasmic compartments is facilitated by large, macromolecular protein assemblies termed nuclear pore complexes (NPCs). NPCs perforate the NE, providing a conduit for the translocation of macromolecules across the NE (reviewed in Refs. 1 and 2).

The translocation of proteins across the NPC is mediated by the association of cargo proteins with soluble nuclear transport receptors, interchangeably referred to as importins/exportins or karyopherins (Kaps). These Kap proteins associate with targeting signals on cargo proteins and mediate their targeting to, and translocation across, the NPCs (see Refs. 3 and 4). Fourteen members of the Kap protein family are expressed in Saccharomyces cerevisiae, with each member mediating the translocation of a distinct (but often overlapping) set of cargo substrates across the NPC (5). Kaps mediating nuclear import recognize and bind specific amino acid sequences, termed nuclear localization signals (NLSs), on their substrates and move through the NPC as a Kap-cargo complex. For example, Kap95 (importin-β in vertebrates) binds acidic “classical” NLS (cNLS) sequences through the Kap60 (importin-α adaptor protein), facilitating import of the Kap95-Kap60-cargo complex into the nucleus (6–8). Kaps involved in nuclear export bind to nuclear export signals on their cargoes and also associate with the GTP-bound form of the Ran-GTPase, forming an obligate heterotrimeric essential for cargo export. The canonical example of a Kap with exportin function is Crm1 (also referred to as Xpol in S. cerevisiae), which binds to a short, non-polar nuclear export signal on its export cargo and mediates cargo export in a Ran-dependent manner (9–14). Of the 14 Kaps in budding yeast, Msn5 (also referred to as Kap142) is the only karyopherin thus far described as having both importin and exportin activity (13). A number of Msn5 nuclear export substrates have been identified (14–20), including the transcription factor Pho4, which shuttles between the nucleus and cytoplasm in response to changes in inorganic phosphate levels (18). Msn5 also associates with the RPA complex of nuclear proteins and is implicated in the nuclear import of the replication protein A (RPA) subunits Rfa1 and Rfa2 (13). Whereas many Kaps and their cargoes have been identified, the mechanism by which they are translocated across the NPC remains unclear.

NPC structure and protein composition are conserved across eukaryotic systems, with both yeast and mammalian NPCs being comprised of multiple copies of ~30 different nucleoporin proteins (Nups) that assemble into a pore with 8-fold radial symmetry around a central channel (21, 22). Each Nup also localizes to a particular subcompartment of the NPC (22). The
localized protein import (28, 30) begins at the cytoplasmic face of the NPC, culminating in the emigration of cytoplasmic filaments from the cytoplasmic face and a fibrous "nuclear basket" from the nucleoplasmic face of each NPC. This asymmetric distribution of specific Nups within the NPC may provide a structural and functional framework for the regulated directional transport of large macromolecules across the NPC. In yeast, for example, Nup1 is localized specifically to the nucleoplasmic face of the NPC (22, 23) where it may function as an initial "docking" site for a subset of macromolecules exiting the nucleus or as a final "release" site for some proteins completing translocation across the NPC into the nucleus.

Two general types of Nups are present within the NPC, as determined by the presence or absence of repeated phenylalanine-n-glycine residues (FG repeats) interspersed with regions of polar residues. Approximately half of the Nups identified in both yeast and metazoans have some FG repeats and are referred to as FG-Nups, whereas the remaining "non-FG-Nups" lack repeat sequences. FG repeats directly associate with karyopherins, presumably providing low affinity binding sites for Kaps to mediate Kap/cargo translocation across the NPC (24). Whereas some combinations of FG-Nups are essential, cells with deletions of significant subsets of FG repeats remain viable, suggesting that FG repeats from different Nups may have redundant functions (25). However, the loss of specific FG repeats results in changes in the transport kinetics of some Nups, but not others (25), suggesting that at least some FG repeats have Kap-specific functions. The role of non-FG sequences in NPC function is less clear. Whereas some non-FG-Nups are likely to have a primarily structural function, other sequences that lack FG repeats may act directly in mediating nucleocytoplasmic transport (26–28). The exact function of specific FG- and non-FG-Nups remains to be elucidated.

Nup82 is an essential non-FG-Nup located specifically at the cytoplasmic face of the yeast NPC (22, 29, 30). Nup82 mutants exhibit nuclear transport defects, including accumulation of poly(A)+ RNA in the nucleus, reduced export of both large and small ribosomal subunits, and reduced kinetics of cNLS-mediated protein import (28, 30–35). However, the transport defects inherent in nup82 mutants are not universal, as the export of mRNAs encoding heat shock proteins and the localization of yeast heterogeneous nuclear ribonucleoprotein proteins Nab2 and Npl3 are not detectably altered upon loss of Nup82 function (36, 37). Nup82 is essential for anchoring the FG-Nups Nup159, Nsp1, and Nup116 to the cytoplasmic face of the NPC (36, 38, 39). However, the deletion of FG repeats from these Nups does not alter Kap-mediated protein transport (25), suggesting that the essential function of Nup82 is not simply to localize FG repeats within the NPC. Nup82 itself closely associates with the karyopherin Msn5 in vivo (40) and deletion of Nup82 does not detectably alter NPC or NE morphology (32, 33), providing evidence that Nup82 may be directly involved in Kap-mediated cargo translocation across the NPC.

Here we report the characterization of a conditional nup82 allele identified using a genetic screen for mutants synthetically lethal with a deletion of NUP1. Our nup82 allele also exhibits synthetic lethality with a deletion of MSN5 and suppresses a growth defect associated with an Msn5-CFP chimera. We observe that a nup82 mutant accumulates the Msn5 export substrate Pho4 in the nucleus, suggesting that Nup82 is essential for Msn5-mediated protein export. We also show that the RPA complex component Rfa2 is imported with reduced kinetics in a nup82 mutant. Interestingly, whereas RPA complex proteins interact physically with Msn5 (13), we do not detect an Rfa2-GFP import defect in strains lacking Msn5. Instead, Rfa2 import is altered most significantly by a loss of the karyopherin Kap95. However, the loss of Msn5 results in the retention of Rfa2 in the nucleus of cells in which Kap-mediated transport is inhibited, suggesting that Msn5 may perform a function in RPA complex transport or release that is distinct from the role of Kap95.

**EXPERIMENTAL PROCEDURES**

**Reagents, Strains, Plasmids, and Media—Enzymes for molecular biology were purchased from New England Biolabs (Beverly, MA) and Sigma and were used as per the manufacturer’s instructions. The yeast strains used in this study are listed in Table I. Synthetic lethal mutants were generated by EMS and UV mutagenesis and identified using a colony sectoring assay as described previously (41). Yeast transformants were performed as described (42), as were genetic manipulations, yeast cell culture, and media preparation (43).**

**Plasmids** pPS1730 ([KAP95-GFP UR3]), pPS1070 ([KAPI23-GFP TRPI]), pPS1912 ([MSN5-CFP TRPI]), and pPS1069 ([KAPI21-GFP TRPI]) were kind gifts from P. Silver (Dana-Farber Cancer Institute, Boston, MA), pLH266 ([MSN5-GFP UR3]) from L. Huang (Boston University, Boston, MA), pEB0347 ([PHO4-GFP UR3]) from E. O’Shea (University of California, San Francisco, CA), and pLDB350 (GFP UR3) from L. Lee (Brandeis University, Waltham, MA). pKBB284 (Rfa2-GFP) was generated by homologous recombination of full-length RFA2 including the endogenous promoter into pLDB350. Full-length RFA2 was amplified by PCR with primers 5’-TACCGGGCCCCCCTTT-CCAGGTCGACCGATATCGATAACCTGAAACTCCTGAGGCCCATTATTAAAACCTCAAGG-3’ and 5’-GAATTCGGAACACTCCAGTTAGAGCTTTCTCTTTGTGACGCAAGCCGATCCCGACGACGGAGCTATTATTGTCG-3’. The RFA2 PCR products were pooled and purified using the Rapid PCR Purification System (Marligen Biosciences, Inc., Ijamsville, MD). pLDB350 was cut with SpeI (New England Biolabs, Beverly, MA) and co-transformed into yeast strain W303 with the purified RFA2 PCR products by lithium acetate/polyethylene glycol (LiAc/PEG) transformation (42). Transformants were selected on SD-Ura and grown at 30 °C. RFA2-GFP fusions were screened by whole cell PCR of potential fusions using primer T3 (5’-ATTTAACCTGACTA-AAG-3’) and a primer internal to GFP (5’-CTGACAGAAAATTTGTCGAGGACCCTGAGGATTTTAAACCTCAAGG-3’ and 5’-GAATTCGGGAACACTCCAGTTAGAGCTTTCTCTTTGTGACGCAAGCCGATCCCGACGACGGAGCTATTATTGTCG-3’). The RFA2 PCR products were pooled and purified using the Rapid PCR Purification System (Marligen Biosciences, Inc., Ijamsville, MD). pKBB284 (Rfa2-GFP) was digested with SnaB1 to confirm insertions and transformed into HMY350 (rfa2-215) (44) to test for complementation.

To clone the wild-type NLE24 gene, a yeast genomic library in pRS314

| Yeast strain | Description | Source |
|-------------|-------------|--------|
| W303       | MATa ade2-1 ura3-1 leu2-3,112 trp1-1 his3-11,15 can1-100 | R. Rothstein |
| EY1069     | MATa msn5-Δ;TRPI ura3-52 ade2-101 met1 leu2 | Ref. 18 |
| PSY1102    | MATa rsi1-3/His3 ura3-52 leu2a1 1 trpΔ63 | F. Silver |
| xpl1-1     | MATa xpo1-1;HIS3 spu4/1 LEU2 ura3 trp1 ade2 | Ref. 12 |
| HMY350     | MATa rfa2-215;HIS3 rfa2Δ;TRPI ura3-1 leu2-3 ade2-1 | Ref. 44 |
| KBY103     | MATa nle4/nup82–3 nup1Δ1:LEU2 trp1a1 1 lys2–801 ade2–101 ade3 ura3–52 | This study |
| KBY147     | MATa nup82–3 ade2–1 ura3–1 ura2–3 leu2–3,112 trp1–1 his3–11,15 can1–100 | This study |
| KBY142     | MATa nup1Δ1:His3 ade2–1 ura3–1 ura2–3 ade2–3,112 trp1–1 his3–11,15 can1–100 | This study |
| KBY1242    | MATa nup82–3 ade2–1 ura3–1 ura2–3 leu2–3,112 trp1–1 his3–11,15 can1–100 | This study |
Nup82 Function in Nuclear Transport

R. S. Sikorski and P. Hieter, unpublished data.

RESULTS

nle4-1 is a Temperature-Sensitive nup82 Allele That Exhibits Synthetic Lethality with NUP1 Mutants—In an effort to identify factors that interact functionally with the FG-nucleoporin Nup1 in nucleocytoplasmic transport, we carried out a screen for mutants that are synthetically lethal with a deletion of NUP1 (41). Among the 16 “nup1 lethal” complementation groups (nle mutants) isolated in this screen, five exhibited temperature-sensitive growth when crossed away from the nle4-1 deletion, including nle4-1. Repeated backcrosses into a W303 strain background revealed that nle4-1 temperature sensitivity and nup1Δ synthetic lethality co-segregated, indicating that both phenotypes resulted from a mutation at a single locus (data not shown). To clone the NLE4 gene, we transformed a nup1Δ nle4-1 double mutant with a yeast genomic library and identified a single centromeric plasmid containing NUP82 that complemented both the synthetic lethality with nup1Δ and the temperature-sensitive phenotype of nle4-1 (Fig. 1). We confirmed that nle4-1 is allelic with NUP82 by integrating NUP82 at its genomic locus and observing that the genomic, tagged NUP82 co-segregated with our nle4-1 temperature-sensitive allele through several back crosses. Thus, we refer to the temperature-sensitive nle4-1 allele as nup82-3.

Sequencing of nup82-3 revealed a C to T substitution in the codon for Gln48, resulting in a nonsense mutation. This stop codon generates a Nup82 truncation lacking the 66 C-terminal amino acids of the protein. nup82-3 mutant cells rapidly accumulate poly(A) RNA in the nucleus upon shift to the non-permissive temperature, as assayed by in situ hybridization (data not shown), similar to the RNA transport defect observed for other nup82 alleles with C-terminal truncations (33, 46). The nup82-3 mutation also results in decreased nuclear import kinetics of cNLS-containing proteins (28).

Nup82 Functionally Interacts with Msn5/Kap142—To determine whether nup82-3 influences protein transport mediated by karyopherins other than Kap60/Kap95, we examined the localization of several Kap-GFP fusions in nup82-3 and wild-type cells at 25 and 37°C (Fig. 2A). Kap95-GFP localized similarly in nup82-3 and wild-type cells under both permissive and non-permissive growth conditions, accumulating at the nuclear envelope while also being present in the cytoplasm and the nucleus. A similar accumulation at the nuclear rim was also seen for Kap121-GFP and Kap123-GFP in wild-type cells and in nup82-3 cells at 25°C. However, accumulation of Kap121-GFP at the NE appeared slightly decreased and Kap123-GFP was not detectable at the nuclear rim in nup82-3 cells shifted to 37°C. Finally, Msn5-GFP was concentrated within the nucleus with little cytoplasmic accumulation in wild-type and nup82-3 cells at both the permissive and restrictive temperatures. The
altered localization of Kap121 and Kap123 in nup82-3 cells implicates Nup82 in Kap121- and Kap123-mediated nuclear transport. The lack of Kap95 and Msn5 localization changes suggest that Nup82 may play a lesser role in the transport of these Kaps. However, nup82-3 was shown previously to have a decreased rate of Kap95-mediated nuclear import (28). In addition, nup82-3 interacts genetically with MSN5 mutant alleles (see below). Finally, none of the Kaps tested here have substantially altered nuclear versus cytoplasmic localization patterns in the nup82-3 mutant. These observations indicate either that subtle defects in nucleocytoplasmic transport kinetics of specific karyopherins, such as those observed previously for Kap95-mediated transport of cNLS-containing cargoes in nup82-3 (28), are not detectable by observing steady-state localization of Kap-GFP fusions or that nup82-3 slows the rate of Kap import and export by equal amounts, thus not altering the relative concentration of nuclear versus cytoplasmic Kaps.

Whereas none of the Kaps exhibited detectable steady-state alterations in nuclear versus cytosolic localization in the nup82-3 mutant, the presence of the nup82-3 allele did noticeably alter the growth rate of cells expressing an Msn5 fusion (Fig. 2B). Whereas the expression of an Msn5-CFP construct (40) confers a slow growth phenotype on wild-type cells, the presence of this Msn5 fusion partially suppresses the growth defect of the nup82-3 allele at 25 °C, suggesting a functional interaction between Nup82 and Msn5. To identify additional genetic interactions between MSN5 and NUP82, we tested for a synthetic phenotype in nup82-3 msn5Δ double mutant cells. To this end, we generated a haploid nup82-3 msn5Δ strain containing a URA3 NUP82 plasmid. This double mutant strain was then plated on 5-FOA to select against the plasmid and was scored for growth compared with wild-type, msn5Δ, nup82-3, and msn5Δ nup82-3 cells containing pKBB329 (CEN URA3 NUP82) were plated on FOA and incubated for 72 h at 25 °C and 48 h at 37 °C.

**Fig. 2.** nup82-3 interacts genetically with MSN5 mutants. A, wild-type (W303) and nup82-3 (KBY417) cells expressing pLH266 (Msn5-GFP), pPS1730 (Kap95-GFP), pFS1069 (Kap121-GFP), and pFS1070 (Kap123-GFP) were assayed by fluorescence microscopy on living cultures in early log phase after incubation at 25 °C or shift to 37 °C for 3 h. B, wild-type (W303) and nup82-3 (KBY417) cells were transformed with pPS1912 (TRP1 MSN5-CFP) and grown overnight in liquid SC-Trp at 25 °C. 5-Fold serial dilutions of a 1.0 A600 overnight culture were plated on YPD at 30 °C and photographed after 48 h. C, 5-fold dilutions of exponentially growing cultures of wild-type, msn5Δ, nup82-3, and msn5Δ nup82-3 cells containing pKBB329 (CEN URA3 NUP82) were plated on FOA and incubated for 72 h at 25 °C and 48 h at 37 °C.
the nucleoporin Nup82 in Msn5 function, but do not differentiate between a potential requirement of Nup82 for Msn5-mediated protein import or export. To determine whether Nup82 is involved in Msn5-mediated nuclear export and/or import, we expressed GFP fusions with Msn5 transport cargoes as markers for Msn5-dependent transport. We then examined the subcellular localization of each cargo-GFP reporter in nup82-3 mutants under both permissive and non-permissive growth conditions.

Msn5 functions as a nuclear export receptor for a number of proteins, including the transcription factor Pho4, which is rapidly exported from the nucleus of cells grown in the presence of inorganic phosphate (47). To determine whether Nup82 is important for efficient nuclear export of Pho4, we expressed Pho4-GFP in wild-type, msn5Δ, and xpo1Δ cells expressing Pho4-GFP were observed by direct fluorescence microscopy after incubation at 25 or shift to 37 °C for 2 h, and prepared for immunoelectron microscopy (see “Experimental Procedures”). Cells were probed with polyclonal anti-GFP directly conjugated to 8-nm colloidal gold. Scale bar represents 200 nm.

Because a loss of Nup82 function resulted in impaired Pho4-GFP nuclear export, we sought to determine whether this export defect was the result of a loss of a specific step in translocation. To this end, we performed immunoelectron microscopy to examine the subcellular distribution of Pho4-GFP in wild-type and nup82-3 cells. As Nup82 is localized to the cytoplasmic face of the NPC (22, 29, 30), Nup82 is likely to function at a late step in substrate translocation out of the nucleus. Thus, we predicted that a loss of Nup82 would result in an accumulation of the Pho4-GFP export substrate within the central channel of the NPC, suggesting that Pho4 began, but was unable to complete, translocation across the NPC. Immunoelectron microscopy revealed an accumulation of Pho4-GFP in the nucleus of nup82-3 cells incubated at 37 °C (Fig. 3B), whereas few Pho4-GFP proteins were detected in nuclei of wild-type cells grown under identical conditions. Surprisingly, the Pho4-GFP was distributed homogeneously within the nucleus of cells lacking Nup82 activity, but sug-
gest that a step prior to entry into the NPC may be influenced by the decreased Nup82 function.

Nucleocytoplasmic Transport of Rfa2-GFP Is Altered in nup82-3, msn5Δ, and kap95ts Mutants—Whereas our data suggest that Nup82 function is required for efficient export of an Msn5 cargo from the nucleus, these data do not address whether the role of Nup82 in Msn5-mediated transport is specific to export or is because of a general loss of Msn5 transport through the NPC. To determine whether the nup82-3 mutation alters the ability of Msn5 to import substrate, we constructed a fusion between GFP and the RPA complex subunit Rfa2, for which Msn5 has been proposed to function as a nuclear import receptor (13). To test the functionality of the Rfa2-GFP fusion, we transformed the RFA2-GFP plasmid into an rfa2-212 temperature-sensitive mutant (44) and assayed for the ability of the Rfa2-GFP fusion to complement the conditional rfa2-212 temperature sensitivity. Because Rfa2 is an essential nuclear protein with functions in DNA replication, recombination, and repair (44), these data suggest that Rfa2-GFP fusion is being efficiently imported into the nucleus.

To analyze whether Nup82 is important for Rfa2 nuclear import, we expressed the Rfa2-GFP fusion in wild-type, nup82-3, msn5Δ, and kap95ts mutant cells and examined Rfa2-GFP localization under steady-state conditions using fluorescence microscopy on living cells. In wild-type cells, Rfa2-GFP was localized primarily to the nucleus but exhibited some cytoplasmic accumulation at all temperatures (Fig. 4B, top row). A similar nuclear and cytoplasmic localization pattern was observed in msn5Δ and nup82-3 mutant cells at both the permissive and restrictive temperatures (Fig. 4B), suggesting that steady-state Rfa2 localization is not significantly altered from wild type in the absence of functional Msn5 and Nup82. In contrast, kap95ts cells accumulated Rfa2-GFP in the cyto-
plasm, with very little nuclear accumulation (Fig. 4B). We observed a similar cytoplasmic localization of Rfa2-GFP in other kap95 mutant alleles (data not shown). Kap95 has been shown previously to be important for the nuclear import of RPA complex proteins into the nucleus in the absence of Msn5 (13). Our observations suggest that Kap95 performs an important role in Rfa2 import, even when functional Msn5 is present.

Whereas these data implicate Kap95 in mediating efficient Rfa2-GFP nuclear import, experiments addressing steady-state localization of a reporter protein may not be sensitive enough to identify subtle alterations in Rfa2-GFP transport kinetics in other nuclear transport mutants, including nup82-3 or msn5. To address the relative rates of Rfa2 import in wild-type, nup82-3, msn5, and kap95 mutant cells, we performed a kinetic import assay (28) using Rfa2-GFP as the import substrate. Briefly, early log-phase cells expressing Rfa2-GFP were incubated at either 25 or 37 °C for 2 h, then treated with sodium azide and 2-deoxyglucose to inhibit nuclear import and allow diffusion of soluble proteins between the nuclear and cytoplasmic compartments. After 1 h in sodium azide, 2-deoxyglucose, cells were washed to remove the drugs, placed in media containing glucose, and incubated at either 25 or 37 °C. Cells were assayed for nuclear accumulation of Rfa2-GFP in the presence of drug (t = 0) and at 2-4-min intervals immediately following drug removal. At 25 °C, both the wild-type and nup82-3 strains exhibited nuclear Rfa2-GFP accumulation in 50% of cells within 5-7 min after drug removal, and reached a maximum level of nuclear accumulation within 10-12 min (Fig. 4C, left panel). In contrast, incubation at 37 °C resulted in decreased kinetics of Rfa2 import in the nup82-3 strain (Fig. 4C, right panel). At 37 °C, 50% of wild-type cells exhibited nuclear Rfa2-GFP accumulation within less than 5 min and greater than 70% of cells had fluorescent nuclei within 10 min. In the nup82-3 strain, 9 min were required to reach 50% fluorescent nuclei, and 70% nuclear Rfa2-GFP accumulation was not attained until 30 min after transport inhibitors were removed. Thus, whereas Rfa2-GFP steady-state localization is not detectably altered in the absence of functional Nup82, the kinetics of Rfa2 import are significantly slowed in the nup82-3 mutant, indicating that Nup82 is important for efficient nuclear import of Rfa2-GFP.

Careful observation of the transport kinetics of Rfa2-GFP in msn5Δ and kap95 mutant cells also confirmed the importance of these karyopherins in Rfa2 transport, but suggested that the role of each Kap in Rfa2 localization may differ. In cells containing a kap95 mutant allele, the absence of wild-type Kap95 resulted in a dramatic loss of Rfa2 import (Fig. 4C, open triangles). The fraction of kap95 cells exhibiting nuclear Rfa2-GFP fluorescence did not rise above 20% at 25 °C or 10% at 37 °C, even after 30 min incubation in the absence of drug. These results, along with our observation of steady-state cytoplasmic localization of Rfa2-GFP in kap95 mutant cells (see Fig. 4B), provide strong evidence that Kap95 is the primary karyopherin functioning in Rfa2-GFP nuclear import. In contrast, msn5Δ cells exhibited a very different nuclear transport defect. Strains lacking Msn5 retained high levels of Rfa2-GFP nuclear fluorescence (>70%) after incubation in sodium azide, 2-deoxyglucose for 90 min (Fig. 4C, closed triangles). This high level of nuclear fluorescence was retained after the metabolic inhibitors were removed. These data suggest that the Kap- and energy-independent diffusion of Rfa2-GFP out of the nucleus that normally occurs upon inhibition of protein import is altered in the absence of Msn5. Yoshida and Blobel (13) have provided evidence that both Msn5 and Kap95 can physically associate with RPA complex proteins. Our results provide further evidence that both Msn5 and Kap95 are important for Rfa2 localization, but suggest that the two karyopherins may have distinct roles in regulating or mediating nucleocytoplasmic localization of Rfa2.

Loss of Msn5 Does Not Alter Rfa2-GFP Import Kinetics, but Does Influence Rfa2 Nuclear Retention—The nuclear localization of Rfa2-GFP in msn5Δ cells after 90 min in deoxyglucose/azide suggested that Rfa2-GFP was diffusing out of the nucleus more slowly than in wild-type cells, Rfa2-GFP was being actively re-imported into the nucleus even in the presence of import-inhibiting drugs, or that some combination of changes in export and import kinetics had occurred. To investigate whether Rfa2-GFP export was occurring at all in the absence of Msn5, we treated wild-type and msn5Δ cells with 2-deoxyglucose and sodium azide as described previously (see also “Experimental Procedures”) and examined Rfa2-GFP localization over a 10-h time course. If Rfa2-GFP was being completely retained in the nucleus or if Rfa2-GFP import remained more rapid than export, a continued high level of nuclear localization would be observed even with longer incubations in drug. On the other hand, if the absence of msn5 simply slowed the kinetics of Rfa2-GFP diffusion out of the nucleus or allowed export to occur at only a slightly faster rate than import, we would expect to see an eventual decrease in the percentage of cells exhibiting nuclear fluorescence over time in the presence of metabolic inhibitors. In wild-type cells, nuclear concentration of Rfa2-GFP was observed in fewer than 50% of cells after only 30 min in the presence of the drugs. The percentage of wild-type cells with nuclear Rfa2-GFP continued decreasing to <20% after 4 h (Fig. 5A, open circles). Rfa2-GFP also exited the nucleus in strains lacking Msn5, but at a much slower rate (Fig. 5A, closed circles). More than 50% of msn5Δ cells retained a nuclear accumulation of Rfa2-GFP after over 5 h in the presence of metabolic inhibitors and almost 40% retained nuclear fluorescence after 10 h. Thus, Rfa2-GFP can still exit the nucleus in the absence of Msn5, but does so at a greatly reduced rate.

To confirm that the nuclear Rfa2-GFP retention observed in the msn5Δ mutant was not due simply to a universal inhibition of diffusion through the NPC, we expressed a cNLS-GFP fusion in wild-type and msn5Δ cells, treated with 2-deoxyglucose and sodium azide, and observed the nuclear versus cytoplasmic localization of the cNLS-GFP reporter. Upon treatment with deoxyglucose and azide, the cNLS-GFP fusion rapidly equilibrated between the nucleus and cytoplasm in both wild-type and msn5Δ cells (Fig. 5B, right panels), with the nuclear concentration of cNLS-GFP disappearing within 30 min of treatment with metabolic inhibitors. In contrast, Rfa2-GFP remained concentrated in the nucleus in msn5Δ cells after treatment with metabolic inhibitors (Fig. 5B, top left). Rfa2-GFP also diffused rapidly out of the nucleus in wild-type cells treated with deoxyglucose and azide and accumulated in small cytoplasmic patches in some cells (Fig. 5B, bottom left).

Having observed that Rfa2-GFP eventually does equilibrate between the nucleus and cytoplasm in msn5Δ cells treated with metabolic inhibitors, we sought to determine whether the loss of Msn5 affected the kinetics of Rfa2-GFP re-import into the nucleus. We grew wild-type and msn5Δ cells to log-phase at 25 °C and treated the cells with deoxyglucose and sodium azide as described above, except that both metabolic inhibitors were washed away after 4 h and cells were retained at 0 °C for an additional 14 h prior to assaying for import. After this long incubation, less than 20% of msn5Δ and 15% of wild-type cells retained nuclear fluorescence (Fig. 6, t = 0). Cells were then resuspended in media containing glucose at 25 °C, and the percent of cells exhibiting nuclear accumulation of Rfa2-GFP was recorded at 2–3-min intervals for 35 min. Both the msn5Δ and wild-type cultures showed nuclear localization of Rfa2-
GFP in /H11022 50% of cells within 10 min and in 70% within 15 min, exhibiting essentially no difference in Rfa2-GFP nuclear import rate. Thus, the non-essential karyopherin Msn5 does not appear to be required for the efficient nuclear import of the RPA complex protein Rfa2, as an msn5Δ strain exhibits Rfa2-GFP import kinetics that are virtually indistinguishable from the Rfa2-GFP import rate in an isogenic wild-type strain.

DISCUSSION

The transport of proteins between the cytoplasm and nucleoplasm of eukaryotic cells requires functional interactions between soluble transport factors (Kaps) and insoluble nuclear pore complex proteins (Nups). Several Nups proposed to be involved in protein translocation across the NPC exhibit asymmetric distribution, including Nup82, which is present at the cytoplasmic face of the NPC. We report a genetic interaction between NUP82 and a deletion of a nucleoplasmically oriented nucleoporin, NUP1. Both Nup82 and Nup1 are asymmetrically distributed within the NPC (22, 29, 30), placing them in physical contexts important for the docking, vectorial movement, and release of substrates being translocated through the NPC. However, their position at opposite faces of the NPC makes a physical interaction between the two unlikely. Indeed, we have been unable to co-precipitate Nup1 and Nup82 from yeast extracts using several different methods (data not shown). However, the genetic interaction between these Nups suggests a functional relationship. Nup1 is a member of a family of nucleoporins containing repeated phenylalanine-glycine sequences (FG repeats). Kap-cargo complexes have been shown to associate directly with FG repeats from a number of distinct nucleoporins (2, 48, 49), including Nup1 (27, 50). However, a C-terminal region of Nup1 that lacks FG repeats appears to be more important for Kap95/Kap60 binding and import than the FxFG repeats (25, 27). Nup82 also does not contain FG repeats and its role in nuclear transport remains unclear. Nup82 physically associates with FG-Nups within the NPC, including Nsp1, Nup100, Nup116, and Nup159 (30, 32, 36, 39), and is essential for the localization of both Nup116 and Nup159 at NPCs (36, 39). These data suggest that one role of Nup82 may be to anchor specific FG-Nups at specific locations on the cytosolic face of the NPC where those Nups can interact with transport factors in mediating translocation. However, the FG repeats of these Nups are also not essential for the translocation of several substrates (25). The synthetic lethal interaction we report between nup82-3 and nup1Δ is likely the consequence of reduced activity of non-FG domains of specific Nups (Nup1 and Nup82 or one or more Nup82-associated Nups) at
the nuclear and cytosolic faces of the NPC, effectively resulting in the loss of an essential transport pathway across the NPC.

Paramount to understanding how Kap-cargo complexes are translocated across the NPC is determining if specific Nups play a role in mediating the translocation of specific Kaps, whether by providing initial docking sites for association of Kap-substrate complexes, participating in translocation through the NPC central channel, or influencing Kap/substrate release after translocation. Our observation of synthetic genetic interactions between nup82-3 and chimeric msn5, combined with the close association between Nup82 and Msn5 in vivo (11), suggests a functional interaction between the Nup and Kap. The recent description of Msn5 as the first yeast karyopherin to mediate nuclear import and export of distinct transport substrates (Refs. 13 and 28; see Introduction) appeared to provide us with an opportunity to examine whether individual Nups are important for the transport of one type of karyopherin (i.e. Msn5) through the NPC specifically in one direction but not the other. We selected Pho4 as a marker for Msn5-mediated export, as Pho4 shuttles between the nucleus and cytoplasm in response to phosphate availability, and Msn5 directs Pho4 nuclear export in the presence of phosphate (18).

We observe that nup82-3 mutants accumulate nuclear Pho4-GFP after shift to the restrictive temperature, whereas relatively few wild-type cells exhibit nuclear accumulation of Pho4-GFP under identical conditions, suggesting that functional Nup82 is important for efficient Msn5-mediated export. The Pho4-GFP export defect in nup82-3 is not as severe as in cells lacking Msn5 (compare cytoplasmic fluorescence in nup82-3 and msn5A, Fig. 3), suggesting either that a less efficient Msn5 export pathway exists, which by-passes Nup82, or that the nup82-3 mutant retains some Nup82 activity, even at the restrictive temperature. Msn5 associates closely with both FG-Nups and non-FG-Nups (40, 48), providing a large number of candidate nucleoporins that may function in mediating Msn5 translocation under conditions of reduced Nup82 activity.

We observe that a functional Rfa2-GFP fusion, which provides an essential component of the nuclear RPA complex, undergoes a reduced rate of nuclear import in nup82-3 mutant cells when compared with wild-type cells at the restrictive temperature. We could not detect a significant change in steady-state localization of Rfa2-GFP between nup82-3 and wild-type strains. These data suggest that Nup82 is also important for protein import of Rfa2-GFP, although significant translocation of Rfa2 into the nucleus continues in the nup82-3 mutant. Previous studies examining the role of Nup82 in protein import have produced similar results with classical NLS (cNLS)-containing substrates. NUP82 C-terminal truncations similar to nup82-3 also do not exhibit changes in steady-state localization of several cNLS-containing substrates (30, 36). However, kinetic measurements previously revealed a significantly decreased rate of nuclear cNLS-GFP accumulation in the absence of functional Nup82 (28). Our observations not only provide further evidence for the importance of Nup82 in nuclear protein import, but also re-emphasize the importance of utilizing kinetic measures to identify subtle alterations in nuclear transport rates.

The inhibition of Pho4-GFP export and Rfa2-GFP import in nup82-3 provides further evidence that Nup82 is critical for bi-directional translocation through the NPC. NUP82 mutants have previously been shown to exhibit defects in the export of poly(A)+ RNA, as well as small and large ribosomal subunits (30–35) and in the import of cNLS-containing proteins (28). Nup82 is not essential for all nucleocytoplasmic transport, however, as heat shock protein-encoding mRNAs and some proteins traverse the NPC in NUP82 mutants (36, 37). Our results identify two additional substrates dependent upon Nup82 for normal transport kinetics. Our observations also add to evidence that Nup82 may be essential for nuclear export, but less important for nuclear import, as we readily detect nuclear accumulation of export substrates under steady-state conditions, but require kinetic analyses to observe import defects.

Yoshida and Blobel (13) observed that RPA complex proteins Rfa1, Rfa2, and Rfa3 coprecipitate with Msn5 and that a deletion of MSN5 results in increased cytoplasmic accumulation of Rfa1-PRA and Rfa2-PRA fusions, suggesting that Msn5 is important for nuclear import of RPA complex proteins. Surprisingly, we did not observe steady-state or kinetic defects in Rfa2-GFP import in msn5Δ mutant strains (Figs. 4 and 6), suggesting that Msn5 may not be the primary karyopherin mediating Rfa2 import. Yoshida and Blobel (13) also reported an association between RPA complex proteins, including Rfa2, and a second karyopherin, Kap95. Our observation of severe Rfa2-GFP transport defects in kap95 mutants (Fig. 4, B and C) confirms the importance of this Kap95-RPA complex interaction and suggests that Kap95 is critical for Rfa2 nuclear import. Rfa2 is a nuclear protein essential for cell viability and Msn5 is a non-essential karyopherin (51), suggesting that at least one Kap in addition to Msn5 is required to transport Rfa2 into the nucleus under some conditions. In addition, the vertebrate homolog of Kap95, importin-β, is essential for the nuclear import of RPA in egg extracts of Xenopus laevis (52), suggesting that Kap95-mediated transport of the RPA complex may represent an evolutionarily conserved transport mechanism.

Our observation that the diffusion of Rfa2-GFP out of the nucleus is hampered in cells lacking Msn5 (Fig. 5) suggests that an interaction between Msn5 and RPA complex proteins independent of nuclear import may regulate RPA localization or activity. Treatment of yeast with metabolic inhibitors results in a loss of karyopherin-mediated nuclear import and export (53). Under these conditions soluble proteins will equilibrate between the nucleus and cytoplasm via diffusion across the NPC. The rate of diffusion of a protein out of the nucleus will be influenced by two primary factors: 1) the size of the protein, including other polypeptides with which it is associated, relative to the diameter of the NPC channel; and 2) the association of the protein with insoluble structures in the nucleus. Thus, the reduced kinetics of Rfa2-GFP diffusion out of the nucleus in cells lacking Msn5 implicates Msn5 in the dissociation of Rfa2 from a multiprotein complex within the nucleus or in the regulation of NPC channel size relative to the Rfa2-containing complex. Further experiments are necessary to differentiate between these two possible nuclear retention mechanisms.

In summary, our results implicate Nup82 in the regulation of both Msn5-mediated protein export and Kap95-mediated protein import through the NPC. Remaining to be determined are whether Nup82 directly associates with Msn5 and/or Kap95 and whether additional Nup82-associated nucleoporins bind these Kaps during nuclear protein import or export. In addition, our data suggest that Msn5 may play a role outside of protein translocation, possibly in regulating complex formation or dissociation in the nucleus or in influencing NPC dynamics. Experiments exploring the influence of Msn5 on the diffusion of additional proteins from the nucleus, characterizing the diffusion channel size in msn5 mutants, and identifying other factors that regulate Msn5 activity should provide insights into Msn5 function.

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