Rnalp, a Ran/TC4 GTPase Activating Protein, Is Required for Nuclear Import

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Abstract. The Saccharomyces cerevisiae gene, RNA1, encodes a protein with extensive homology to the mammalian Ran/TC4 GTPase activating protein. Using indirect immunofluorescence microscopy, we have demonstrated that rnal-1 mutant cells are defective in nuclear import of several proteins. The same result is obtained when nuclear import is examined in living cells using a nuclear protein fused to the naturally green fluorescent protein. These findings suggest a role for the Rnalp in trafficking of proteins across the nuclear membrane. To investigate this role more directly, an in vitro import assay that monitors the import of a fluorescently labeled substrate into the nuclei of semi-intact yeast cells was used. Import to the nucleus requires the addition of exogenous cytosol. Results indicate that, in contrast to wild-type cytosols, extracts made from rnal-1 mutant cells are unable to support import of the fluorescently labeled substrate into competent nuclei. Immunoblotting demonstrates that these mutant-derived extracts are depleted of Rnalp. However, when purified Rnalp is added back to these extracts the import activity is restored in a dose-dependent manner. These results demonstrate that Rnalp plays a direct role in the import of proteins into the nucleus.

Macromolecular transport across the nuclear envelope occurs at nuclear pores (Forbes, 1992; Rout and Wente, 1994) and consists both of the import of protein and snRNAs and the export of mature RNA and ribosomes (Garcia-Bustos et al., 1991; Nigg et al., 1991; Osborne and Silver, 1993; Fabre and Hurt, 1994). Since these processes are linked by their site of action it is likely that a number of components of the transport system are directly involved in both processes. For example, the nucleoporins, which are the structural components of the pore complex (Panté and Aebi, 1993; Rout and Wente, 1994), are likely to participate directly in both import and export. Although the nuclear pores embedded in the nuclear membrane serve as the site of translocation, proteins found both in the cytoplasm and the nucleus are required for the bi-directional transport of macromolecules (Powers and Forbes, 1994). It is not clear whether these accessory proteins function in a single concerted cycle of import and export or comprise two independent cycles, one that mediates the import of proteins to the nucleus and one that mediates the export of RNA from the nucleus.

Recent work has focused on the role of the small GTP-binding protein Ran/TC4 in nuclear import. This protein, which was originally identified on the basis of its homology to Ras (Drivas, 1990), has been shown to be a cytosolic factor that is absolutely required for import into isolated nuclei (Melchior et al., 1993a; Moore and Blobel, 1993, 1994a). Highly conserved homologues of Ran/TC4 have been identified in a number of organisms including Xenopus (Moore and Blobel, 1993), fission yeast (Matsuzato and Beach, 1991), and budding yeast (Belhumeur et al., 1993). In Saccharomyces cerevisiae, there are two copies of the Ran/TC4 homologue, GSP1 and GSP2, which differ only by two amino acids. GSP1 is an essential gene which is expressed at a higher level than the nonessential GSP2 (Belhumeur et al., 1993).

A recent study demonstrated that in budding yeast, overexpression of mutant Gsp1p locked in the GTP-bound form causes defects both in protein import and poly(A)+ RNA export (Schlenstedt et al., 1995). These results indicate that the ratio of GTP-GDP-bound Gsp1p within the cell must be meticulously regulated. Biochemical studies have shown that isolated Ran/TC4 hydrolyzes GTP very slowly (Bischoff et al., 1994; Schlenstedt et al.,...
The rate of GTP hydrolysis is enhanced in the presence of cytosolic extracts. Fractionation of cytosolic extracts has resulted in purification of a GTPase activating protein or (GAP) activity for Ran/TC4 and identified it as a mammalian homologue of S. cerevisiae Rnalp (Bischoff et al., 1995a). Other studies have demonstrated that Rnalp from Schizosaccharomyces pombe, which complements the rna-1 mutation in S. cerevisiae (Melchior et al., 1993b), also functions as a GTPase activating protein (Bischoff et al., 1995a).

The RNA1 gene was initially identified in S. cerevisiae in a genetic screen for mutants defective in RNA processing (Hartwell, 1967) and mutations in this gene have subsequently been shown to result in defective pre-rRNA splicing, processing of pre-rRNA, and production and export of poly(A) RNA from the nucleus (Hopper et al., 1978; Amberg et al., 1992). As localization studies of Rnalp determined that it was a cytoplasmic protein that was excluded from the nucleus (Hopper et al., 1990), it was not clear how defects in nuclear events such as RNA processing and transport were manifested. Its identification as a GAP suggests that Rnalp may be a component of a multifaceted system that uses a cycle of GTP hydrolysis to regulate the transport of macromolecules both into and out of the nucleus. Thus, the phenotypes observed in the mutants might result from alterations in the regulation of this hydrolysis cycle rather than from a direct involvement of the Rnalp in RNA processing.

The loss of a functional GAP protein would be reflected in a higher ratio of GTP/GDP-bound protein within the cell. Experiments that artificially altered this ratio by overexpression of the GTP-bound form of Gsp1p (Schlenstedt et al., 1995) demonstrated that the localization of proteins to the nucleus, as well as the export of poly (A) RNA from the nucleus (Hopper et al., 1978; Amberg et al., 1992). Since RNA transport defects in the rna-1 mutant have been characterized, we were interested in determining whether this mutant would also display defects in protein import as would be expected if it is involved in regulation of GTP hydrolysis by Gsp1p.

Results presented here indicate that the temperature-sensitive mutant rna-1 is unable to efficiently target proteins to the nucleus at the nonpermissive temperature. To determine whether Rnalp is directly involved in protein import, we use an in vitro import assay (Schlenstedt et al., 1993) and demonstrate that cytosols prepared from rna-1 mutant cells are unable to support import of a fluorescently labeled nuclear localization sequence (NLS)-containing substrate. Addition of purified Rnalp is able to rescue the defective cytosols. These findings strongly suggest that Rnalp plays a direct role in the import of macromolecules to the nucleus.

1. Abbreviations used in this paper: ECL, enhanced chemiluminescence; GAP, GTPase activating protein; GFP, green fluorescent protein; GST, glutathione-S-transferase; GT, glutathione; HSA, human serum albumin; IPTG, isopropylthio-β-D-galactoside; NLS, nuclear localization sequence; RT, room temperature.

Materials and Methods

Strains and Plasmids

The wild-type strain employed for indirect immunofluorescence microscopy was the haploid strain FY86 ([MATu, ura3-52, leu2Δ1, his3A200]; a gift from F. Winston, Harvard University Medical School). Strain PSY714 ([MATu, rna-1 ura3-52, leu2Δ1]) was created by crossing the original strain EEL1 (Atkinson et al., 1985) twice to FY86. For in vitro import assays a wild-type sister spore of PSY714 (PSY715 [MATu, ura3-52, leu2Δ1, his3A200]) was used as the control. The diploid wild-type strain PSY223 (MATu/MATα, ura3-52, leu2Δ3, leu2Δ3, his3Δ1, his3Δ1, his4Δ1, his4Δ4) was used to prepare semi-intact cells for the in vitro assay.

The glutathione-S-transferase (GST)-Rnalp fusion plasmid was constructed by ligating PCR-amplified RNA1 DNA with engineered BglII-EcoRI ends into the BamHI-EcoRI sites of pGEX-2T (Pharmacia, Piscataway, NJ). For experiments that examined the localization of a reporter protein, two constructs were employed. The first was a LEU2 GAL1 promoter plasmid encoding the SV40-NLS fused to the SUC2 gene (Nelson and Silver, 1989), and the second was a URA3 GAL1 promoter plasmid that consists of a portion of the nuclear protein Npl3p (Bossie et al., 1992) fused to the NH2-terminus of the naturally fluorescent green fluorescent protein (GFP) (Chalfie et al., 1994) (Lee, M. S., and P. A. Silver, unpublished data).

Protein Localization

Indirect immunofluorescence microscopy was used to examine the localization of either an endogenous nuclear protein, Npl3p (Bossie et al., 1992; Flach et al., 1994), or an artificial reporter protein. For experiments that examined the cellular localization of Npl3p, cells were grown in YEPD at room temperature (RT) to a density of 107 cells/ml. Cultures were then split and half was maintained at RT and half was shifted to 36°C for 2 h. For inducible reporter proteins the cells were grown to a density of 5 × 106 cells/ml in media lacking uracil or leucine (depending on the plasmid employed) with 2% raffinose. Cultures were then induced by the addition of 2% galactose and growth was continued at RT for 2–4 h. Following this induction, cultures were shifted to 36°C. Samples were then either prepared for immunofluorescence microscopy as previously described (Flach et al., 1994) or viewed directly by microscopy using the FITC channel to examine the cellular localization of the fusion to GFP. For immunofluorescence microscopy, incubation with antibodies against Npl3p (1:500 dilution) (Bossie et al., 1992) or invertase (1:20,000 dilution) (Nelson and Silver, 1989) was followed by incubation with FITC-labeled antibodies (Jackson ImmunoResearch Laboratories, West Grove, PA) at a 1:1,000 dilution and with DAPI.

In Vitro Import Assay

This assay was performed as described previously (Schlenstedt et al., 1993). Semi-intact cells were prepared from the wild-type strain PSY223. Cytosols were made from rna-1 mutant cells (strain PSY714) or a wild-type spore from the same tetrad (PSY715) grown at RT or 36°C. Cultures were grown to a density of 107 cells/ml at RT and then shifted to the nonpermissive temperature (36°C) (or maintained at the permissive temperature) for 2 h prior to the preparation of cytosols. Cells were pelleted, washed in buffer A (0.25 M sorbitol, 20 mM Pipes-KOH, pH 6.8, 150 mM K-acetate, 5 mM Mg-acetate), and resuspended in 1/75 vol buffer A containing 1 mM DTT, 0.5 mM PMSF, 3 μg/ml leupeptin, aprotinin, chymostatin, and pepstatin. Acid washed 0.5 mm glass beads were added at 1/3 vol of the cell suspension. Cells were lysed by a series of six to eight 120-s pulses in an ice-cold mini-bead beater. The resulting lysate was centrifuged for 30,000 g for 10 min. The resulting supernatant was then centrifuged at 100,000 g for 45 min. Samples were frozen in liquid nitrogen and stored at −80°C. The final protein concentration was ~50 μg/ml as determined by Bio-Rad protein assay reagent (Bio-Rad Laboratories, Inc., Hercules, CA) using BSA as a standard.

Import assays (30 μl total reaction vol) were performed in buffer A and contained 5 × 107 semi-intact cells/ml, 1 mM ATP, 0.1 mM creatine kinase, 10 mM creatine phosphate, 1.5–2 mg/ml cytosolic proteins, and 5 μg/ml of a fluorescent substrate consisting of NLS peptide (CTPPKKKRKV) conjugated to human serum albumin (HSA). Some experiments also contained purified GST-Rnalp at a concentration of 10–50 μg/ml. Reactions were incubated at 30°C for 10 min, stained with DAPI (final concentration 0.25 μg/ml), and viewed by fluorescence microscopy. For all import data,
experiments were performed at least three times and 200 cells were scored in each experiment. The means and standard deviations shown were calculated from three independent experiments.

**Purification of GST-Rnalp**

The GST-Rnal1 fusion protein was expressed in *Escherichia coli* DH5α cells. Cultures were grown overnight in Luria broth containing 100 μg/ml ampicillin and 37°C and then diluted into fresh media and grown until an OD 600 reached 0.6-0.7. Cultures were then induced with 0.5 mM isopropyl-β-D-galactoside (IPTG) for 2 h at 30°C. Cells were pelleted at 6000 g at 4°C for 15 min and pellets were stored at -20°C overnight. The cells were then thawed and resuspended in 1 ml (per 50 ml culture) lysis buffer (200 mM NaCl, 50 mM Tris, pH 8.0, 2.5 mM EDTA, 0.1% Tween 20) containing protease inhibitors (0.5 mM PMSF, 3 μg/ml leupeptin, aprotinin, chymostatin, and pepstatin). Cells were lysed by sonication with six to eight 30-s pulses at a medium setting on a Heat Systems Ultrasonicator (Heat Systems Inc. Farmingdale, NY). The lysate was centrifuged at 30,000 g at 4°C for 15 min to pellet cell debris. The soluble fraction was diluted to a concentration of 5 mg/ml and bound to GT-sepharose (Pharmacia) (1 ml bed vol/500 ml culture). The binding was carried out for 1-3 h at 4°C. 10-min washes of 10 vol were performed at 4°C in the following order: 1× lysis buffer; 1× wash buffer (50 mM Tris, pH 8.0, 200 mM NaCl); 1× high salt wash buffer (50 mM Tris, pH 8.0, 500 mM NaCl); 2× wash buffer. The fusion protein was eluted in three 10-min washes (1 vol) of 10 mM glutathione in 50 mM Tris, pH 8.0. The pooled eluates were then thawed and resuspended in 1 ml (per 50 ml culture) lysis buffer. The fusion protein was eluted to the purified protein to a final concentration of 10% and aliquots were stored at -80°C. Final protein concentration was 0.2-0.3 mg/ml as determined by Bio-Rad protein assay employing BSA as a standard.

GAP Assay

Purification of GST-Gsplp from yeast lysates was performed as previously described (Schlenstedt et al., 1995). Gsplp was further purified after cleavage with thrombin (Sigma, St. Louis, MO) as previously described (Smith and Johnson, 1988). Gsplp (160 nM) was labeled with 1.4 μl [γ-32P]GTP (6,000 Ci/mmol; NEN DuPont, Boston, MA), 1 mM DTT, 1 mg/ml BSA in a volume of 10 μl at 20 min at room temperature. GTPase assays were performed in 50-μl reactions in the presence of 40 nM Gsplp-[γ-32P]GTP with or without GST-Rnal1p or GST-Rnal-1p diluted in GAP buffer (20 mM Tris, pH 7.5, 50 mM NaCl, 10 mM MgCl₂, 1 mM DTT, 1 mg/ml BSA) (Smith and Johnson, 1988). The amount of non-hydrolyzed GAP was determined by a filter binding assay. After incubation for 10 min at 30°C, 1 ml ice-cold GAP buffer was added. Triplicate samples were vacuum filtered through nitrocellulose filters, were washed 10 times with 0.5 ml GAP buffer. The remaining Gsplp-bound GTP was determined by scintillation counting. No radioactivity was removed under identical conditions when [γ-32P]GTP-labeled Gsplp was used.

**Immunoblotting**

Protein samples were resolved by SDS-PAGE (Laemmli, 1970) on 10% gels. Proteins were then transferred by standard methods to nitrocellulose (Towbin et al., 1979). The anti-Rnalp antibody (Hopper et al., 1990) was used at a concentration of 1:10,000. Immunoactive bands were detected with the enhanced chemiluminescence (ECL) detection kit (Amersham, Arlington Heights, IL).

**Results**

**Rnal-1 Mutant Cells Are Defective in Protein Import In Vivo**

To investigate the role of Rnal1p in protein import to the nucleus, we used the well-characterized mutant *rnal-1*. This temperature-sensitive, conditionally lethal mutation prevents growth at temperatures of 30°C and above. Mutant cells display pleiotropic defects in RNA processing and transport. The defect in RNA transport occurs rapidly following a shift to the nonpermissive temperature and results in the accumulation of approximately 60% of poly(A)+ RNA within the nucleus (Hutchinson et al., 1969; Amberg et al., 1992).

Initial experiments examined the localization of the nuclear protein Npl3p (Bossie et al., 1992). As evidenced by co-localization with the DAPI staining of chromatin, this protein is efficiently targeted to the nucleus in wild-type cells maintained at RT or shifted to 36°C (Fig. 1, a–f). While *rnal-1* cells target Npl3p to the nucleus when maintained at RT (Fig. 1, g–i), they are unable to do so following a two hour shift to the nonpermissive temperature (Fig. 1, j–l). The same results were obtained when cells were shifted to the nonpermissive temperature for one hour (data not shown).

Since Npl3p is an RNA binding protein that shuttles between the nucleus and the cytoplasm (Flach et al., 1994; Wilson et al., 1994), we considered the possibility that the aberrant targeting of this protein in the *rnal-1* mutant

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Figure 1. The endogenous nuclear protein, Npl3p, is mislocalized in *rnal-1* mutant cells at the nonpermissive temperature. Wild-type (a–f) or *rnal-1* mutant cells (g–l) were maintained at RT (a–c and g–i) or shifted to 36°C for 2 h (d–f and j–l) and prepared for immunofluorescence microscopy as described in experimental procedures. Cells were stained with anti-Npl3p followed by FITC-labeled anti-rabbit antibody (a, d, g, and j), stained with DAPI (b, e, h, and k), or viewed by Nomarski optics (c, f, i, and l).
might be a reflection of the function of Npl3p in RNA metabolism rather than an indication of a general defect in protein localization. To address this point, we examined the localization of a galactose-induced reporter protein containing an NLS. Cells were grown at the permissive temperature in selective media containing 2% raffinose. Cultures were then induced for 3–4 h by the addition of 2% galactose followed by a one to 2-h shift to 36°C. Since rna1-1 mutants are defective in the export of poly(A)⁺ RNA from the nucleus, it was critical that reporter constructs be induced at the permissive temperature and shifted to the nonpermissive temperature only after sufficient induction. Experiments in which cultures were induced and shifted simultaneously showed no signal for the reporter protein (data not shown) as would be expected if the reporter construct is transcribed but message is unable to be efficiently transported to the cytoplasm for translation.

Results are shown for an inducible reporter construct that encodes the SV-40–NLS fused to invertase. As observed with the endogenous Npl3p, the protein is efficiently localized to the nucleus in wild-type cells at both RT and 36°C (Fig. 2, a–f). This is not the case for the rna1-1 mutants which localize the protein predominantly to the nucleus at the permissive temperature (Fig. 2, g–i) but show marked mislocalization of the reporter protein to the cytoplasm following a shift to the nonpermissive temperature (Fig. 2, j–l). Similar results were observed when another reporter construct, H2B-NLS β-galactosidase (Moreland et al., 1987), was employed for this experiment (data not shown). Since the RNA export defect in rna1-1 cells can be observed following a 20-min shift to the nonpermissive temperature (Amberg et al., 1992), we examined the time of onset of the protein import defect. Using the SV-40–invertase reporter protein expressed as described above, mislocalization to the cytoplasm was observed as early as 15 min following a shift to 36°C (data not shown). These data suggest that cells containing a conditional allele of RNAI are unable to efficiently target proteins to the nucleus at the nonpermissive temperature and that this defect occurs on the same time scale as the previously reported RNA transport defect.

Nuclear protein import was also examined directly in living cells. To accomplish this, GFP (Chalfie et al., 1994) was targeted to the nucleus by constructing a fusion to a small portion of Npl3p. These experiments have the advantage over indirect immunofluorescence microscopy that cells are not fixed, thus results obtained cannot be attributed to fixation procedures or to processing of the cells. For these experiments, cells were grown and induced at RT as described above. Cultures were shifted to the nonpermissive temperature when the GFP signal of the fusion protein became visible by fluorescence microscopy (approximately three to four hours after induction). Following the shift to the nonpermissive temperature, samples were examined directly by fluorescence microscopy. The GFP-Npl3 fusion protein was properly targeted to the nucleus in wild-type cells at either RT or 36°C (Fig. 3, a–d) and in mutant cells maintained at the permissive temperature (Fig. 3, e and f). However, when mutant cells were shifted to the nonpermissive temperature, protein mislocalization to the cytoplasm was observed in a manner similar to that observed by indirect immunofluorescence microscopy (Fig. 3, g and h). Cells containing the GFP-Npl3 fusion protein were also stained with DAPI to demonstrate that the fusion protein co-localized with DAPI staining of the nucleus (data not shown). Taken together, the results presented above show that cells containing mutant Rna1p are unable to efficiently target proteins to the nucleus.

**Rna1-1 Mutant Cytosols Are Unable to Support Nuclear Import of a Fluorescently Labeled Substrate In Vitro**

In order to examine the role of the Rna1p in protein import more directly, we employed an in vitro import assay (Schlenstedt et al., 1993). The assay faithfully reproduces all aspects of nuclear import. Both exogenous cytosol and an energy regenerating system are required for import of a fluorescently labeled NLS-containing substrate into the nuclei of semi-intact yeast cells. Hence, this assay can be combined with yeast genetics to investigate the role of nuclear components in import by examining the function of mutant semi-intact cells or to investigate the function of cytosolic factors by examining cytosols derived from mutant cells. Using this approach mutations in several nucleoporins have been shown to block import to the nucleus (Schlenstedt et al., 1993); however, no mutants with import defective cytosols had previously been described.

Since Rna1p is located in the cytoplasm (Hopper et al., 1990), we prepared cytosols from both rna1-1 mutant (PSY714) and wild-type (PSY715) cells either maintained at the permissive temperature or shifted to the nonpermis-
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**Figure 3.** An Npl3p-GFP fusion protein is mislocalized in living *rnal-1* cells shifted to the nonpermissive temperature. Both wild-type (*a*-*d*) and *rnal-1* (*e*-*h*) cells expressing a fusion protein consisting of a portion of Npl3 fused to GFP are shown. Wild-type cells efficiently target this protein to the nucleus at either RT (*a*) or following a 2-h shift to 36°C (*c*). Corresponding Nomarski images are shown in panels *b* and *d*, respectively. In contrast, *rnal-1* cells which localize the protein to the nucleus efficiently at RT (*e*) are unable to do so after a 2-h shift to 36°C (*f*). Nomarski images are shown in *g* and *h*, respectively.

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**Figure 4.** (A) Cytosols made from *rnal-1* mutant cells are unable to support import in vitro. Cytosolic extracts made from wild-type and *rnal-1* cells were included in an in vitro assay that monitored the uptake of a fluorescently labeled NLS-containing substrate. In the absence of exogenous cytosol import does not occur, although binding to the nuclear envelope is observed. When cytosol prepared from wild-type cells maintained at RT or shifted to 36°C is added to import reactions to a final concentration of 2 mg/ml, ~40% of the cells accumulate substrate within the nucleus. In contrast, at the same concentration cytosols made from *rnal-1* mutant cells at RT or at 36°C were unable to support import. The height of each bar represents the mean of three separate experiments in which 200 cells were counted. Standard deviations are indicated by the error bars. (B) Examples of the binding and import reactions observed in the in vitro assay are shown. In the presence of mutant *rnal-1* cytosols (final protein concentration 1.5 mg/ml) binding of the NLS-containing fluorescently-labeled substrate is observed (*rnal-1*). As previously demonstrated (Schlenstedt et al., 1993), in the presence of wild-type cytosol import to the nucleus is observed (WT). Import is also observed when mutant *rnal-1* cytosol is supplemented with 50 µg/ml purified GST-Rnalp (*rnal-1 + RNA1*). The rhodamine signal indicates the NLS-containing fluorescent substrate and the nucleus is indicated by DAPI staining of chromatin. Nomarski images of the corresponding cells are shown.
Rnalp Is Absent from Mutant Cytosols

To address the inability of rnl-1 mutant cytosols to support nuclear import, an analysis of Rnalp in wild-type and mutant extracts was carried out. Immunoblot analysis with anti-Rnalp antibody (Hopper et al., 1990) demonstrated that, as previously reported (Hopper et al., 1990; Tung et al., 1992), the amount of Rnalp present in rnl-1 mutant cells (Fig. 5, lanes 3 and 4) is similar to the amount found in wild-type cells (Fig. 5, lanes / and 2). While cytosolic extracts made from wild-type cells contain as much Rnalp as found in whole cells (Fig. 5, lanes 5 and 6), cytosolic extracts made from rnl-1 mutant cells and utilized in the in vitro assay almost completely lack Rnalp (Fig. 5, lanes 7 and 8) which is found exclusively in the pelleted material (data not shown). Quantitation of the amount of protein by scanning densitometry of immunoblots and comparison to known amounts of purified protein indicates that Rnalp is very abundant in cytosol. The protein is present at a level that is approximately 1% of total soluble protein in wild-type cytosolic extracts used for these experiments. Mutant cytosols, however, have at least 50-fold less Rnalp than found in wild-type cytosols when prepared from cells grown at the permissive temperature. The protein is virtually undetectable in cytosolic extracts made from cells grown at the nonpermissive temperature.

Addition of Purified Rnalp Restores the Import Activity of rnl-1 Mutant Cytosols

Since rnl-1 mutant cytosols that were not competent to stimulate import into semi-intact cells were depleted of Rnalp, we wanted to determine whether adding back purified Rnalp would rescue the defective cytosols. To facilitate purification of Rnalp, a GST-fusion protein was expressed in E. coli. In yeast, expression of a galactose-inducible GST-Rnalp fusion protein rescues the temperature-sensitive phenotype of the rnl-1 mutant (data not shown), indicating that Rnalp can function as a GST fusion. In addition, the GST-Rnalp fusion protein is localized to the cytoplasm and excluded from the nucleus in a manner similar to that observed for endogenous Rnalp (data not shown). A purification profile for the E. coli fusion protein is shown in Fig. 6. Briefly, the protein was induced in DH5α cells (lane 2), a soluble lysate was made (lane 3), and the protein was purified by incubation with glutathione (GT)-Sepharose (lane 4). The GST fusion protein was eluted with excess glutathione, dialyzed against buffer, and concentrated to 0.2 mg/ml. The purified fusion protein was specifically recognized by antibodies directed toward either Rnalp or GST (data not shown).

Purified GST-Rnalp was then added to import assays at various concentrations (Fig. 7). Addition of 50 μg/ml (final concentration) of purified GST-Rnalp had no effect on the stimulation of import observed with 1.5 mg/ml wild-type cytosol (data not shown). A final concentration of 50 μg/ml of the fusion protein did not stimulate import in the absence of exogenous cytosol (data not shown). However, when GST-Rnalp fusion protein was added in conjunction...
with 1.5 mg/ml rna1-1 mutant cytosol, a dose-dependent increase in protein import was observed. As the amount of import to the nucleus increases, the amount of binding at the nuclear envelope decreases (Fig. 7, inset; also see Fig. 4 B). The assumption is that although in the absence of functional Rna1p import to the nucleus is blocked, binding to the nuclear envelope can still occur. Thus, upon addition of functional Rna1p a portion of the nuclear envelope-bound substrate is imported to the nucleus and a subsequent decrease in the overall level of binding results. Maximal stimulation (approximately equal to that observed with wild-type cytosol) was observed at a final concentration of 50 μg/ml of the fusion protein. Import of the fluorescently-labeled substrate observed in the presence of 50 μg/ml GST-Rna1p in conjunction with mutant cytosol is shown in Fig. 4 B (rna1-1 + RNA1). The maximal amount of GST-Rna1p added back to experiments (50 μg/ml) corresponds to ~2% of total cytosolic protein in these reactions, an amount that correlates well with the observed level of Rna1p found in wild-type cytosols, approximately 1% of total protein. Slightly less import was observed in the experiments described in Fig. 7 than in those presented in Fig. 4. This is due to the fact that cytosol was added at a final concentration of 1.5 mg/ml (rather than 2 mg/ml) in these experiments to allow for the addition of appropriate concentrations of purified GST-Rna1p. This amount of cytosol is limiting for import and thus results in the observed decrease in the percentage of cells importing substrate to the nucleus. Purified GST alone had no effect on protein import at either concentration. Finally, addition of the purified mutant protein, GST-Rna1-1p, to a concentration of 50 μg/ml did not stimulate import in the presence of mutant cytosol (data not shown).

To examine the biochemical activity of both GST-Rna1p and GST-Rna1-1p, a GAP assay was performed. This assay monitors the stimulation of GTP hydrolysis by a GTPase in the presence of a GAP (Schlenstedt et al., 1995). It has recently been demonstrated that homologues of Rna1p serve as GAPs for Ran/TC4 (Coutavas et al., 1993; Bischoff et al., 1994, 1995a). Thus, Gsp1p purified from yeast was labeled with [γ-32P]GTP as described in Materials and Methods. A GST fusion to either wild-type or mutant Rna1p was added to reactions and the rate of GTP hydrolysis was monitored by following the loss of the labeled γ-phosphate. This is plotted as the percent radioactive GTP that remained bound to Gsp1p (Fig. 8) vs. concentration of Rna1p (or Rna1-1p) added to reactions. GST-Rna1p induces GTP hydrolysis by Gsp1p in a manner comparable to that previously reported for the mammalian proteins (Bischoff et al., 1994, 1995a). As determined by three independent titrations, 0.5 ± 0.2 nM wild-type Rna1p was required to hydrolyze 50% of the bound GTP in a 10-min incubation. In contrast, the mutant protein purified from E. coli was unable to stimulate GTP hydrolysis by Gsp1p under conditions identical to those used for the wild-type protein. Even at a concentration of 90 nM, mutant protein was unable to significantly stimulate GTP hydrolysis (data not shown). Thus, data obtained from the GAP assay confirms the biochemical functionality of the wild-type Rna1p fusion protein employed for these stud-

Figure 7. Addition of purified GST-Rna1p fusion protein from E. coli to rna1-1 cytosols results in the stimulation of import in the in vitro assay. The in vitro assay was performed and quantitated as described in Fig. 4 with the exception that cytosols were added to a final concentration of 1.5 mg/ml. No import was observed in the absence of cytosol (not shown) or in the presence of rna1-1 mutant cytosol prepared from cells shifted to 36°C. Addition of purified GST (final concentration of 50 μg/ml) to rna1-1 cytosol did not stimulate import. However, when purified GST-Rna1p was added to rna1-1 cytosol at 10 μg/ml, 30 μg/ml, or 50 μg/ml, a dose-dependent increase in import of the fluorescently labeled substrate was observed. The height of each bar represents the mean of three separate experiments in which 200 cells were counted. Standard deviations are indicated by the error bars. (Inset) As import of the fluorescent substrate increases a consequent decrease in the level of binding to the nuclear envelope is observed. The percentage of cells showing either binding (■) or import (○) is plotted versus the amount of purified GST-Rna1p added to the reaction.

Figure 8. Rna1p is a GTPase activating protein for Gsp1p. [γ-32P]GTP-labeled Gsp1p was incubated for 10 min with various concentrations of GST fused to either wild-type (○) or mutant (□) Rna1p as indicated. Experiments were carried out as described in Materials and Methods. Results are plotted as the percent [γ-32P]GTP that remained bound to Gsp1p. Each point shown on the graph represents the mean of three experiments. Standard deviations are indicated by the error bars.
ies. These results also provide a probable explanation for the inability of the mutant fusion protein to induce import of the NLS-containing substrate to the nucleus and suggest that GAP activity is required for nuclear import.

Discussion

The results presented above demonstrate that Rnalp is involved in the import of proteins into the nucleus. The temperature-sensitive mutant rnal-1 exhibits defects in protein localization at the nonpermissive temperature. Furthermore, an in vitro assay demonstrates that cytosols lacking Rnalp are unable to support import into the nuclei of semi-intact yeast cells. Only when purified Rnalp is added back to rnal-1 cytosols do they become competent to support import.

The rnal-1 strain was originally isolated as a yeast mutant with defects in RNA processing and transport (Hartwell, 1967; Hopper et al., 1978; Amberg et al., 1992). The subsequent observation that Rnalp was located in the cytoplasm and excluded from the nucleus (Hopper et al., 1990) made it difficult to assign a cellular function to Rnalp. However, recent biochemical studies have identified a role for this protein in the regulation of the small GTP-binding protein Ran/TC4. The Rnalp homologues from both HeLa cells (Coutavas et al., 1993; Bischoff et al., 1994) and S. pombe (Bischoff et al., 1995a) were purified as stimulators of Ran GTPase activity. Data presented here demonstrate that the S. cerevisiae protein also functions as a GAP for Gsp1p. Furthermore, it has been shown that the S. pombe homologue of Rnalp is able to function in S. cerevisiae (Melchior et al., 1993b). Thus, Rnalp is a highly conserved protein that regulates the ratio of GTP/ GDP-bound Ran/TC4 within the cell by enhancing the rate of Ran/TC4/Gsp1p-mediated GTP hydrolysis. This explains the similar phenotypes observed for rnal-1 mutants shifted to the nonpermissive temperature and wild-type cells overexpressing a GTP-bound form of the budding yeast Ran/TC4 homologue, Gsp1p (Schlenstedt et al., 1995). In both cases, cells are defective in the localization of nuclear proteins and in the export of poly(A)^+ RNA from the nucleus. Taken together, these findings suggest that the cytoplasmic Rnalp does not function solely in RNA processing but rather affects other aspects of cellular metabolism. Thus, Rnalp may play two distinct roles within the cell, one that affects export of RNA and one that affects import of proteins. Conversely, it may function through a more general role in regulation of macromolecular transport, a suggestion that is supported by its identification as the GAP for Ran/TC4.

To address the possibility that the Rnalp functions directly in nuclear import we employed an in vitro import assay. The in vitro assay has the advantage over in situ methods that protein import can be separated from RNA export; the assay monitors uptake of substrate into nuclei of semi-intact cells and requires the addition of exogenous cytosol. Since cells and cytosol can be derived from different sources (i.e., wild-type or mutant), it is possible to determine whether mutant extracts directly affect protein import in the absence of improperly processed or transported RNA molecules. It is difficult to uncouple protein import and RNA export in vivo as they are both mediated by the nuclear pores, a fact that complicates the determination of which phenotype is direct and which is indirect. For this reason, the in vitro assays are critical. In experiments that examine the function of mutant cytosols, the nuclei employed are derived from wild-type cells; thus, the results obtained in this assay are not clouded by the possibility that poly(A)^+ RNA is aberrantly accumulated in the nucleus or within the nuclear pore resulting in an indirect blockage of protein import. In addition, the GAP assay directly examines the biochemical function of Rnalp.

Results indicate that cytosolic extracts that lack Rnalp are unable to support import into wild-type nuclei; however, in the presence of wild-type cytosol, rnal-1 mutant cells are able to support import. Furthermore, addition of purified Rnalp to mutant cytosols depleted of Rnalp restores the import activity. This is not the case for mutant protein. A GAP assay confirms the biochemical activity of the wild-type protein purified from E. coli but also demonstrates that the mutant protein does not serve as a functional GAP when purified from E. coli. The rnal-1 mutant consists of two point mutations, both of which are required to obtain the conditional phenotype (Traglia et al., 1989). Further structure-function analyses will be carried out to determine whether either or both of these mutations specifically alter the GAP activity of Rnalp. Thus, results obtained for the GAP activity of Rnalp parallel those obtained in the in vitro import assay. Wild-type Rnalp which functions as a GAP in vitro is able to efficiently restore protein import activity to depleted cytosols whereas the nonfunctional mutant protein is not. These findings strongly support the hypothesis that Rnalp is an essential cytosolic factor required for nuclear import.

A recent study that demonstrated that the rnal-1 mutant was synthetically lethal with a conditional allele of the nucleoporin NUP1 provides further evidence that Rnalp is involved in transport of macromolecules across the nuclear envelope (Bogerd et al., 1994). In contrast to data presented in this report, Bogerd et al. (1994) cited unpublished data that found no protein import defect in rnal-1 mutant cells under conditions in which RNA export is defective. Since the experimental methods were not detailed, it is impossible to make a direct comparison to the results presented in this manuscript. In order to explain the RNA export defect and the synthetic lethality with nup1, the authors invoke a model where the cytoplasmic Rnalp might transiently associate with the nuclear pore to facilitate RNA export. However, our finding that Rnalp plays a direct role in import of proteins into the nucleus raises the possibility that this synthetic lethality results from a loss of an essential interaction between cytosolic components of the protein import system and the nuclear pore. Although the rnal-1 mutant was originally isolated on the basis of defective RNA processing, it has not yet been demonstrated that Rnalp has a primary function in the maturation and export of RNA. It is plausible, in light of the role of Rnalp in protein import, that RNA export defects observed in the rnal-1 mutant may be indicative of the loss of some short-lived nuclear factor that is required for RNA processing/transport rather than a direct function in RNA export. Until an in vitro assay for the export of poly(A)^+ RNA from the nucleus is developed, it will not be feasible to examine RNA export in the absence of
protein import and distinguish between these two possibilities.

RnAlp restores import activity to mutant cytosols but alone is not able to substitute for wild-type cytosol in import reactions. This suggests that RnAlp is part of a multicomponent system that functions in protein import through regulation of the nucleotide-bound state of Ran/TC4. Other members of this system have been identified (Coutavas et al., 1993; Lee et al., 1993; Moore and Blobel, 1993, 1994b; Bischoff et al., 1994; Lounsbery et al., 1994; Powers and Forbes, 1994; Bischoff et al., 1995b; Ousponski et al., 1995; Schlenstedt, G., D. H. Wong, D. M. Koeppe, and P. A. Silver, manuscript submitted). For example, the nuclear protein RCC1 (Ohtsubo et al., 1989; Dasso, 1993) serves as the nucleotide exchange factor for Ran/TC4 mediating the interconversion of the GDP-bound state to the GTP-bound state (Bischoff and Ponstingl, 1991a). In fact, in the nucleus Ran/TC4 exists as a complex with RCC1 (Bischoff and Ponstingl, 1991b). Interestingly, the yeast homologue of RCC1, PRP20, was identified as a temperature-sensitive mutant with pleiotropic defects in RNA processing/transport (Aebi et al., 1990; Fleischmann et al., 1991; Kadowaki et al., 1992). Like nml-1, the prp20-1 mutant is unable to efficiently localize NLS-containing proteins to the nucleus (Corbett, A. H., D. M. Koeppe, and P. A. Silver, unpublished results). In support of a direct role for Prp20p/RCC1 in nuclear import it has also been demonstrated that in mammalian cells loss of RCC1 causes a defect in protein import to the nucleus in vivo (Tachibana et al., 1994).

Previous fractionation studies have not identified RnAlp as an essential cytosolic factor required for nuclear import (Melchior et al., 1993a; Moore and Blobel, 1993, 1994b). It is possible that at sufficiently high Ran/TC4 concentration, the level of GTP hydrolysis obtained is able to compensate for the lack of GAP activity. This might also be the case if mechanistic differences between Ran/TC4 and Gsp1p result in different intrinsic rates of GTP hydrolysis. Alternatively, wild-type nuclei employed for other in vitro assays could contain an amount of RnAlp that is adequate to support import under the conditions employed.

Several models can be constructed based on the present data regarding RnAlp, Ran/TC4, and RCC1. Since Ran/TC4 is found both in the cytoplasm and in the nucleus and interacts with proteins in both the nucleus and the cytoplasm, it is possible that: (a) a single pool of protein shuttles between the nucleus and cytoplasm; or that (b) distinct protein pools function separately in the different compartments. In either case, a role for RnAlp can be envisioned. If Ran/TC4 functions by shuttling between the nucleus and the cytoplasm, GTP hydrolysis could be required for transport into the nucleus. Ran/TC4 would be targeted to the nuclear membrane in its GTP-bound state, and then upon hydrolysis it would be transported into the nucleus. Since a nucleotide exchange factor, RCC1, is located in the nucleus, it is likely that import would be followed by an interconversion between the GDP-bound state of Ran/TC4 and the GTP-bound state. It is possible that an as of yet unidentified nuclear GAP is required for export of Ran/TC4 from the nucleus which may or may not be accompanied by the export of poly(A)+ RNA or proteins. In this model, a cytoplasmic nucleotide exchange factor would be required to return GDP-bound Ran/TC4 to its GTP-bound state and complete the cycle. Alternatively, it is possible that Ran/TC4 is simply exported in its GTP-bound state. A similar scenario would occur if Ran/TC4 exists as separate, compartmentalized pools. In this case, GTP hydrolysis would support transport of the substrate alone rather than a substrate-Ran/TC4 complex, and the Ran/TC4 would simply cycle to and from the nuclear membrane rather than across it.

At this point it is not possible to distinguish between these models. Based on the data presented in this manuscript it is, however, possible to ascribe a direct role in protein import to RnAlp. Thus far the combination of biochemical and genetic approaches has been extremely useful in the identification of factors involved in nuclear trafficking and will continue to be effective in the elucidation of the mechanism of transport across the nuclear envelope.

We would like to thank Darren Wong, Mike Henry, Praveen Kesheva, and Tom Rapoport for extensive discussions and critical reading of the manuscript.

This work was supported by grants from the National Institutes of Health to P. A. Silver and A. K. Hopper and an Established Investigator Award from the American Heart Association to P. A. Silver. A. H. Corbett was supported by a postdoctoral fellowship from NIH, D. M. Koeppe by an NIH training grant, and M. S. Lee by an American Cancer Society postdoctoral fellowship.


Received for publication 15 May 1995 and in revised form 22 June 1995.

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