Dynamic Localization of the Nuclear Import Receptor and Its Interactions with Transport Factors

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Abstract. Characterization of the interactions between soluble factors required for nuclear transport is key to understanding the process of nuclear trafficking. Using a synthetic lethal screen with the *rna1-1* strain, we have identified a genetic interaction between Rna1p, a GTPase activating protein required for nuclear transport, and yeast importin-β, a component of the nuclear localization signal receptor. By the use of fusion proteins, we demonstrate that Rna1p physically interacts with importin-β. Mutants in importin-β exhibit in vivo nuclear protein import defects, and importin-β localizes to the nuclear envelope along with other proteins associated with the nuclear pore complex. In addition, we present evidence that importin-α, but not importin-β, mislocalizes to the nucleus in cells where the GTPase Ran is likely to be in the GDP-bound state. We suggest a model of nuclear transport in which Ran-mediated hydrolysis of GTP is necessary for the import of importin-α and the nuclear localization signal–bearing substrate into the nucleus, while exchange of GDP for GTP on Ran is required for the export of both mRNA and importin-α from the nucleus.

TRANSPORT of proteins from the cytoplasm to the nucleus is a regulated process that depends not only on an intrinsic signal in the protein to be imported, but also on a number of cellular proteins. Many nuclear proteins have a nuclear localization signal (NLS)[1] that is required for their proper targeting to the nucleus (Hall et al., 1984; Kalderon et al., 1984; Silver et al., 1984). Alternatively, nuclear proteins lacking an NLS may be carried into the nucleus in a complex with an NLS-bearing protein (Dingwall et al., 1982). Generally, nuclear protein import can be divided into two steps: (*a*) binding of the NLS-bearing protein to a cytosolic NLS receptor and translocation to the nuclear pore complex (NPC) and (*b*) transport of the NLS-bearing protein into the nucleus.

The initial binding step of nuclear protein import requires at least two proteins in addition to the NLS-bearing protein substrate. The first is a 60-kD protein that will be referred to here as importin-α (also known as karyopherin-α in vertebrates and as Kap60p and Srp1p in yeast) (Adam and Gerace, 1991; Görlich et al., 1994; Yano et al., 1994; Radu et al., 1995). The yeast importin-α was originally identified as a suppressor of RNA polymerase I (Yano et al., 1992). Mutants in importin-α show defects in nuclear protein import and nuclear structure and also exhibit a G2/M cell cycle arrest (Yano et al., 1994; Küssel and Frasch, 1995; Loeb et al., 1995). Purified importin-α binds NLSs and targets an NLS-bearing substrate to the nuclear pore in in vitro import assays in the presence of a second NLS receptor subunit, importin-β (also known as karyopherin-β in vertebrates and Kap95p in yeast) (Görlich et al., 1995*b*; Imamoto et al., 1995; Moroianu et al., 1995). The importin-β protein has a molecular weight of ~95,000 and has a weaker affinity for NLSs than importin-α (Chi et al., 1995; Görlich et al., 1995*a*; Imamoto et al., 1995; Moroianu et al., 1995). Importin-β is most likely responsible for targeting of the complex to the NPC, as it has been shown that importin-α cannot deliver NLS-containing substrates to the NPC in the absence of importin-β in vitro (Enenkel et al., 1995; Görlich et al., 1995*b*; Moroianu et al., 1995). In addition, importin-β binds specific nucleoporins (Iovine et al., 1995; Kraemer et al., 1995; Radu et al., 1995; Rexach and Blobel, 1995).

The second step of import requires the small GTPase, Ran (the yeast homolog is called Gsp1p), and a number of proteins that associate with it (Drivas, 1990; Belhumeur et al., 1993; Moore and Blobel, 1993, 1994). In in vitro assays, the addition of Ran stimulates transport of NLS substrate already bound at the pore (Melchior et al., 1993; Moore and Blobel, 1993, 1994). Overexpression of mutant Ran locked in the GTP-bound state in yeast leads to defects in both nuclear protein import and mRNA export (Schlenstedt et al., 1995*a*). Rna1p, a cytoplasmic protein, has recently been shown to be directly involved in nuclear import and to act as a GTPase activating protein (GAP) for

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1. *Abbreviations used in this paper*: ECL, enhanced chemiluminescence; FOA, 5′ fluoro-orotic acid; GAP, GTPase activating protein; GST, glutathione-*S*-transferase; NLS, nuclear localization signal; NPC, nuclear pore complex; Ran-GTP, GTP-stabilized form of Ran.

Table I. Strains Used in This Study

Name	Genotype	Reference
PSY137	*ade2 ade3 can1 leu2 lys2 ura3 MAT a*	Kranz and Holm, 1990
EE1b (PSY544)	*rna1-1 his4 his7 tyr1 ura3 MAT a*	Traglia et al., 1989
FY23 (PSY580)	*leu2Δ1 trp1Δ63 ura3-52 MAT a*	Winston et al., 1995
FY86 (PSY581)	*his3Δ200 leu2Δ1 ura3-52 MAT α*	F. Winston, unpublished data
PSY615	*ade2/ade2 ade8/ADE8 his3/his3 leu2/leu2 lys1/lys1 ura3/ura3 MAT a/α*	Henry et al., 1996
2bx3b (PSY667)	*rna1-1/rna1::LEU2 ade2/ade2 MAT a/α*	Traglia et al., 1989
PSY713	*prp20-1 leu2 trp1 ura3 MAT α*	This study
PSY714	*rna1-1 leu2 trp1 ura3 MAT a*	Corbett et al., 1995
PSY868	*rna1-1 ade2 leu2 leu2 ura3 MAT α*	This study
PSY869	*rna1-1 ade2 ade3 leu2 ura3 MAT α* carrying pPS714	"
PSY870	*rna1-1 ade2 ade3 leu2 ura3 MAT a* carrying pPS714	"
PSY871	*rna1-1 rsl1-1 ade2 ade3 leu2 ura3 MAT a* carrying pPS714	"
PSY872	*rna1-1 rsl1-2 ade2 ade3 leu2 ua3 MAT a* carrying pPS714	"
PSY873	*rna1::LEU2 ade2 ura3* Tyr⁻ Gal⁻ *MAT α* carrying YCp*RNA1*	"
PSY874	*rna1-1 ade2 ade3 leu2 ura3 MAT α*	"
PSY875	*rna1-1 ade2 ade3 leu2 lys2 ura3 MAT α*	"
PSY876	*rna1-1 rsl1-1 ade2 ade3 leu2 lys2 ura3* carrying pPS714	"
PSY877	*rna1-1 rsl1-2 ade2 ade3 leu2 lys2 ura3* carrying pPS714	"
W303 (PSY878)	*ade2/ade2 his3/his3 leu2/leu2 trp1/trp1 ura3/ura3 MAT a/α*	J. Loeb, unpublished data
PSY879	*rna1Δ359-397-HIS3 ade2 ade8 leu2 lys1 ura3 MAT α*	This study
PSY880	*rna1Δ359-397-HIS3 ade2 leu2 trp1 ura3 MAT α*	"
PSY881	*rna1Δ359-397-HIS3 ade2 ade3 leu2 ura3 MAT a*	"
PSY882	*Δrsl1::HIS3/RSL1 ade2/ade2 leu2/leu2 trp1/trp1 ura3/ura3 MAT a/α*	"
PSY883	*Δrsl1::HIS3 ade2 leu2 trp1 ura3 MAT α* carrying pPS890	"
PSY885	*rna1-1 RSL1-LEU2 ade2 ade3 ura3 MAT a*	"

All strains used for this study are shown. For detailed information on the construction of strains refer to the Materials and Methods section.

yeast Ran (Becker et al., 1995; Bischoff et al., 1995*a*; Corbett et al., 1995). In in vivo assays, mutants in Rna1p exhibit defects in both protein import and mRNA export (Hopper et al., 1978; Amberg et al., 1992; Corbett et al., 1995), similar to the defects seen when the GTP-stabilized form of Ran (Ran-GTP) is overexpressed. These results are consistent, since both situations lead to an excess of Ran-GTP in the cell. A second protein associated with Ran is the cytosolic 34-kD protein, Yrb1p (yeast Ran binding protein 1, known as RanBP1 in vertebrates) (Coutavas et al., 1993; Lounsbury et al., 1994; Bischoff et al., 1995*b*; Ren et al., 1995; Schlenstedt et al., 1995*b*). Yrb1p specifically associates with the GTP form of Ran and acts as a GAP activating protein, stimulating the GAP activity of Rna1p up to 10-fold (Bischoff et al., 1995*b*; Schlenstedt et al., 1995*b*). Mutants in Yrb1p have both import and export defects (Schlenstedt et al., 1995*b*). Taken together, these results suggest that Ran-mediated hydrolysis of GTP is important for proper nuclear protein import and mRNA export.

The molecular details of translocation of an NLS-bearing substrate into the nucleus are unclear. It appears that importin-α migrates into the nucleus with the NLS-bearing substrate during the translocation step (Görlich et al., 1995*b*). Presumably, importin-α is later exported to the cytoplasm to repeat its role in carrying NLS-containing proteins to the NPC. In addition, the localization of Ran to the cytoplasm as well as the nucleus suggests that Ran may also move in and out of the nucleus in a manner similar to importin-α (Ren et al., 1993). In fact, Ran appears to enter the nucleus during translocation in at least one in vitro system (Melchior et al., 1995). Interestingly, the only known exchange factor for Ran, RCC1 (yeast name: Prp20p) is located in the nucleus (Ohtsubo et al., 1989). As no additional exchange factors or GAPs for Ran have been identified (i.e., a nuclear GAP or cytoplasmic exchange factor), it is possible that exchange occurs only in the nucleus and hydrolysis occurs only in the cytoplasm if Ran is free to move between the nucleus and cytoplasm.

It is not clear how the proteins required for the binding of an NLS-bearing protein interact with proteins required for translocation, or indeed, if they directly interact at all. Only very recently has it been shown that Ran associates with the NLS receptor (Rexach and Blobel, 1995). In this study we report both a genetic and a physical interaction between Rna1p and yeast importin-β. We show that the importin-β protein localizes to the nuclear envelope and colocalizes with importin-α and Nup159p. Importin-β mutants exhibit defects in nuclear protein import in vivo. In addition, by investigating the localization of importin-α and importin-β in a number of mutants defective for nuclear protein import and/or mRNA export, we have found that when exchange of GDP for GTP on Ran is blocked, importin-α is mislocalized to the nucleus. This supports a model in which hydrolysis of GTP by Ran occurs only in the cytoplasm, and exchange of GDP for GTP occurs only in the nucleus.

Materials and Methods

All chemicals were obtained from Sigma Chemical Co. (St. Louis, MO) unless otherwise noted. All DNA manipulations were performed according to standard methods (Sambrook et al., 1989), and all yeast media were prepared by standard procedures (Rose et al., 1990). All strains used are described in Table I, and all plasmids novel to this work are described in Table II.

Synthetic Lethal Screen

Strain Construction. The parental strains for the screen were created by

Table II. Plasmids Generated for This Study

pPS709	5.5-kb Bam HI–SalI *ADE3* fragment from pRDK225 (Johnson and Kolodner, 1995) inserted into the BamHI–SalI sites of YEp352
pPS714	2.3-kb *RNA1* SacI fragment of YCpRNA1 (Traglia et al., 1989) inserted into SacI site of pPS709
pPS779	5.5-kb BamHI–SalI *ADE3* fragment from pRDK225 (Johnson and Kolodner, 1995) inserted into the BamHI–SalI sites of YEp351
pPS783	0.7-kb coding region of yeast Ran amplified with BamHI–HindIII oligonucleotides inserted into pAlter-1; threonine 26 was changed to arginine using a mutated oligonucleotide
pPS888	2.3-kb *RNA1* SacI fragment of YCpRNA1 (Traglia et al., 1989) inserted into SacI site of pPS779
pPS889	Original clone identified in screen, 10-kb Sau3AI fragment inserted into the BamHI site of YCp50 (Rose et al., 1987)
pPS890	3.5-kb *RSL1* EcoRV–KpnI fragment of pPS889 inserted into SmaI–KpnI sites of pRS316 (Sikorski and Hieter, 1989)
pPS891	33-bp c-myc oligonucleotide inserted into AflII site of pPS890
pPS892	XbaI oligonucleotide inserted into pGEX.2TB (Lee et al., 1994) to convert XbaI site to BglII; 0.7-kb Bgl II–BamHI fragment of pGEX.2TB inserted into BamHI site of pPS293 (Lee et al., 1996)
pPS893	2.5-kb coding region of *RSL1* amplified with BamHI–XhoI oligonucleotides inserted into BamHI–SalI sites of pPS892
pPS894	2.5-kb coding region of *RSL1* amplified with BamHI–XhoI oligonucleotides inserted into BamHI–XhoI sites of pGEX.4T-1
pPS898	3.4-kb *RSL1* BamHI–PstI fragment of pPS890 inserted into pRS305 (Sikorski and Hieter, 1989)
pPS900	2.3-kb *RNA1* SacI fragment of YCp*RNA1* (Traglia et al., 1989) inserted into SacI site of YEp351
pPS909	1.0-kb coding region of *HIS3* using NcoI–PstI oligonucleotides inserted into NcoI–PstI sites of pRUΔ9 (Traglia et al., 1989)

All plasmids generated for this study are described.

crossing an *rna1-1* strain EE1b (PSY544, gift of A. Hopper, Pennsylvania State University, Hershey, PA) to the wild-type strain FY86, (PSY581, gift of F. Winston, Harvard Medical School, Boston, MA) to produce PSY868. PSY868 was crossed to PSY137 (gift of C. Holm, University of California, San Diego) to create PSY869 and PSY870, carrying *ade2* and *ade3* alleles. The diploid 2bx3b (PSY543, gift of A. Hopper), containing a *LEU2* disruption of the *RNA1* locus and the YCp50*RNA1* plasmid (pPS350, gift of A. Hopper), was sporulated to generate PSY873.

Plasmid Construction. pPS709 was generated by inserting a 5.5-kb BamHI–SalI *ADE3* fragment from pRDK255 (Johnson and Kolodner, 1995) into the BamHI and SalI sites of YEp352 (New England Biolabs, Beverly, MA). The same strategy was used to create pPS779, except that YEp351 was the vector used. pPS714 was then created by inserting a 2.3-kb *RNA1* SacI fragment into the SacI site of pPS709. Similarly, pPS884 was generated by insertion of the *RNA1* SacI fragment into the SacI site of pPS779. pPS900 was created by insertion of the *RNA1* SacI fragment into YEp351 (New England Biolabs).

Screen. PSY869 and PSY870 transformed with pPS714 were grown to 1×10^8 cells per ml and mutagenized with ethyl methanesulfonate as described (Lawrence, 1991) to produce 50% cell death. Approximately 100,000 colonies were screened for a nonsectoring phenotype ($Sect^-$). $Sect^-$ colonies were streaked three times to yeast extract/peptone/dextrose medium and once to synthetic complete media containing 1 mg/ml 5′ fluoro-orotic acid (FOA). 30 consistently $Sect^-$- and FOA-sensitive (FOA^s) strains were transformed with pPS900. 15 of those 30 became $Sect^+$ and FOA-resistant (FOA^r). These mutants were backcrossed to determine dominance/recessiveness. The backcrosses were sporulated to determine if the mutants had single mutations. They were also crossed to PSY873 to eliminate any *RNA1* null alleles created in the mutagenesis. The two remaining mutants, PSY871 and PSY872, were crossed to each other to determine complementation groups. Both mutants were also streaked to yeast extract/peptone/dextrose medium at 16°, 25°, 30°, 34°, and 36°C to determine temperature sensitivity. Backcrosses generated the strains PSY875 and PSY876.

Cloning. Both PSY871 and PSY872 were transformed with pPS884 and streaked to FOA to eliminate pPS714. The mutants were then transformed with a *URA3 CEN* genomic library (Rose et al., 1987) and selected on Ura^- low Ade (6 mg/l) synthetic complete plates. $Sect^+$ colonies were restreaked on Ura^- low Ade plates as well as on FOA. Plasmid DNA was recovered from strains that became $Sect^-$ on FOA and retransformed into PSY871 and PSY872. Plasmids that rescued the $Sect^-$ phenotype were used for subcloning. Several HindIII fragments of one of the plasmids (pPS889) were subcloned into pRS316 (pPS329). The subclones were subjected to DNA sequencing using the T3 and T7 universal primers. Sequence analysis indicated that the chromosome corresponding to the subclone inserts (XII) was already sequenced by the Yeast Genome Project. Several open reading frames were identified on the original clone. Subclones designed to carry single open reading frames were created in pPS329 and transformed into PSY871 and PSY872. pPS890 (3.5-kb EcoRV–KpnI genomic fragment inserted into SmaI–KpnI sites of pPS329) rescued the $Sect^-$ phenotype.

Linkage. An integration construct, pPS898, was generated by insertion of the 3.5-kb BamHI–PstI fragment of pPS890 into the BamHI and PstI sites of pPS749 (pRS305). pPS898 was linearized by digestion with NsiI and transformed into PSY874. Correct integration of the construct in Leu^+ transformants was verified by Southern blotting using enhanced chemiluminescence (ECL) as a detection method (Amersham Life Science, Inc., Arlington Heights, IL). The strain bearing the integration construct was backcrossed once to PSY869 to generate PSY885, which was then crossed to PSY871 and PSY872.

Construction of rna1Δ359-397 Strains. A strain containing the *rna1Δ359-397* allele was constructed using pPS909. pRUΔ9 (pPS908, gift of A. Hopper) contains *rna1Δ359-397* linked to the *URA3* gene. To generate pPS909, the 1.0-kb PstI–NcoI fragment of *URA3* was removed and replaced with the *HIS3* gene, which was amplified using the oligonucleotides DK7 5′CGCATGCTGCAGCCACGACGCTTTGTC 3′ and DK8 5′ CATGCCATGGCACATGTATATATATCGTATGGTGCA 3′. pPS909 was digested with SacI and XbaI to release the *HIS3*-tagged *rna1Δ359-397* allele, which was then transformed into PSY615. Correct integration in His^+ transformants was verified by Southern blotting using ECL as described above. PSY615 was sporulated to produce PSY879, which was then crossed to PSY581 to eliminate the *ade8* allele and to generate PSY880. PSY880 was crossed to PSY137 to create PSY881, carrying both *ade2* and *ade3* alleles. PSY881 was crossed to PSY871 and PSY872 to determine synthetic lethality.

Generation of RSL1 Deletion Strain

The *RSL1* gene was replaced in the W303 diploid, PSY878 (gift of J. Loeb,

Fink Lab, Massachusetts Institute of Technology, Cambridge, MA), using a PCR method (Baudin et al., 1993). Oligonucleotides were generated that contained 19-bp DNA complementary to the *HIS3* coding region and 45-bp DNA complementary to *RSL1* flanking sequence. They are DK11: 5′ AATAAAACTGATTTAACAGTAATCCATCATATCACACAAGGAGCAGGCCTCCTCTAGTACACTC 3′ and DK12: 5′CAAAGATGGAAAAGAACCAAAATCAGCTTGTAAGTTCTATCGTAAGCG CGCCTCGTTCAGAATG 3′. DNA was amplified from pPS729 using DK11 and DK12 and transformed into PSY878. Correct integration of the *HIS3* gene was verified by PCR analysis using DK17 5′ CTGGAGAATTATCTGTCCTTTTTC 3′ and the internal *HIS3* oligonucleotide 5′ GCCTCATCCAAAGGCGC 3′, and by Southern blotting using ECL for detection as described above.

Generation of Myc-tagged Importin-β

Oligonucleotides encoding the c-myc epitope with AflII-compatible ends were generated. They are DK15 5′ TTAAGTCCCGAGCAGAAGCTGAATAGCGAGGAAGATCTGAATGCC 3′ and DK16 5′ TTAAGGCATTCAGATCTTCCTCGCTAATCAGCTTCTGCTCGGGAC 3′. The oligonucleotides were mixed 1:1 and denatured for 2 min at 65°C followed by a shift to 37°C for 5 min to anneal. The double-stranded oligonucleotide was then inserted into the AflII site of pPS890. Correct orientation was verified by DNA sequencing.

Indirect Immunofluorescence Microscopy

Cells requiring galactose induction were first grown in glucose-supplemented medium overnight, and then diluted into 2% raffinose medium overnight. Cells were induced with 2% galactose for 3 h, and cells without a galactose-inducible construct were grown overnight in the appropriate medium. In experiments with cells shifted to the nonpermissive temperature, the cells were shifted to 36°C for 2 h.

All cells were washed in 0.1 M potassium phosphate buffer, pH 6.5, and resuspended in P solution (0.1 M potassium phosphate buffer, pH 6.5, 1.2 M sorbitol). Cell walls were digested with 15–30 μg of zymolyase (10 mg/ml in P solution plus 0.5% β-mercaptoethanol) at 30°C until the cells appeared dark under phase microscopy. Cells were adhered to slides treated with 0.3% poly-L-lysine. Fixation conditions differed depending on the experiment.

Formaldehyde Fixation. 0.1-vol 37% formaldehyde was added to cultures 30 min before digestion with zymolyase. Cells adhered to slides were also fixed in methanol for 6 min at −20°C and dried in cold acetone for 30 s.

Methanol Fixation. No formaldehyde was added to cells before washing. After cells were adhered to slides, the cells were fixed in methanol for 30 min at −20°C. Slides were dried in cold acetone for 30 s before incubation with antibody. In all experiments, primary antibody was incubated overnight in PBS plus 0.5% BSA, while secondary antibody was incubated for 2 h in PBS plus 0.5% BSA. Antibody dilutions for all experiments are as follows: anti-myc (9E10) tissue-culture supernatant was used undiluted; anti-importin-α, 1:1,000 (gift of D. Görlich, Wellcome/CRC Institute, Cambridge, UK); anti-Nup159, 1:2,000 (Gorsch et al., 1995); anti-β-galactosidase, 1:800 (Bossie et al., 1992); α-invertase, 1:20,000 (Nelson and Silver, 1989); and anti-importin-β, 1:500. All secondary antibodies (Jackson Immunoresearch Laboratories, Inc., West Grove, PA) were used at 1:1,000. 4′, 6-diamidino-2-phenylindole was used at a final concentration of 1 μg/ml to label DNA.

Immunoprecipitation

Lysates were made from cells grown to 1×10^8 cells per ml in PBSMT (PBS, 5 mM $MgCl_2$, 0.5% Triton X-100) plus protease inhibitors (0.5 mM PMSF, 3 μg/ml each leupeptin, aprotinin, chymostatin, and pepstatin) by glass bead lysis. Lysates were centrifuged at high speed in a microfuge for 10 min at 4°C to pellet cell debris. Lysates were incubated with an anti-myc agarose conjugate (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) for 1 h at 4°C. The beads were washed as follows: once in PBSMT and twice in PBSM. Sample buffer was added directly to the agarose conjugate, and samples were resolved by SDS-PAGE analysis using 10% gels (Laemmli, 1970).

Purification of Glutathione-S-transferase (GST)–importin-β from Yeast

Plasmid Construction. The *Escherichia coli* GST expression vector, pGEX.2TB (Lee et al., 1994), was digested with XbaI, and the oligo 5′ CTAGTAGAATCTA 3′ was inserted to convert the XbaI site to BglII. A 675-bp BglII–BamHI fragment containing the GST open reading frame was then inserted into pPS293 (Lee et al., 1996) downstream of the Gal1 promoter to generate pPS892. The *RSL1* coding region was amplified by PCR with VENT polymerase (New England Biolabs) using oligonucleotides DK13 5′ CGCGGATCCATGTCCACCGCTGAATTTGCT 3′ and DK14 5′ CGGCTCGAGCTCTCCTATGACGGAGAAGGT 3′. The 2.5-kb PCR fragment was digested with BamHI and AvaI and inserted into the BamHI–SalI sites of pPS892.

Purification of Protein. Cells were grown overnight in Ura⁻ synthetic complete medium with 2% glucose at room temperature. Cells were diluted into Ura⁻ synthetic complete medium with 2% raffinose. Cultures were induced at $\sim 2 \times 10^7$ cells per ml with 2% galactose for 3 h at room temperature. Lysates were made in PBSMT by glass bead lysis, and then centrifuged in a microfuge at 13,000 *g* for 10 min at 4°C. Lysates were incubated with GT-Sepharose (Pharmacia Biotech, Piscataway, NJ) at 4°C for 1 h. The beads were washed once in PBSMT and twice in PBSM. Sample buffer was added directly to the beads, and samples were resolved by SDS-PAGE analysis on 10% gels (Laemmli, 1970). For immunoblots, protein was transferred to nitrocellulose by standard methods (Towbin et al., 1979). Anti-importin-α was used at 1:5,000. Immunoreactive bands were detected by ECL (Amersham Life Science, Inc.).

Construction of Ran Mutants

The GDP-bound form of yeast Ran, T26N, was constructed using the Altered Sites II mutagenesis kit (Promega, Madison, WI). Briefly, the coding region of Ran was PCR amplified as a BamHI–HindIII fragment and cloned into the pAlter-1 vector (Promega). The mutagenic oligo DHW1 (5′-GGTACTGGTAAGAACACTTTCGTCAAGAGACATTTG-3′), where the underlined A and C originally were C and T, respectively, was used to change threonine-26 to arginine and to create an XmnI restriction site for screening through the clones. This mutant corresponds to the mammalian GDP-bound form of human Ran (Kornbluth et al., 1994). After mutagenesis, the coding region was sequenced to confirm the point mutation. The T26N mutant was cloned downstream of the GAL1 promoter in plasmid pPS310 (Schlenstedt et al., 1995*a*) as a BamHI–HindIII fragment. The GTP-bound form of yeast Ran, G21V, has been described (Schlenstedt et al., 1995*a*).

mRNA Export Assay

This assay is a modification of the original mRNA in situ hybridization method described by Amberg et al. (1992). Cells were grown in Ura⁻ synthetic complete media with 2% glucose overnight at room temperature, and then diluted into media with 2% raffinose. Expression of the yeast Ran and mutants was induced by the addition of galactose to 2% for 4 h at room temperature. Cells were collected, washed once in 0.1 M potassium phosphate buffer, pH 6.5, and resuspended in P solution. Cell walls were digested with zymolyase (ICN Pharmaceuticals, Costa Mesa, CA) as for cells prepared for immunofluorescence. Cells were adhered to slides coated with 0.3% polylysine and permeabilized with 0.1% Triton X-100 in P solution for 10 min. After washing in P solution, cells were equilibrated in 0.1 M triethanolamine, pH 8.0, for 2 min. Polar groups were blocked by incubation in 0.1 M triethanolamine and 0.25% acetic anhydride for 10 min. Cells were incubated in prehybridization solution (50% deionized formamide, 4× SSC, 1× Denhardt's solution, 125 μg/ml tRNA, 10% dextran sulfate, 500 μg/ml denatured salmon sperm DNA) for at least 1 h at 37°C. The digoxigenin-labeled oligo dT probe (50 mer) was made using a Genius 6 kit (Boehringer Mannheim, Indianapolis, IN). Hybridization was carried out overnight at 37°C in hybridization buffer (prehybridization buffer plus probe). Slides were washed once quickly in 2× SSC, 2× SSC for 1 h at room temperature, 1× SSC for 1 h at room temperature, 0.5× SSC for 30 min at 37°C, and 0.5× SSC for 30 min at room temperature. Cells were blocked for 1 h in Antibody blocking buffer (0.1 M Tris, pH 9.0, 0.15 M NaCl, 5% heat-inactivated FCS, 0.3% Triton X-100). Anti-importin-α (1:1,000 in Antibody blocking buffer) was incubated on the cells overnight. Slides were washed three times with Antibody blocking buffer. Anti-rabbit secondary antibody (1:1,000) and FITC-conjugated anti-digoxigenin (1:200) were incubated in Antibody blocking buffer for 2 h. Slides were washed once quickly in Antibody wash 1 (Ab1: 0.1 M Tris, pH 9.0, 0.15 M NaCl), once in Ab1 for 10 min, once in Ab1 for 30 min, once in Antibody wash 2 (Ab2: 0.1 M Tris, pH 9.5, 0.1 M NaCl, 50 mM $MgCl_2$) for

10 min, and once in Ab2 for 30 min. 4',6-diamidino-2-phenylindole (1 μg/ml in Ab2) was used to visualize DNA.

Generation of Anti-importin-β and Anti-Rna1p Antibodies

The open reading frame of *RSL1* was amplified using the oligonucleotides DK13 and DK14. The 2.5-kb PCR product was digested with BamHI and AvaI and inserted into the BamHI and XhoI sites of pGEX4T-1 (Pharmacia Biotech) to create pPS894. The GST–*RNA1* fusion construct has already been described (Corbett et al., 1995).

DH5α carrying the fusion constructs were grown overnight in Luria Bertani broth containing ampicillin (100 μg/ml) at 37°C, and then diluted 1:200 in fresh Luria Bertani broth with ampicillin (100 μg/ml) and grown at 30°C until OD_{600} = 0.6–0.7. Cultures were induced with 0.5 mM isopropyl β-D-thiogalactopyranoside for 2 h, cells were pelleted at 6,000 *g* at 4°C for 15 min, and pellets were stored at −20°C overnight. The cells were resuspended in (1 ml per 50 ml culture) lysis buffer (200 mM NaCl, 50 mM Tris, pH 8.0, 2.5 mM EDTA, pH 8.0, 0.1% Tween-20, 0.5 mM PMSF, 3 μg/ml each leupeptin, aprotinin, chymostatin, and pepstatin). Cells were lysed by sonication with six to eight 30-s bursts at a medium setting on a Heat Systems Ultrasonicator (Heat Systems, Inc., Farmingdale, NY). The lysate was centrifuged at 30,000 *g* for 15 min at 4°C to pellet cell debris. The soluble fraction (at concentration of 5 mg/ml) was bound to GT-Sepharose (Pharmacia Biotech) (at 1-ml bed vol per 500 ml culture) previously washed with lysis buffer. The binding step was performed at 4°C for 1–3 h. 10-min washes (10 vol) were performed at 4°C in the following order: once in lysis buffer, once in wash buffer (200 mM NaCl, 50 mM Tris, pH 8.0), once in high salt wash buffer (500 mM NaCl, 50 mM Tris, pH 8.0), and twice in wash buffer. The fusion protein was eluted in three 10-min washes (1 vol) 10 mM glutathione in 50 mM Tris, pH 8.0, at room temperature.

The proteins were concentrated using Centriprep-30 (Amicon Inc., Beverly, MA). Purification and concentration was monitored by SDS-PAGE. Final protein concentration was determined by the Bio-Rad protein assay (Richmond, CA) using BSA as a standard. Purified protein was sent to HRP, Inc. (Denver, PA) for injection into New Zealand White rabbits. Titration of antisera was performed using previously described immunoblotting techniques (Towbin et al., 1979).

Results

The Rna1-1 Protein Accumulates at the Nuclear Envelope

The *rna1-1* mutation exhibits temperature-sensitive defects in RNA processing, mRNA export, and nuclear protein import (Hopper et al., 1978; Amberg et al., 1992; Corbett et al., 1995). Rna1p is a normally cytoplasmic protein; however, Rna1-1p is not observed in immunoblots of cytosolic extracts, even though the protein is present at wild-type levels in whole cell lysates (Hopper et al., 1990; Tung et al., 1992; Corbett et al., 1995). We generated antisera specific for the Rna1 protein using an *E. coli*–purified GST fusion. When this antisera is used for indirect immunofluorescence on wild-type cells, the Rna1 protein is found throughout the entire cytoplasm but is absent from the nucleus, as has been previously reported (Fig. 1, *top row*) (Hopper et al., 1990). However, when the anti-Rna1p antibody is used on *rna1-1* cells, the mutant protein is found to be associated with the nuclear envelope with only slight cytoplasmic staining (Fig. 1, *bottom row*; only cells at permissive temperature are shown). This localization of the *rna1-1* mutant protein suggests that it is binding with high affinity to a protein associated with the nuclear envelope, thus explaining the absence of Rna1-1p from cytosolic extracts. Given the interesting phenotypes of the *rna1-1* mutant, we chose to identify other factors with which it might interact by performing a synthetic lethal screen (Kranz and Holm, 1990; Bender and Pringle, 1991).

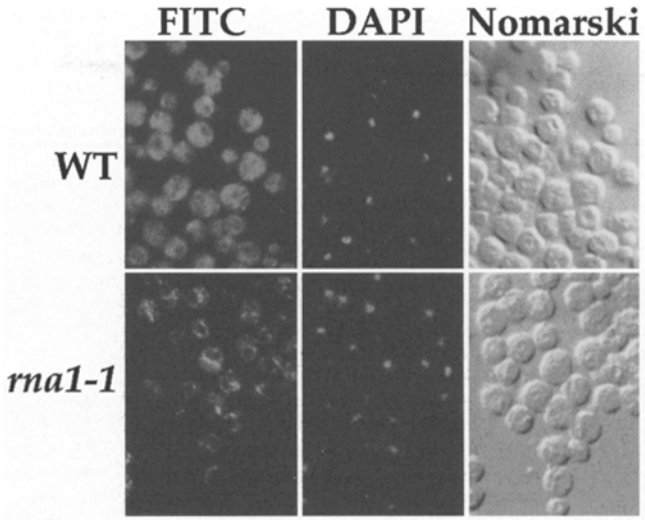

Figure 1. The Rna1-1 protein accumulates at the nuclear envelope. Wild-type (*top row*) and *rna1-1* (*bottom row*) cells were grown to log phase, and cultures were split in half and either shifted to 36°C for 3 h or kept at room temperature. Cells were prepared for immunofluorescence without formaldehyde fixation. The α-Rna1p antibody was used at 1:1,000 dilution.

Genetic Interactions with Mutant Alleles of RNA1

Synthetic lethality refers to the phenomenon observed when two conditional mutations are lethal in combination under otherwise normally permissive conditions for each mutant allele. An example is when the double mutant of two temperature-sensitive mutations is dead at permissive temperature, conditions under which each allele is viable. One interpretation of this phenomenon is that the cell can survive a slight malfunction in the first gene, but upon a defect in an interacting gene, the cell dies. This strategy can be used to identify potential genetic interactions.

Toward this end, a synthetic lethal screen was performed with the *rna1-1* allele using the yeast sectoring assay (Kranz and Holm, 1990; Bender and Pringle, 1991). Two *rna1-1 ade2 ade3* strains of opposite mating type transformed with a plasmid carrying wild-type alleles of *RNA1* and *ADE3* were sufficiently mutagenized with ethyl methanesulfonate to kill 50% of the cells (Lawrence, 1991). After screening ~100,000 colonies, 30 nonsectoring ($Sect^-$), FOA-sensitive mutants were identified. Of those 30, 15 became $Sect^+$ and FOA resistant when an additional plasmid carrying the *RNA1* allele was introduced into the mutants, indicating that they were dependent on *RNA1* for viability. These 15 mutants were backcrossed to the parental strain to determine if the mutations were dominant or recessive. The backcrosses were also sporulated to determine if the mutation segregated as a single gene. In addition, the 15 mutants were crossed to an *RNA1* disruption strain to identify any null alleles of *RNA1*.

Two mutants, PSY871 and PSY872, were identified that were not *RNA1* null alleles. Both were recessive, and their $Sect^-$, FOA^s phenotypes segregated as a single locus. When the mutants were crossed to each other, the diploid was $Sect^-$ and FOA^s, indicating noncomplementation. When tetrads were dissected from this diploid, only $Sect^-$, FOA^s colonies were obtained, indicating that PSY871 and

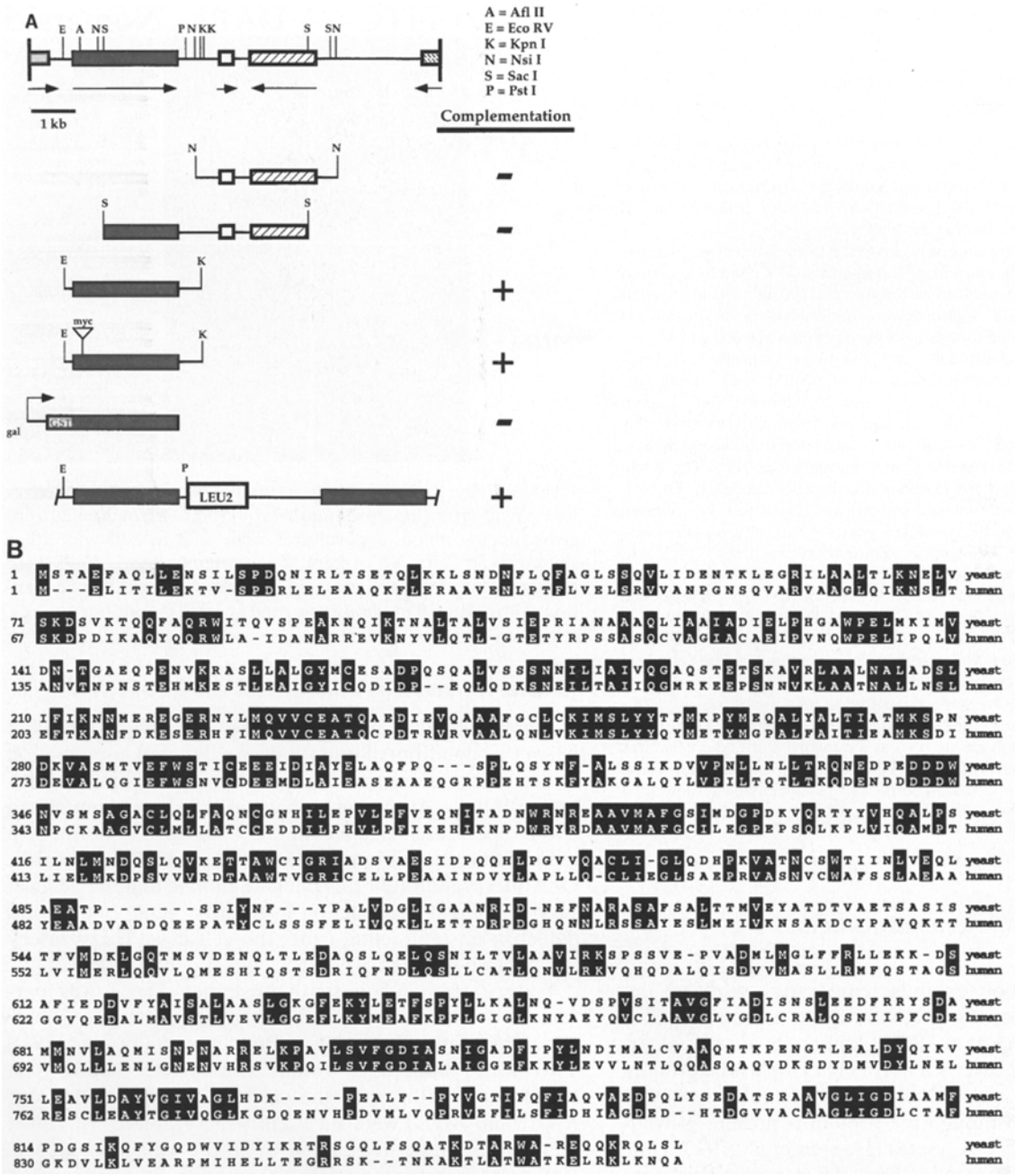

Figure 2. (*A*) The top figure is a schematic representation of the genomic clone that rescued the *rsl1* mutant phenotypes. (*Box*) Open reading frame. (*Arrows*) Direction of transcription. Restriction sites used to make each construct are shown, except for the GST fusion protein, which is described in Table II. Complementation of the Sect$^-$ phenotype of both *rsl1* mutants and the temperature sensitivity of *rsl1-1* were tested. +, both defects were rescued. (*B*) The alignment of the amino acid sequences of yeast importin-β with human importin-β. The proteins are 34% identical. (*Black outline*) Identical residues. These sequence data are available from EMBL/Gen Bank/DDBJ under accession number U19028.

PSY872 were mutated in the same gene. PSY871 was unable to grow at 36°C, while PSY872 did not demonstrate any temperature sensitivity. We refer to these mutants as *rsl1-1* (PSY871) and *rsl1-2* (PSY872) for *rna1-1* synthetic lethals.

Rsl Mutants Are Not Allele Specific

To determine allele specificity, both mutants were crossed to a strain containing another temperature-sensitive allele of *rna1*, *rna1Δ359-397*. The *rna1Δ359-397* allele is missing amino acids 359–397 and exhibits defects in RNA processing (Traglia et al., 1989). When the diploids were sporulated, two Sect$^-$ and two Sect$^+$ spores were obtained in every complete tetrad, indicating that the *rsl* mutants are also synthetically lethal with the *rna1Δ359-397* allele (data not shown).

Cloning of the RSL1 Gene

The gene responsible for the mutations was cloned by complementing the nonsectoring phenotype. Both *rsl1*

mutants were transformed with a yeast genomic library (Rose et al., 1987). Approximately 10,000 transformants were screened for a Sect$^+$ phenotype, and plasmid DNA was recovered from three that were identified as Sect$^+$ and FOA^r. When the plasmid DNA was retransformed into *rsl1-1* and *rsl1-2* strains, only two of the clones rescued the nonsectoring phenotype. Restriction analysis indicated that the clones contained overlapping sequences and that neither clone contained the *RNA1* gene. Several HindIII fragments of the genomic clone were subcloned and subjected to DNA sequence analysis. Sequences obtained indicated that the clone mapped to chromosome XII, whose sequence had already been completed by the Yeast Genome Sequencing Project. Several open reading frames were identified on the clone, and subclones designed to isolate specific open reading frames were created (Fig. 2 *A*). Only the open reading frame between the EcoRV and KpnI sites rescued the nonsectoring phenotype of *rsl1-1* and *rsl1-2*, as well as the temperature sensitivity of *rsl1-1* (data not shown).

The open reading frame identified was 2.5 kb long and predicted a protein of ~95 kD. When a database search was performed with the protein sequence, the most homologous protein was the human importin-β protein, with 34% identity (Fig. 2 *B*). Given the reasonably high percent identity between human importin-β and the *RSL1* gene product, this protein has been identified as the yeast importin-β homolog (Görlich et al., 1995*a*). The yeast importin-β protein also shows a limited region of homology to both vertebrate and yeast importin-α in the first arm repeat of these proteins. In addition, there are two uncharacterized yeast proteins and the previously identified protein Pse1p (Chow et al., 1992) that exhibit a moderate level of identity to importin-β, all of which might have a similar function to importin-β (Görlich et al., 1995*a*).

To show that the *RSL1* gene is the same gene that is mutated in the synthetic lethal mutants, the *LEU2* gene was integrated next to the *RSL1* gene. Correct integration of the *LEU2* gene was verified by Southern blotting. After crossing to the *rsl1-1* and *rsl1-2* mutants, tetrads were dissected. Of eight complete tetrads, none of the Leu$^+$ colonies exhibited a nonsectoring phenotype, indicating that *RSL1* was the gene mutated in the mutants identified in the synthetic lethal screen (data not shown).

The RSL1 Gene Is Essential for Cell Viability

The *RSL1* coding region was replaced with the *HIS3* gene using a PCR method (Baudin et al., 1993). The *HIS3* gene was amplified using oligonucleotides with flanking *RSL1* sequence. The PCR product was transformed into a wild-type diploid and His$^+$ transformants were recovered. Correct integration at the *RSL1* locus was determined by Southern blotting and PCR analysis (data not shown). The diploid strain was sporulated, and tetrads were dissected. Only two spores were recovered from each of the twelve tetrads, and these spores were His$^-$, indicating that the *RSL1* gene is essential for viability. When the diploid transformed with a plasmid carrying the *RSL1* gene was sporulated, four spores were recovered in each of the twelve tetrads. Only His$^-$ spores were FOA resistant, indicating that the *RSL1* gene was rescuing the lethality of the His$^+$ spores. Similar results have recently been shown (Iovine et al., 1995).

rsl1 Mutants Exhibit Import Defects In Vivo

Given the similarity of the *RSL1* gene product with mammalian importin-β, we predicted that the *RSL1* gene product would be involved in nuclear protein import. Because importin-β has only been shown to function in import using in vitro methods, we conducted in vivo import assays on the *rsl1* mutants. The *rsl1* mutants and the parental strain from the mutagenesis were transformed with a construct that expresses a nuclear reporter protein under a galactose-inducible promoter. This reporter protein is a fusion of the SV40 large T antigen NLS to yeast invertase, a normally secreted protein (Nelson and Silver, 1989). Cells were grown in media that selected for a plasmid with the *RNA1* gene as well as the reporter construct, as all three strains carry an *rna1-1* allele, which shows import defects in vivo (Corbett et al., 1995). Cells expressing the reporter

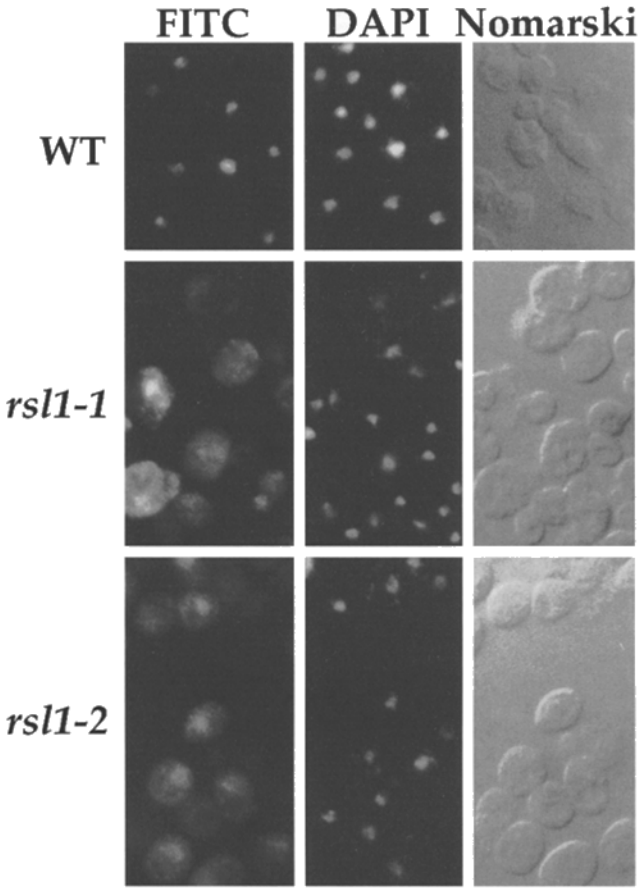

Figure 3. The *rsl1* mutants exhibit nuclear protein import defects in vivo. Expression of the reporter protein SV40–invertase was induced by the addition of 2% galactose to log-phase cultures of wild-type (*top row*), *rsl1-1* (*middle row*), and *rsl1-2* (*bottom row*) cells for 2 h at room temperature. Cultures were split in half and shifted to 36°C for 2 h. Cells were fixed with formaldehyde and prepared for immunofluorescence. The α-invertase antibody was used at 1:20,000, and FITC-conjugated secondary antibodies were used to visualize the reporter protein (*left column*). 4′,6-diamidino-2-phenylindole staining indicates the location of DNA in the cells (*middle column*). Only cells shifted to 36°C are shown.

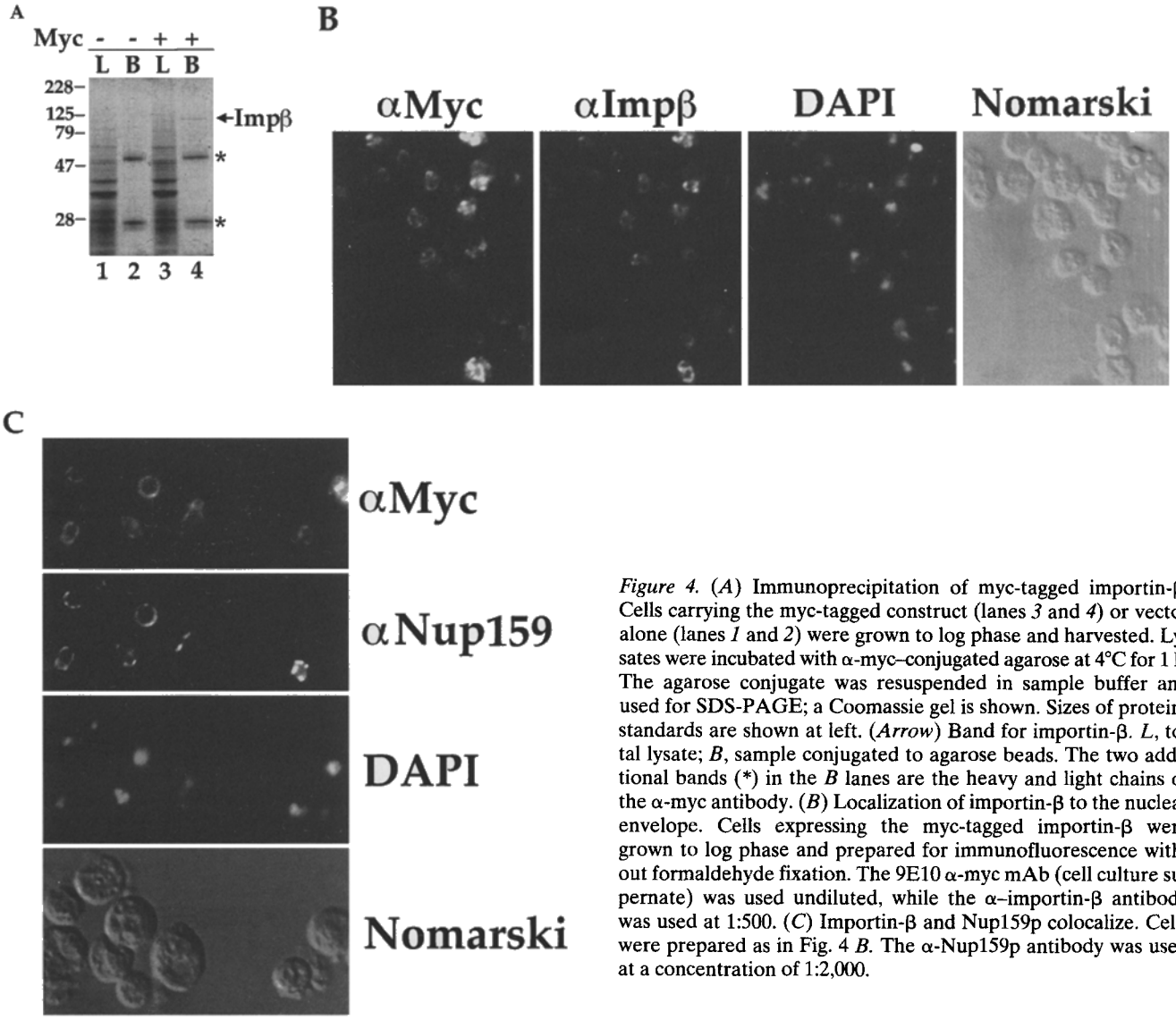

Figure 4. (*A*) Immunoprecipitation of myc-tagged importin-β. Cells carrying the myc-tagged construct (lanes *3* and *4*) or vector alone (lanes *1* and *2*) were grown to log phase and harvested. Lysates were incubated with α-myc–conjugated agarose at 4°C for 1 h. The agarose conjugate was resuspended in sample buffer and used for SDS-PAGE; a Coomassie gel is shown. Sizes of proteins standards are shown at left. (*Arrow*) Band for importin-β. *L*, total lysate; *B*, sample conjugated to agarose beads. The two additional bands (*) in the *B* lanes are the heavy and light chains of the α-myc antibody. (*B*) Localization of importin-β to the nuclear envelope. Cells expressing the myc-tagged importin-β were grown to log phase and prepared for immunofluorescence without formaldehyde fixation. The 9E10 α-myc mAb (cell culture supernate) was used undiluted, while the α–importin-β antibody was used at 1:500. (*C*) Importin-β and Nup159p colocalize. Cells were prepared as in Fig. 4 *B*. The α-Nup159p antibody was used at a concentration of 1:2,000.

protein were split in half, with half remaining at room temperature and the other half shifted to 36°C for 2 h. In the parental strain, the reporter protein is nuclear at both room temperature and at 36°C (Fig. 3, *top row*; only cells at 36°C are shown). However, both *rsl1* alleles show gross mislocalization of the SV40NLS–invertase protein to the cytoplasm at both room temperature and 36°C (Fig. 3, *middle and bottom rows*; only cells at 36°C are shown).

Since other nuclear import mutants also exhibit mRNA export defects (Amberg et al., 1992; Schlenstedt et al., 1995*a*), we tested the *rsl1* mutants for nuclear accumulation of polyA$^+$ RNA. The mRNA export phenotype of both *rsl1* mutants was determined using in situ hybridization with a digoxigenin-labeled poly dT probe (Amberg et al., 1992). No mRNA export defect was observed in either mutant after a 2-h shift to 36°C, a time at which they did exhibit protein import defects (data not shown). This is consistent with what is seen with importin-α mutants, which exhibit protein import defects both in vivo and in vitro but do not show mRNA export defects (Loeb et al., 1995).

Importin-β Localizes to the Nuclear Envelope

To determine the intracellular localization of importin-β, the importin-β protein was epitope tagged by inserting an oligonucleotide coding for the c-myc epitope between codons 14 and 15 in the *RSL1* gene. When transformed into *rsl1-1* and *rsl1-2* strains, the myc-tagged construct was able to rescue the nonsectoring phenotype of both mutants and the temperature sensitivity of *rsl1-1*, indicating that the myc-tagged protein is functional. When immunoprecipitations using anti-myc–conjugated agarose are performed on cells expressing the myc-tagged protein, a single band of ~95 kD is observed on SDS gels (Fig. 4 *A*, lane *4*). This band is not seen in immunoprecipitations from cells expressing the untagged importin-β protein (Fig. 4 *A*, lane *2*). The other two bands present in the immunoprecipitations correspond to the heavy and light chains of the anti-myc antibody. When the monoclonal anti-myc antibody (9E10) is used for indirect immunofluorescence microscopy, the importin-β protein localizes to the nuclear envelope in a punctate pattern similar to nucleoporin stain-

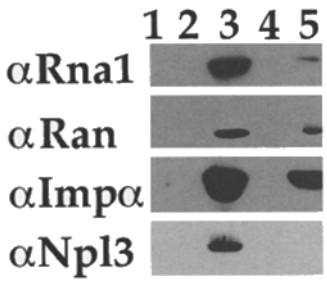

Figure 5. GST–importin-β binds to Rna1p, Ran, and importin-α. A GST–importin-β fusion was purified from *E. coli* as for anti–importin-β antibody production (described in Materials and Methods), omitting the elution step. The fusion protein bound to glutathione-Sepharose was used to probe an excess of wild-type lysate (lanes *3–5*) for 1 h at room temperature. The conjugate was resuspended in Laemmli buffer before SDS-PAGE. Protein samples were transferred to nitrocellulose and immunoblotted with antibodies to Rna1p, Ran, importin-α, and Npl3. (Lanes *1* and *2*) Purified GST and GST–importin-β protein, respectively. (Lane *3*) Sample of a lysate from wild-type cells; (lane *4*) sample of GST incubated with wild-type lysate; (lane *5*) sample of GST–importin-β mixed with wild-type lysate. It is important to note that the sample of wild-type lysate shown in lane *3* was used only as a positive signal and bears no relation to how much protein binds the GST–importin-β fusion.

ing (Fig. 4 *B*). To confirm this observation, antibodies specific for importin-β were generated using a GST–importin-β fusion protein purified from *E. coli.* As shown in Fig. 4 *B*, when cells expressing the myc-tagged importin-β are co-stained with anti-myc and anti–importin-β antisera, both antibodies give the same localization (Fig. 4 *B*).

Importin-β Colocalizes with Nup159p

To establish that the localization of importin-β was indeed consistent with nuclear pore staining, localization of both the nucleoporin Nup159p and importin-β in cells expressing the myc-tagged importin-β was observed. Cells prepared for immunofluorescence were incubated with both anti-myc and anti-Nup159p (Gorsch et al., 1995). As shown in Fig. 4 *C*, both Nup159p and importin-β are found at the nuclear envelope, although the overlap of the two proteins is not complete. Similar results are seen when the anti–importin-β antisera is used (data not shown). These results indicate that importin-β localizes to the nuclear pore complex, as would be expected of a component of the NLS receptor. In addition, it has been shown that importin-β interacts with the repeats of Nup159p in vitro (Enenkel et al., 1995); our observation of colocalization of importin-β and Nup159p suggests a possible in vivo interaction as well.

Importin-β Interacts with Import Factors In Vitro

As the *RSL1* gene was obtained in a *rna1-1* synthetic lethal screen, it is possible that importin-β and Rna1p physically interact. To investigate this possibility, a GST–importin-β fusion was expressed under a galactose-inducible promoter in wild-type yeast cells. The fusion protein was purified from yeast cell lysates using glutathione-Sepharose, and samples were resolved by SDS-PAGE, transferred to nitrocellulose, and blotted with an anti-Rna1p antibody. However, no copurifying Rna1p was seen (data not shown).

Conversely, when a wild-type yeast lysate is mixed with *E. coli*–purified GST–importin-β protein bound to glutathione-Sepharose, immunoblotting indicates that Rna1p and Ran are bound, as well as importin-α (Fig. 5, compare lanes *3* and *5*). No Rna1p or Ran is seen when GST is incubated with wild-type lysates, indicating that the interaction is dependent on importin-β (Fig. 5, lane *4*). We did not observe binding of importin-β to Npl3p, another factor implicated in nuclear transport, indicating that the interaction between importin-β and Rna1p and Ran is specific (Fig. 5).

Importin-β Colocalizes with Importin-α

To better understand the interaction between importin-β and importin-α, we more closely investigated the localization of the two proteins. In cells expressing the myc-tagged importin-β, both proteins localize to the nuclear envelope, but the overlap of the two proteins did not appear to be complete (Fig. 6 *A*). To investigate this localization more thoroughly, confocal microscopy was used on cells labeled for both importin-α and importin-β (Fig. 6 *B*). In the confocal micrographs of Fig. 6 *B*, importin-α is represented by red, importin-β is represented by green, and yellow is the overlap of the two proteins. The two proteins do indeed overlap to a significant extent at the nuclear envelope, although they are not completely coincident. Some cells show more importin-α staining at what appears to be inside the nucleus, but it is possible that this staining is actually at the surface of the nuclear envelope.

Importin-α Is Mislocalized to the Nucleus in prp20-1 Cells

The importin-α protein is predicted to enter the nucleus with the NLS substrate in the process of nuclear import and then presumably exit the nucleus at a later time. Recently, it has been demonstrated that importin-α accumulates in the nucleus of *nup120Δ* cells, a mutant that exhibits an mRNA export defect (Aitchinson et al., 1995). This result suggests that when mRNA export is blocked, importin-α cannot exit the nucleus. Therefore, we chose to investigate the localization of importin-α in a number of mutants that exhibit transport defects.

In wild-type cells, importin-α is primarily cytoplasmic at room temperature and at 36°C (Fig. 7 *B*, *top row*) when cells are prepared for indirect immunofluorescence microscopy using formaldehyde fixation. However, when cells are not fixed with formaldehyde before immunofluorescence microscopy, importin-α localizes primarily to the nuclear envelope, as was shown in the previous section (Fig. 6 *A*). The difference in importin-α localization depending on fixation conditions may reflect the possibility that there are distinct pools of importin-α inside the cell. For example, a population of importin-α may be in the cytoplasm awaiting binding of an NLS protein, while another population is complexed with importin-β and is bound to the NPC. Similar differences in localization depending on fixation conditions are also seen with importin-β, but not with other transport factors such as Rna1p (data not shown), indicating that this phenomenon may be specific to components of the NLS receptor.

When the localization of importin-α was investigated in a number of transport mutants that exhibit either a nuclear protein import (*srp1-31*, *srp1-49*, *rsl1-1*, *rsl1-2*) defect or both a nuclear protein import defect and an mRNA export defect (*rna1-1*, *nup49-313*), importin-α staining ap-

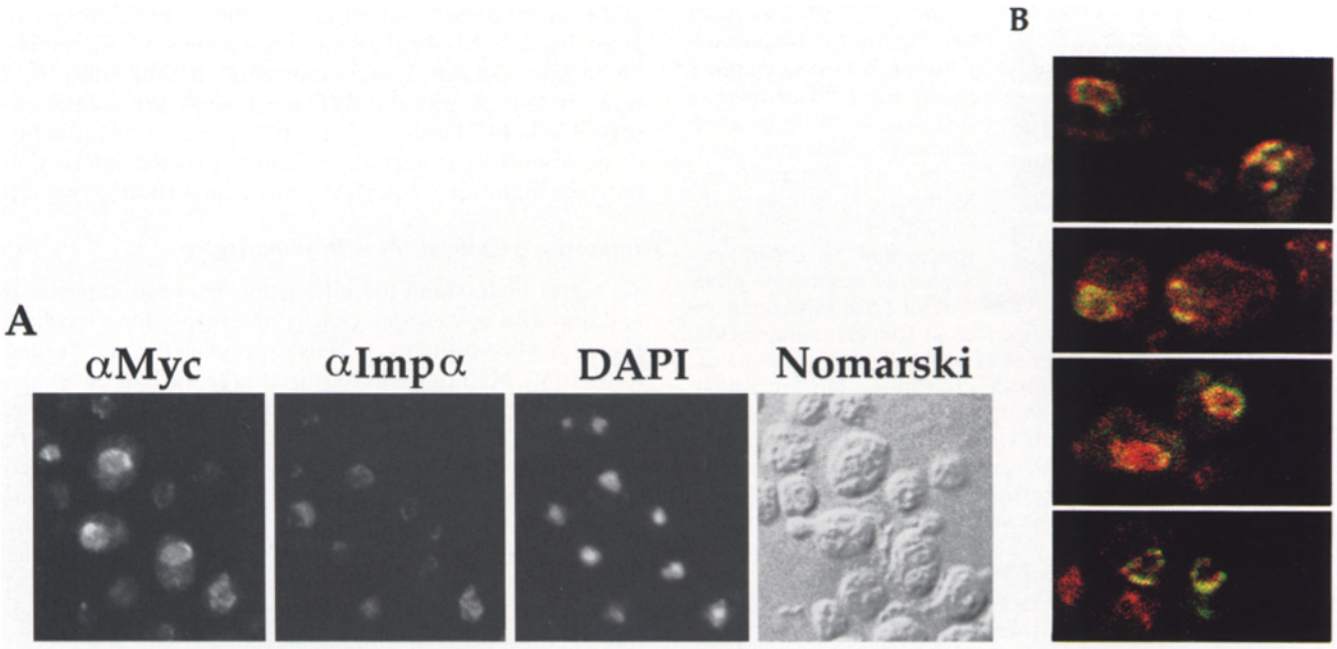

Figure 6. (*A*) Importin-β colocalizes with importin-α. Cells expressing myc-tagged importin-β were prepared for immunofluorescence without formaldehyde fixation. The α-myc antibody was used undiluted, while the anti–importin-α antibodies were used at a concentration of 1:1,000. (*B*) Importin-α and importin-β overlap at the nuclear envelope. Confocal microscopy was used on cells prepared as in Fig. 6 *A*. Importin-β (*green*), importin-α (*red*), and overlap of the two proteins (*yellow*).

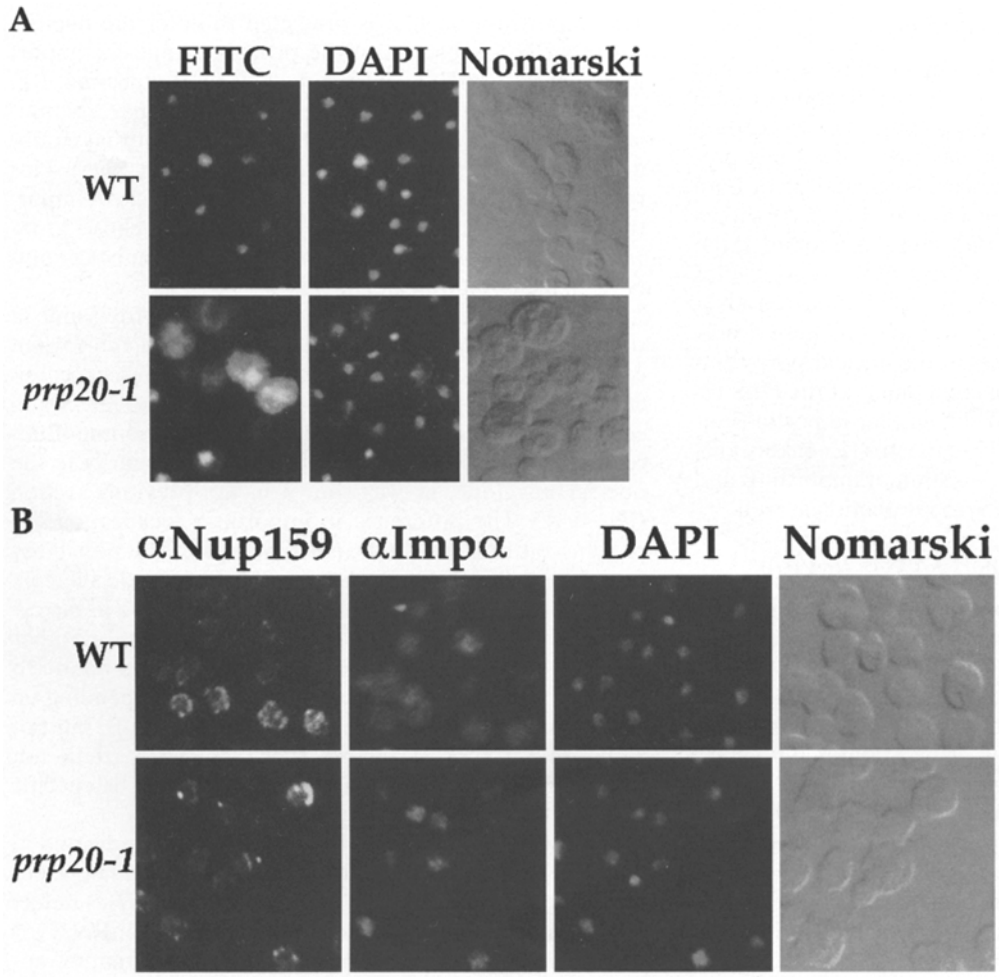

Figure 7. (*A*) *prp20-1* cells exhibit a protein import defect in vivo. Wild-type (*top row*) and *prp20-1* (*bottom row*) cells carrying the SV40–invertase reporter construct were induced to express the reporter protein with 2% galactose for 2 h at room temperature. Cultures were then shifted to 36°C for 2 h and fixed with formaldehyde. Cells were then prepared for immunofluorescence. Anti-invertase was used at 1:20,000 dilution. Only cells shifted to 36°C are shown. (*B*) *prp20-1* cells mislocalize importin-α to the nucleus. Wild-type (*top row*) and *prp20-1* (*bottom row*) cells were grown to log phase, and cultures were split in half and then either shifted to 36°C or kept at room temperature. After formaldehyde fixation, cells were prepared for immunofluorescence. Antibodies to importin-α were used at 1:1,000 dilution, while α-Nup159p was used at 1:2,000.

peared identical to that observed in wild-type cells (data not shown). The only transport mutant which exhibited mislocalization of importin-α to the nucleus was *prp20-1* (see Fig. 7 *B*, *bottom row*). Costaining of importin-α and the nucleoporin Nup159p in these cells indicates that importin-α is indeed accumulating inside the nucleus and not simply at the nuclear envelope. Importin-β was not mislocalized in any of the mutant strains tested (data not shown).

The *prp20-1* allele shows both a nuclear protein import and an mRNA export defect in vivo (Aebi et al., 1990; Fleischmann et al., 1991). Fig. 7 *A* shows the results of an in vivo nuclear protein import assay using the SV40NLS–invertase reporter protein. The reporter protein is properly localized to the nucleus in wild-type cells (*top row*) but is grossly mislocalized in *prp20-1* cells after 2 h at 36°C (*bottom row*). Additional nuclear reporter proteins (i.e., Npl3p and H2B-β-galactosidase) are mislocalized in *prp20-1* cells at the nonpermissive temperature (data not shown). It is difficult to determine which defect, import or export, occurs first in *prp20-1* cells; thus, it is difficult to determine which defect causes the accumulation of importin-α in the nucleus.

Importin-α Is Nuclear in Cells Expressing Ran-GDP

Because Prp20p is the homolog of RCC1 (Fleischmann et al., 1991), the exchange factor for Ran, we reasoned that if importin-α accumulates in the nucleus when the *prp20-1* protein is nonfunctional, then perhaps importin-α accumulates in the nucleus of *prp20-1* cells because of the lack of exchange of GTP for GDP on Ran. To further investigate this idea, we localized importin-α in cells expressing mutant forms of Ran. It has been shown that expression of Ran-GTP (RanG21V) causes defects in both nuclear import and mRNA export (Schlenstedt et al., 1995*a*) (Fig. 8 *A*, *middle row*). In addition, when Ran is expressed in GDP form (RanT26N), an mRNA export defect results (Fig. 8 *A*, *bottom row*). In cells expressing wild-type Ran, no mRNA export defect results (Fig. 8 *A*, *top row*). Importin-α was also localized in these cells. In cells expressing Ran-GTP (RanG21V) or wild-type Ran, importin-α is found primarily in the cytoplasm (Fig. 8 *B*, *top and middle rows*), while in cells expressing Ran-GDP (RanT26N), importin-α accumulates in the nucleus (Fig. 8 *B*, *bottom row*). This result suggests that the lack of exchange results in the accumulation of importin-α in the nucleus. The importin-β protein is not mislocalized in cells expressing the GDP form of Ran (data not shown).

Discussion

In this study we have shown that mutants of importin-β are synthetically lethal with mutant *RNA1* alleles, and importin-β interacts physically with importin-α, Rna1p, and Ran. Mutants in importin-β show defects in nuclear protein import in vivo, as would be expected for mutant al-

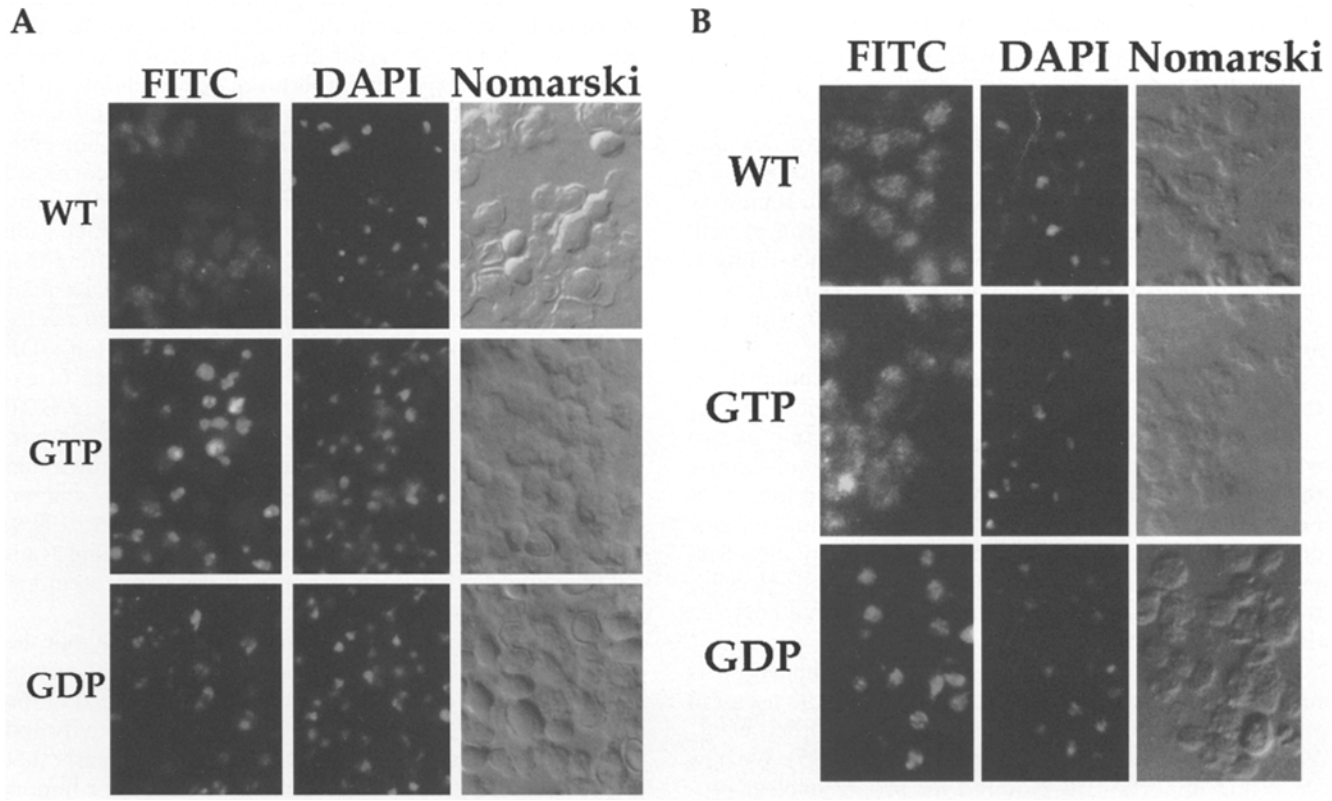

Figure 8. (*A*) Ran mutants exhibit an mRNA export defect. Wild-type cells expressing wild-type Ran (*top row*), Ran-GTP (*middle row*), and Ran-GDP (*bottom row*) were fixed with formaldehyde and prepared for the in situ mRNA export assay. Cells were probed with a digoxigenin-labeled oligonucleotide followed by incubation with FITC-conjugated α-digoxigenin antibodies. (*B*) Cells expressing Ran-GDP mislocalize importin-α to the nucleus. Cells prepared as in Fig. 7 *A* were incubated with α–importin-α (1:1,000) followed by FITC-conjugated secondary antisera.

leles of components of the NLS receptor. Importin-β localizes to the nuclear envelope and colocalizes with other proteins that are known to associate with the nuclear pore complex, namely importin-α and Nup159p. We also find that importin-α mislocalizes to the nucleus in *prp20-1* cells and in cells expressing Ran in the GDP-bound form.

Importin-β corresponded to the single complementation group identified in the *rna1-1* synthetic lethal screen. The *rsl1* mutants identified in the screen were also synthetically lethal with another temperature-sensitive allele of *RNA1*, *rna1Δ359-397*. Why would mutations in importin-β lead to lethality when present in combination with mutant *RNA1* alleles? Synthetic lethality can result from a number of situations. For example, mutations in two genes with redundant functions might lead to cell death, or mutations in two genes that function in the same pathway, but do not physically interact, could lead to cell death. We have tested synthetic lethality between the *rna1-1* allele and alleles of other transport factors (Prp20, Yrb1) and have found that in each case the double mutants were viable (Koepp, D.M., and P.A. Silver, unpublished observations). Thus, we believe that a more specific interaction between *rna1-1* and the *rsl1* mutants is the cause of their synthetic lethality. Mutations in two genes that cause their respective gene products to no longer physically interact might also lead to lethality. We have shown that importin-β is able to physically interact with Rna1p in vitro using a GST–importin-β fusion protein to probe wild-type lysates. Given the result of the *rna1-1* synthetic lethal screen, it seems likely that importin-β and Rna1p also interact in vivo. However, it is also possible that Rna1p and importin-β interact using Ran as an intermediary. Investigation of the nature of the in vitro interaction between Rna1p and importin-β is currently underway.

There are several possible reasons why Rna1p does not copurify with GST–importin-β in yeast. One alternative is that the yeast importin-β protein is modified in some way that prevents interaction with Rna1p; the *E. coli* protein would not have this modification. Another possibility is that the interaction between Rna1p and importin-β is either weak or transitory; thus, it can only be seen with large amounts of GST–importin-β.

Given the interaction between Rna1p and importin-β, the synthetic lethality between mutant alleles of *RNA1* and importin-β mutants can be explained in one of two ways. First, mutations in both Rna1p and importin-β cause the two proteins to no longer interact (or to no longer interact with the appropriate intermediary), leading to a lack of nuclear protein import, which results in lethality. Second, the mutations in both Rna1p and importin-β cause the two proteins to associate in a nonfunctional complex that also results in cell death.

Previously, it has only been shown that importin-β is necessary for nuclear protein import through the use of in vitro import assays (Enenkel et al., 1995; Görlich et al., 1995*b*; Imamoto et al., 1995; Moroianu et al., 1995). We now show that importin-β is required for proper nuclear protein import in vivo as well. The *rsl1* mutants grossly mislocalize the nuclear reporter protein, SV40NLS–invertase, at both room temperature and at 36°C. Interestingly, the strength of the import defect depends on the reporter protein used in the in vivo import assay. For example, neither *rsl1-1* nor *rsl1-2* mislocalizes the RNA-binding protein Npl3p (Koepp, D.M., and P.A. Silver, unpublished observations), which is known as the shuttle between the nucleus and cytoplasm (Flach et al., 1994). When the reporter is β-galactosidase fused to the histone H2B NLS, *rsl1-1* mislocalizes the protein only at the nonpermissive temperature, while *rsl1-2* slightly mislocalizes it at both room temperature and 36°C (Koepp, D.M., and P.A. Silver, unpublished observations). Such differences in import defects with respect to reporter proteins are not seen with other transport mutants such as *prp20-1* and *rna1-1* (Corbett et al., 1995). It is tempting to speculate that the reason for this difference in import activity lies in the differences in the types of NLS present in the reporter proteins. This could also explain why there appears to be more than one importin-β–like protein in yeast.

We have shown that importin-β localizes to the nuclear envelope by two methods, using a myc-tagged protein and by generating importin-β–specific polyclonal antibodies. Importin-β also colocalizes with two nuclear envelope–associated proteins, Nup159p and importin-α, although in each case the overlap of two proteins is not complete. Using confocal microscopy, importin-α appears to localize to the nuclear envelope and to the cytoplasm. This is consistent with importin-α moving from the cytoplasm with the NLS-bearing substrate into the nucleus during nuclear protein import.

Further evidence for this movement of importin-α into the nucleus comes from the localization of importin-α in cells expressing Ran-GDP (RanT26N). In these cells, importin-α is found to accumulate in the nucleus. It is possible that Ran-GDP (RanT26N) is titrating an essential factor that is required for the export of importin-α; one candidate might be Prp20p. When *PRP20* on a high copy plasmid is introduced into cells expressing Ran-GDP (RanT26N), however, the aberrant localization of importin-α is not corrected (Koepp, D.M., and P.A. Silver, unpublished observations). This suggests that there might be another factor, aside from Prp20p, that Ran-GDP (Ran T26N) is titrating that causes the mislocalization of importin-α. We find a similar accumulation of importin-α in the nucleus of *prp20-1* cells. Because both the *prp20-1* mutation and the Ran-GDP (RanT26N) mutant presumably result in the lack of exchange activity, these data suggest that exchange of GDP for GTP on Ran is necessary for the proper exit of importin-α from the nucleus. In addition, it has been shown that a mutant that has defects in mRNA export also accumulates importin-α in the nucleus (Aitchinson et al., 1995). However, the *prp20-1* mutant and cells expressing Ran-GDP exhibit both mRNA export and nuclear protein import defects.

To put this data in a broader perspective, recall that the GAP for Ran, Rna1p, is located exclusively in the cytoplasm, while the exchange factor, Prp20p, is located in the nucleus. Evidence concerning the lack of GAP activity and the effects of nonhydrolyzable GTP analogs suggests that hydrolysis of GTP by Ran is directly required for import into the nucleus (Melchior et al., 1993; Corbett et al., 1995; Schlenstedt et al., 1995*a*). When importin-α is localized in either *rna1-1* strains or cells expressing Ran-GTP (Ran G21V), it is found to be in the cytoplasm. However, both the *rna1-1* mutant and cells expressing Ran in the GTP

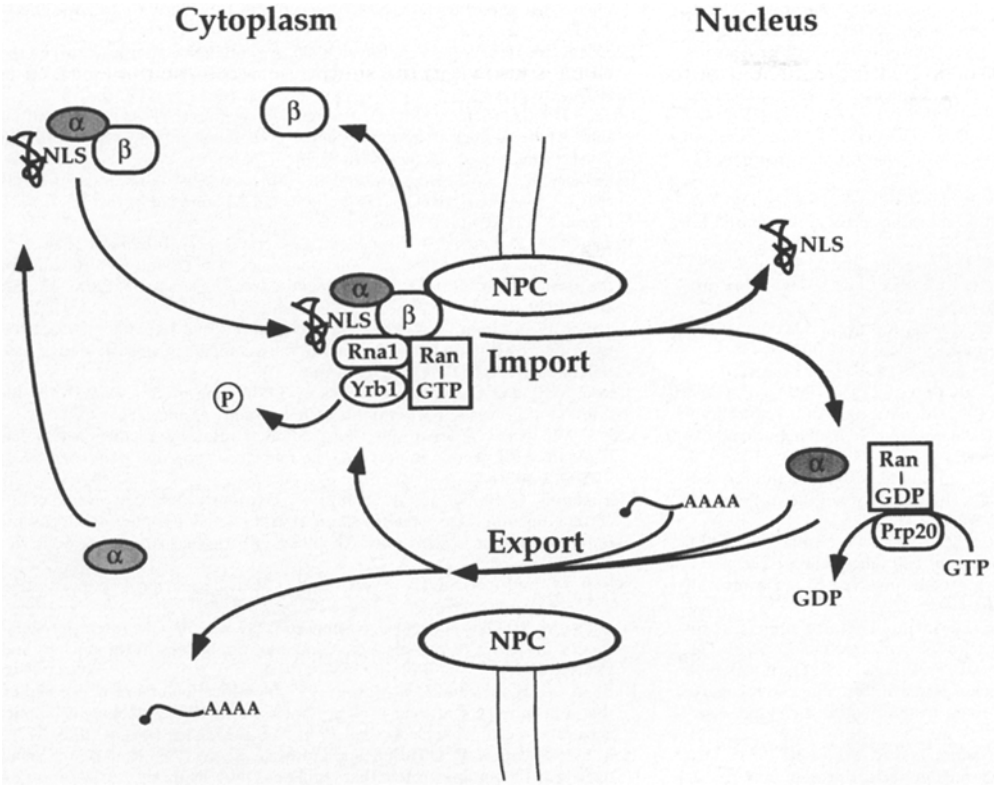

Figure 9. Model of nuclear transport. Importin-α and importin-β cooperate to target the NLS-bearing protein to the NPC. Ran-mediated hydrolysis of GTP, activated by Rna1p and Yrb1p, occurs, and importin-α, Ran-GDP, and the NLS-bearing protein move into the nucleus. Exchange of GDP for GTP on Ran, effected by Prp20p, allows the exit of importin-α, Ran-GTP, and mRNA from the nucleus.

form also exhibit both protein import and mRNA export defects. How then might we explain the aberrant localization of importin-α in only certain transport mutants? Perhaps importin-α is found in the cytoplasm of cells that lack GAP activity because the import defect is the primary defect, and the mRNA export defect is a result of an import blockage. Similarly, in cells where exchange activity is compromised, the primary defect might be in mRNA export. If this is the case, then importin-α would accumulate in the nucleus of such cells because the import defect takes place later as a result of the blockage of mRNA export. Taken together, this suggests a model in which hydrolysis of GTP by Ran is necessary for the import of NLS-bearing proteins complexed with importin-α, and exchange of GDP for GTP on Ran is necessary for the exit of importin-α and mRNA from the nucleus (Fig. 9). Further investigation will be necessary to test the many predictions of this model.

How does the interaction between Rna1p and importin-β fit into such a model for nuclear transport? There are two simple ways that this interaction might be explained: (*a*) the Ran-mediated hydrolysis of GTP, which is activated by Rna1p, causes a conformational change that leads to dissociation of importin-α from importin-β. This dissociation then allows importin-α to move into the nucleus with the NLS-bearing substrate. (*b*) The Ran-mediated hydrolysis of GTP is not necessary for the dissociation of importin-α from importin-β, but is instead necessary for the recycling of importin-β from the NPC back to the cytoplasm (Rexach and Blobel, 1995). The combined use of genetics and biochemistry should allow us to distinguish between these two alternatives.

We thank Praveen Keshava for help with the initial synthetic lethal screen and Margaret Lee for a critical reading of the manuscript.

This work was supported by National Institutes of Health (NIH) grants to P.A. Silver. D.M. Koepp was supported an NIH training grant to Harvard Medical School, D.H. Wong by a Sandoz Drug Discovery postdoctoral fellowship, and A.H. Corbett by an NIH postdoctoral fellowship.

Received for publication 30 January 1996 and in revised form 2 April 1996.

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