Interaction between Ran and Mog1 Is Required for Efficient Nuclear Protein Import*

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Mog1 is a nuclear protein that interacts with Ran, the Ras family GTPase that confers directionality to nuclear import and export pathways. Deletion of MOG1 in Saccharomyces cerevisiae (∆mog1) causes temperature-sensitive growth and defects in nuclear protein import. Mog1 has previously been shown to stimulate GTP release from Ran and we demonstrate here that addition of Mog1 to either Ran-GTP or Ran-GDP results in nucleotide release and formation of a stable complex between Mog1 and nucleotide-free Ran. Moreover, MOG1 shows synthetic lethality with PRP20, the Ran guanine nucleotide exchange factor (RanGEF) that also binds nucleotide-free Ran. To probe the functional role of the Mog1-Ran interaction, we engineered mutants of yeast Mog1 and Ran that specifically disrupt their interaction both in vitro and in vivo. These mutants indicate that the interaction interface involves conserved Mog1p residues Asp62 and Glu65, and residue Lys136 in yeast Ran. Mutations at these residues decrease the ability of Mog1 to bind and release nucleotide from Ran. Furthermore, the E65K-Mog1 and K136E-Ran mutations in yeast cause temperature sensitivity and mislocalization of a nuclear import reporter protein, similar to the phenotype observed for the ∆mog1 strain. Our results indicate that a primary function of Mog1 requires binding to Ran and that the Mog1-Ran interaction is necessary for efficient nuclear protein import in vivo.

The nucleotide state of the Ras family GTPase, Ran, governs the interactions between nuclear transport receptors and protein substrates. Import receptors bind cargo in the absence of Ran-GTP whereas export receptors bind cargo in a trimeric complex with Ran-GTP (reviewed in Ref. 1). The directionality of transport is based on nuclear Ran being primarily GTP-bound and cytoplasmic Ran mainly GDP-bound. Ran-GTP is generated in the nucleus by the Ran guanine nucleotide exchange factor (RanGEF),1 RCC1 (2, 3). Conversely, the primary function of Mog1 requires binding to Ran and its interaction with Ran is evolutionarily conserved in other organisms.

Ran GTase-activating protein, RanGAP1 (4), is localized in the cytoplasm (5, 6) and is believed to maintain cytoplasmic Ran in the GDP-bound state. NTF2 recycles Ran-GDP from the cytoplasm to the nucleus (7, 8) where Ran-GTP is regenerated by RCC1. Thus, the asymmetric distribution of the two nucleotide states of Ran, vital to the directionality of nuclear transport, is maintained through the concerted efforts of several Ran-binding proteins, including RCC1, RanGAP1, and NTF2.

Mog1, a recently identified Ran-binding protein, appears to have a novel role in regulating the Ran GTPase cycle. Mog1p was first isolated in yeast as a suppressor of temperature-sensitive alleles of the Saccharomyces cerevisiae Ran homologue, Gsp1p (9). Mog1p is predominantly nuclear and both Mog1 and its interaction with Ran are evolutionarily conserved (9–12). The yeast ∆mog1 strain is temperature-sensitive for growth and shows defects in signal-mediated protein import, suggesting a role for Mog1p in nuclear protein import (9). The temperature sensitivity of ∆mog1 is suppressed by overexpression of either GSP1 or NTF2 and many of the temperature-sensitive gsp1 mutants suppressed by MOG1 are also suppressed by NTF2 (9). The genetic interaction between MOG1 and NTF2 implies that the functions of these two proteins may at least partially overlap. Recently, Mog1p has been reported to stimulate nucleotide release from Ran-GTP (11, 13). Although these observations suggest an involvement of Mog1 in regulating the nucleotide state of Ran, the precise function of Mog1 in Ran-coordinated nuclear transport has not been defined.

We previously described the 1.9-Å resolution crystal structure of S. cerevisiae Mog1p and identified a highly conserved cluster of surface residues as a putative Ran-binding site (10). Here we have used structure-based targeted mutagenesis of yeast Mog1p and Gsp1p to probe the interaction interface of the protein complex and the cellular function of this interaction. We show that the conserved acidic surface residues Asp62 and Glu65 of Mog1p are important in maintaining the Mog1-Ran interaction. Although Mog1p and Ntf2p cannot bind Gsp1p simultaneously (10), they do not appear to bind to the same site. Hence, mutations in the switch I and II loop regions of Ran that disrupt NTF2 binding (14) do not prevent Mog1 binding and conversely, residue Lys136 in Gsp1p is required for interaction with Mog1p but not with Ntf2p. The E65K-Mog1p and K136E-Gsp1p mutations decrease the strength of the Mog1-Ran interaction and cause defects in growth and nuclear protein import in yeast, indicating that a direct Mog1-Ran interaction is important for these functions in vivo.

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The atomic coordinates and structure factors (code 1JJH) have been deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ (http://www.rcsb.org/)

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2 The abbreviations used are: RanGEF, Ran guanine nucleotide exchange factor; RanGAP, Ran GTase-activating protein; PCR, polymerase chain reaction; mantGDP/GTP, 2′,3′-O-methylanthraniloyl GDP/GTP; PAGE, polyacrylamide gel electrophoresis; GST, glutathione S-transferase; NLS, nuclear localization signal; GFP, green fluorescent protein.
EXPERIMENTAL PROCEDURES

Recombinant Protein Expression and Purification—Yeast Mog1p was expressed in Escherichia coli and purified as described (15). His-tagged human Mog1, which is 29% identical and 47% similar to yeast Mog1p, was expressed and purified as described (12). Yeast Ntf2p was prepared as previously described for vertebrate NTF2 (16) and the yeast homologue of importin-β, Kap85p, was purified according to the protocol used for vertebrate importin-β (17).

The open reading frame of GSP1 was amplified by PCR and cloned into the NdeI/BamHI sites of pET15b (Novagen) to generate an N-terminal His-tag. His-tagged Gsp1p was expressed in E. coli strain BL21 (DE3) and purified by Ni-NTA affinity and gel filtration chromatography (12). The nucleotide state of Gsp1p was modulated by incubating the bacterially expressed protein with GDP, GTP, mantGDP, or mantGTP at 100-fold molar excess in the presence of 20 mM EDTA for 30 min at room temperature. Nucleotide exchange was completed by the addition of 40 mM MgCl₂. Unbound nucleotide was removed and the Gsp1p exchanged into the desired buffer using a PD-10 gel filtration column (Amersham Pharmacia Biotech). Wild-type canine Ran and Ran mutants were expressed as described (14), purified by ion exchange chromatography on a Q-Sepharose column (Amersham Pharmacia Biotech), and charged with GDP or GTP as described above.

Mutagenesis of Mog1p and Gsp1p—To improve protein expression and stability of recombinant Mog1p, we created an NH₂-terminal truncation construct that uses Met19 as the translation start site. Amino-terminal truncated Mog1p retained the ability to bind Gsp1p in vitro and complemented the temperature-sensitive phenotype of Δmog1 (data not shown). Amino acid substitutions were introduced into Mog1p and Gsp1p with construct by site-directed mutagenesis (QuickChange Site-directed Mutagenesis Kit, Stratagene). For expression in yeast, MOG1 and GSP1 open reading frames cloned in the pBluescript (Stratagene) were mutagenized and then subcloned into yeast plasmids. For each mutant, the presence of the desired mutations and absence of extraneous mutations was confirmed by sequencing the open reading frame. Gsp1p mutants were screened for binding to the open reading frame. Gsp1p mutants were screened for binding to yeast plasmids. For each mutant, the presence of the desired mutations and absence of extraneous mutations was confirmed by sequencing the open reading frame cloned in the HIS3 integrating plasmid, pRS303 (24). E65K-Mog1p was then integrated at the endogenous MOG1 locus by linearization of E65K-Mog1/pRS303 (pAC688) and transformation into the wild-type haploid ACT193 (25) to create ACT545. Transformants that grew on plates lacking histidine were selected and the presence of the Δmog1 mutation was confirmed by PCR and sequencing. To generate cells carrying K136E-Gsp1p or Myc-tagged Gsp1p plasmids as the sole source of Gsp1p, Δgsp1Δmyc2 cells (ACY212) (26) were transformed with centromeric plasmids encoding K136E-Gsp1p (pAC838), myc-Gsp1p (pAC827), or myK136E-Gsp1p (pAC839) and streaked on 5-fluoroorotic acid to remove the wild-type GSP1 plasmid (27).

In Vivo Analysis—For growth analysis, yeast cells were grown to saturation in selective media at 25 °C. Cells were diluted to 5 × 10^6 cells/ml and shifted to 37 °C. Growth was monitored by measuring the optical density (Aₑ₀₂₅ nm) at 2-h intervals. Growth on plates was assessed by growing cultures to saturation as above, serial diluting them (1:10), and spotting onto plates. Plates were incubated at either 25 or 37 °C for 5 days.

GST Binding Experiments/Immunoblotting—Yeast expressing myc-Gsp1p or myc-K136E-Gsp1p as the only source of Gsp1p were transformed with 2 μg galactose inducible plasmids (28) encoding GST alone (pAC403), GST-Mog1p (pAC590), GST-D62K-Mog1p (pAC877), or GST-E65K-Mog1p (pAC878). GST binding experiments were performed as described previously (12) except that cells were induced with galactose from 25 °C to 37 °C in selective media. One μg of total protein was loaded on SDS-PAGE gels, and unbound samples and 30 μl of the bound sample were resolved on a 12% SDS-PAGE gel, then transferred to nitrocellulose and probed with monoclonal α-Myc antibody (1:2000 dilution) or α-GST antibody (1:1500 dilution). To analyze the mutant proteins and confirm their expression at equivalent levels to wild-type protein, equal concentrations of lysates were loaded with either monoclonal α-Myc antibody (1:2000 dilution) or a polyclonal α-Mog1p antibody (1:5000 dilution).

Nuclear Protein Localization—An NLS-GAL4AD-GFP reporter plasmid (pAC697) (29) was transformed into wild-type (ACY193), Δmog1 (ACY547), E65K-Mog1p (ACY548), and K136E-Gsp1p (ACY570) yeast cells. Cells were grown to phase at 25 °C and shifted to 37 °C for 4 h. For all experiments, cells were stained with 4',6-diamidino-2-phenylindole to visualize the DNA and confirm the location of the nucleus (data not shown). The localization of the NLS reporter was monitored by directly viewing the GFP signal in living cells through a GFP optimized filter (Chroma Technology) using an Olympus BX60 epifluorescence microscope equipped with a Photometrics Quantix digital camera.

RESULTS

Engineered Mog1p Mutants with Decreased Affinity for Ran—The crystal structure of Mog1p showed a cluster of highly conserved residues (Fig. 1A) on a ∼25-Å diameter surface patch (10). Many of these residues are charged and could potentially form intermolecular salt bridges with oppositely charged residues on Ran. We targeted these charged residues on Mog1p for mutagenesis and compared the mutant proteins to wild-type Mog1p for their ability to bind Gsp1p-GDP and Gsp1p-GTP. Wild-type Mog1p bound to Gsp1p in either nucleotide state (Fig. 1B), although the amount of Gsp1p-GTP bound was greater than Gsp1p-GDP. The binding of Gsp1p in both nucleotide states to Mog1p beads is consistent with recent reports that Mog1 binds not only Ran-GTP as initially suggested (9) but Ran-GDP as well (10, 12). Although mutations at Arg385 (R58A or R58E) did not reduce Gsp1p binding (data not shown), mutations at either Asp392 (D62K) or Glu405 (E65K and E65A) significantly reduced the affinity of Mog1p for both nucleotide states of Gsp1p in vitro (Fig. 1B). The effect of the Mog1p mutations was even more pronounced in vivo since the amount of Gsp1p that co-purified with GST-tagged Mog1p from yeast cells was decreased to undetectable levels for both D62K-Mog1p and E65K-Mog1p (Fig. 1C).

Since residues Asp62 and Glu45 are on the surface of Mog1p, it is unlikely that mutating them to lysine would cause an overall conformational change in the molecule. To investigate the effect of mutations in these residues, we used crystallography and CD spectroscopy. We solved the crystal structure of the E65A-Mog1p mutant by molecular replacement to 2.0-Å reso-
Located within this cluster are two conserved acidic residues, Asp 62 and Glu 65, respectively. Wild-type Mog1p binds Gsp1p in both nucleotide states of Gsp1p. A cluster of conserved residues (Table I) suggested that they too retained the same three-dimensional structure as wild-type Mog1p, although a severe twinning problem prevented us from obtaining high-resolution crystal structures for these two mutants. The CD spectra for wild-type Mog1p and the mutants were identical (data not shown), consistent with the mutants having the same conformation as wild-type Mog1p.

Mog1p-stimulated Nucleotide Release from Ran-GDP and Ran-GTP—Our binding experiments (Fig. 1B) suggested that, in contrast to previous reports (11, 13), Mog1 might bind and stimulate nucleotide release from both the GDP- and GTP-bound states of Ran. To investigate this possibility in more detail, we developed a guanine nucleotide release assay using the fluorescent nucleotide analogues, mantGDP and mantGTP, which are comparable to unmodified nucleotides in their affinity for Ran (30). Following incubation of Mog1p with Gsp1p-mantGDP or Gsp1p-mantGTP, samples were resolved by gel filtration and fractions were examined for protein absorbance and fluorescence emission. This allowed the relative amounts of Mog1p-Gsp1p complex, free protein monomers, and free nucleotide to be compared. This assay demonstrated that addition of Mog1p to either nucleotide state of Gsp1p generated a 1:1 complex between wild-type Mog1p and nucleotide-free Gsp1p with the concomitant release of either GDP or GTP, whereas in the absence of Mog1p, the fluorescent nucleotide remained stably bound to Gsp1p (Fig. 2, A and B). The Mog1p mutants were also assayed for their ability to bind Gsp1p and stimulate nucleotide release. Compared with wild-type Mog1p, significantly reduced levels of complex formation and nucleotide release were observed when the Mog1p mutants were incubated with either nucleotide state of Gsp1p (Fig. 2, A and B).

In Vivo Analysis of Mog1p Mutants—Because E65K-Mog1p displayed a more dramatic decrease in Ran binding than D62K-Mog1p in vitro, we chose to examine the effects of the E65K mutation in S. cerevisiae. To assess the in vivo function of E65K-Mog1p, we integrated E65K-Mog1 into the endogenous MOG1 locus making E65K-Mog1p the only form of Mog1p expressed in these cells. Cells lacking MOG1 are temperature sensitive for growth at 37 °C (9). To determine if the E65K-Mog1p mutant also conferred a temperature-sensitive growth phenotype, we compared the growth of E65K-Mog1p cells to wild-type and Δmog1 cells (Fig. 3). We found that E65K-Mog1p cells grew more slowly than wild-type cells at 37 °C. The phenotype observed was not as profound as in cells lacking Mog1p (Fig. 3B), suggesting that either the Mog1p-Gsp1p interaction was not completely disrupted by the E65K-Mog1p mutation in vivo or that Mog1p performs additional functions in the cell that do not involve its interaction with Gsp1p. Mog1p levels in wild-type and E65K-Mog1p cells were comparable, confirming that the growth phenotype observed for E65K-Mog1p was not due to a decreased level of E65K-Mog1p (data not shown).

Identification of a Putative Mog1-binding Site on Ran—Since Gsp1p-GDP binding by Mog1p and Ntf2p is mutually exclusive, the two proteins may have a common or at least overlapping binding site on Ran (10). The NTF2-Ran interaction is mediated by residues in the switch I and II loops of Ran (14, 31, 32), the regions of Ras family GTPases that change conformation in response to nucleotide state (33, 34). To determine if the same residues were involved in the Mog1-Ran interaction, we tested the Ran switch I mutant T42A and switch II mutants Q69L, F72W, and R76E for binding to His-tagged human Mog1. Con-
Mog1p stimulates nucleotide release from Gsp1p-mantGDP/GTP. Gsp1p charged with a fluorescent guanine nucleotide analogue, mantGDP (A) or mantGTP (B), was incubated with equimolar wild-type Mog1p, D62K-Mog1p, E65K-Mog1p, or as a control, with buffer alone for 1 h at room temperature and then filtered through a Superdex 75 FPLC column (Amersham Pharmacia Biotech). The protein absorbance at 280 nm (black circles) and mantGDP fluorescence emission at 450 nm (red triangles) were measured for each fraction. In the absence of Mog1p, Gsp1p elutes bound to nucleotide with overlapping A 280 and E 450 peaks in fraction 24. In reactions with wild-type Mog1p and Gsp1p in either nucleotide state, the major peak of protein absorbance elutes at fraction 20 with an apparent molecular mass of 50 kDa, which corresponds to a 1:1 complex between Mog1p and Gsp1p. Minor peaks of absorbance at 280 nm are also observed, corresponding to free protein monomers and free nucleotide. No fluorescence emission is associated with fractions containing the Mog1p-Gsp1p complex. Instead, the main peak of fluorescence elutes in fractions containing free nucleotide, with a second minor peak co-eluting with the Gsp1p monomers. The protein absorbance and fluorescence emission profiles for reactions between Gsp1p-mantGDP and the D62K-Mog1p or E65K-Mog1p mutants are similar to those of the control reaction without Mog1p. Complex formation and nucleotide release is observed for reactions between Gsp1p-mantGTP and the Mog1p mutants, but at reduced levels compared with reactions with wild-type Mog1p.

**Table I**

Crystallographic data for P2 12121 E65A-Mog1p

| Data collection | Value |
|-----------------|-------|
| Resolution range (Å) | 31.2–1.9 (2.0–1.9) |
| Number of observations/unique reflections | 62794/18191 |
| Completeness (%) | 99.9 (99.9) |
| Multiplicity | 3.5 (3.6) |
| Rmerge | 0.046 (0.076) |
| l/σ | 6.4 (8.9) |

| Refinement | Value |
|-----------|-------|
| Number of residues/water molecules | 180/198 |
| R-factor/free R-factor | 0.208/0.248 |
| RMS bond length (Å/angle (°)) | 0.009/1.46 |

| Ramachandran plot | Value |
|------------------|-------|
| Most favored (%) | 88.7 |
| Allowed (%) | 9.5 |
| Generously allowed (%) | 1.4 |
| Forbidden (%) | 0.5 |

* Highest resolution shell in parentheses.

Rmerge = \[\sum_{hkl} \left| \frac{I_{hkl}}{I_{obs}} - \frac{I_{calc}}{I_{calc}} \right| \sum_{hkl} \left| I_{calc} \right| \], where \( I_{obs} \) is the mean of the observations \( I_{calc} \) of reflection \( hkl \).

R-factor = \[\sum_{hkl} \left( \frac{\left| F_{hkl} \right|}{\sum_{hkl} \left| F_{hkl} \right|} - \frac{\left| F_{calc} \right|}{\sum_{hkl} \left| F_{calc} \right|} \right) \sum_{hkl} \left| F_{calc} \right| \], where \( F_{obs} \) and \( F_{calc} \) are the observed and calculated structure factors, respectively.

Free R was computed using 5% of the data assigned randomly.

**Fig. 2.** Mog1p stimulates nucleotide release from Gsp1p-mantGDP/GTP. Gsp1p charged with a fluorescent guanine nucleotide analogue, mantGDP (A) or mantGTP (B), was incubated with equimolar wild-type Mog1p, D62K-Mog1p, E65K-Mog1p, or as a control, with buffer alone for 1 h at room temperature and then filtered through a Superdex 75 FPLC column (Amersham Pharmacia Biotech). The protein absorbance at 280 nm (black circles) and mantGDP fluorescence emission at 450 nm (red triangles) were measured for each fraction. In the absence of Mog1p, Gsp1p elutes bound to nucleotide with overlapping A 280 and E 450 peaks in fraction 24. In reactions with wild-type Mog1p and Gsp1p in either nucleotide state, the major peak of protein absorbance elutes at fraction 20 with an apparent molecular mass of 50 kDa, which corresponds to a 1:1 complex between Mog1p and Gsp1p. Minor peaks of absorbance at 280 nm are also observed, corresponding to free protein monomers and free nucleotide. No fluorescence emission is associated with fractions containing the Mog1p-Gsp1p complex. Instead, the main peak of fluorescence elutes in fractions containing free nucleotide, with a second minor peak co-eluting with the Gsp1p monomers. The protein absorbance and fluorescence emission profiles for reactions between Gsp1p-mantGDP and the D62K-Mog1p or E65K-Mog1p mutants are similar to those of the control reaction without Mog1p. Complex formation and nucleotide release is observed for reactions between Gsp1p-mantGTP and the Mog1p mutants, but at reduced levels compared with reactions with wild-type Mog1p.
sistent with the yeast Mog1 binding assays (see Fig. 1B), the amount of Ran-GDP bound to human Mog1 was lower than observed with Ran-GTP (Fig. 4A). Importantly, the Ran switch loop mutants in both nucleotide states displayed wild-type levels of binding to Mog1, with the possible exception of Q69L-loop mutants in both nucleotide states displayed wild-type binding to the switch II mutants Q69L, F72W, and R76E charged with GDP or GTP. As a control, GDP-bound wild-type Ran and mutants were also assayed for binding to NTF2-Sepharose beads. B, schematic representation of the Ran-GDP structure showing the positions of residues targeted for mutagenesis. Lys136 of Ran (red), located on helix α4 (dark pink) is equivalent to Lys134 in Gsp1p, which is required for efficient Mog1p binding. Mutation of two residues (green) decrease binding of Gsp1p to Ntf2p without affecting the Mog1-Ran interaction. The positions of the switch I loop (yellow), switch II loop (orange), and guanine nucleotide (indigo) are indicated. Conserved residues Lys136 and Asp138 (violet) stabilize binding of the guanine base. The COOH-terminal helix α6 (light pink) packs against helix α4 in the Ran-GDP structure.

In Vitro and In Vivo Analysis of K136E-Gsp1—Mog1p was assayed for its ability to interact with His-tagged K136E-Gsp1p in either nucleotide state. Compared with wild-type Gsp1p, only a weak residual interaction with K136E-Gsp1p was observed for wild-type Mog1p (Fig. 5A, lanes 1–2 and 7–8). A similar low level of binding to K136E-Gsp1p was detected for the D62K-Mog1p and E65K-Mog1p mutants, even when present in 5-fold excess over the level of wild-type Mog1p (Fig. 5A, lanes 3–4 and 9–10). In contrast, neither Ntf2p nor Kap95p binding to K136E-Gsp1p was decreased compared with wild-type Gsp1p (Fig. 5A, lanes 5–6 and 11–12) indicating that the K136E mutation did not introduce a major conformational change. The effect of the K136E substitution on the strength of the Mog1-Ran interaction in yeast was also examined. Unlike wild-type Gsp1p, which co-purified with GST-tagged Mog1p, we were unable to detect any K136E-Gsp1p associated with GST-tagged Mog1p (Fig. 5B).

To analyze the function of K136E-Gsp1p in vivo, we expressed this mutant as the only source of Gsp1p in yeast. The growth of wild-type, Δmog1, and K136E-Gsp1p cells was monitored by A600 (Fig. 5C). This analysis showed that K136E-Gsp1p cells were viable, indicating that K136E-Gsp1p can functionally replace wild-type Gsp1p. However, the growth of K136E-Gsp1p was slower than wild-type cells and similar to Δmog1.
FIG. 5. The K136E-Gsp1p mutation disrupts the Mog1p-Gsp1p interaction and causes a temperature-sensitive phenotype in vivo. A, His-tagged wild-type Gsp1p or K136E-Gsp1p charged with GDP or GTP was bound to Ni-NTA-agarose and then incubated with wild-type Mog1p, D62K-Mog1p, or E65K-Mog1p. Control reactions show wild-type levels of binding between Ntf2p and K136E-Gsp1p-GDP (compare lanes 5 and 6) and between Kap95p and K136E-Gsp1p-GTP (compare lanes 11 and 12). The D62K-Mog1p and E65K-Mog1p mutants were present at 5-fold molar excess compared with wild-type Mog1p. B, yeast expressing myc-Gsp1p or myc-K136E-Gsp1p as the only functional copy of Gsp1p were transformed with plasmids encoding GST or GST-Mog1p. Binding assays were carried out as described under “Experimental Procedures.” An immunoblot probed with an anti-Myc antibody to detect Myc-tagged Gsp1p is shown. Lanes are designated lysate (L), unbound (U), or bound (B). The expression of wild-type Gsp1p or K136E-Gsp1p was similar and the amount of GST fusion protein in the bound (B) sample was approximately equal for each sample (data not shown). The negative control GST alone does not interact with Gsp1p or K136E-Gsp1p. C, wild-type Gsp1p, K136E-Gsp1p, or an empty vector were expressed in Δgsp1Δgsp2 yeast cells that were maintained by a wild-type Gsp1p URA plasmid (see “Experimental Procedures”). K136E-Gsp1p function was analyzed by streaking the transformants on 5-fluoroorotic acid to remove the wild-type GSP1 plasmid. The growth of cells expressing K136E-Gsp1p (▲) was compared with wild-type (●), and Δmog1 (■) at 37 °C by measuring A600 at 2-h intervals as described under “Experimental Procedures.”

FIG. 6. Nuclear protein import is impaired by the E65K-Mog1 and K136E-Gsp1p mutations. Wild-type, Δmog1, E65K-Mog1, and K136E-Gsp1p cells were transformed with a plasmid encoding an NLS-GAL4AD-GFP reporter protein. The cells were grown to log phase at 25 °C and shifted to 37 °C for 4 h. The localization of the reporter protein was detected by direct fluorescence of the GFP. In wild-type cells the reporter protein is concentrated in the nucleus. Δmog1, E65K-Mog1, and K136E-Gsp1p cells all show diffuse localization of the reporter protein throughout the cell indicative of a nuclear protein import defect. Wild-type, Δmog1, E65K-Mog1, and K136E-Gsp1p are defective in nuclear protein import (Fig. 6), which may indicate that other functions of Gsp1p, in addition to Mog1p binding, were affected by the K136E-Gsp1p mutation.

Genetic Interaction between MOG1 and PRP20—Mog1p binds tightly to the nucleotide-free form of Gsp1p (11, 13). The only other protein that binds to the nucleotide-free form of Ran is the RanGEF, RCC1 (2) in vertebrates or Prp20p (35) in yeast. As a genetic test for functional overlap between MOG1 and PRP20, we tested for any synthetic growth defects in a double mutant. Since PRP20 is essential, we used a well characterized temperature-sensitive allele, prp20-1 (36, 37), which shows reduced binding to Gsp1p (38), suggesting that nucleotide exchange by this mutant protein is also reduced. If Mog1p is involved in modulating the nucleotide state of Gsp1p within the nucleus, as has been demonstrated for the mammalian exchange factor, RCC1 (2, 3), cells with mutations in both MOG1 and PRP20 might be more growth compromised than either of the single mutants. We therefore deleted MOG1 in the prp20-1 mutant to generate the prp20-1Δmog1 double mutant. The prp20-1Δmog1 double mutant was unable to grow under conditions that supported the growth of each single mutant (Fig. 7), demonstrating a genetic interaction between MOG1 and PRP20.
FIG. 7. Δmo11 and prp20-1 mutants are synthetically lethal. The Δmo11 and prp20-1 mutants were combined by deleting the MOG1 gene in a prp20-1 background as described under “Experimental Procedures.” The double mutant cells were maintained by a PRP20 plasmid. The growth of prp20-1Δmo11 was compared when transformed with both PRP20 and MOG1 (wild-type), MOG1 (prp20-1), PRP20 (Δmo11), or empty vector (prp20-1Δmo11). For analysis of the double mutant, the wild-type PRP20 maintenance plasmid was expelled using 5-fluoroorotic acid. Cells were grown to saturation in synthetic media before serial dilution (1:10) and spotting. The plate was incubated at 25 °C for 5 days. The wild-type, Δmo11, and prp20-1 cells all grew at similar rates. prp20-1Δmo11 cells are unable to grow, demonstrating a synthetic lethal interaction between these two mutations.

DISCUSSION

The Ran-binding protein, Mog1, has emerged as a novel factor involved in regulating the nucleotide state of Ran. Previous reports showed that Mog1 is a guanine nucleotide release factor for Ran-GTP (11, 13) and we have demonstrated that Mog1 also stimulates the release of nucleotide from Ran-GDP. Thus, an interaction between Mog1 and either nucleotide state of Ran results in nucleotide release and formation of a stable complex between Mog1 and nucleotide-free Ran. The RanGGEF, RCC1, can also catalyze nucleotide release from either nucleotide state of Ran (30) but unlike RCC1, Mog1 does not promote rebinding of nucleotide (11). Therefore, Mog1 binding may cause Ran to adopt a unique conformation that releases the nucleotide and stabilizes the molecule in this nucleotide-free state. We have used structure-based mutagenesis of Mog1 and Ran to identify residues required for formation of this complex and to assess the importance of the Mog1-Ran interaction in vivo.

From the crystal structure of S. cerevisiae Mog1p, we predicted that a highly conserved cluster of surface residues might constitute a binding site for Ran. Mutagenesis of this conserved surface patch revealed that residues Asp122 and Glu125 are important for the Mog1-Ran interaction. Replacement of wild-type Mog1p in yeast with the E65K-Mog1p mutant caused temperature-sensitive growth and defects in nuclear protein import, similar to the phenotype observed for Δmo11 cells. These results suggest that a primary function of Mog1p in the cell relies on its ability to bind Ran.

The switch loops of Ran (involved in the NTF2-Ran interaction) were possible candidates for a Mog1-binding site because Mog1p and Ntf2p cannot bind Gsp1p simultaneously (10). However, several switch I and II loop mutants retarded the ability to bind Mog1, suggesting that this region of Ran is not involved directly in the interaction with Mog1. Although reduced binding to Mog1 was previously reported for the switch II mutant, Q69L-Ran-GTP (11), we found that Mog1 had a wild-type level of affinity for Q69L-Ran-GTP. Because the Q69L-Ran mutant is prone to denaturation, we always carried out our assays using freshly prepared protein.

In a mutagenesis screen of Gsp1p charged surface residues, we observed reduced Mog1p binding to the K136E-Gsp1p mutant. The conformation of Gsp1p was not likely to have been perturbed by this mutation because wild-type levels of binding to both Ntf2p and Kap95p were observed for K136E-Gsp1p. Like Δmo11 and E65K-Mog1, the K136E-Gsp1 mutant showed protein import defects, consistent with Lys134 being involved in the Mog1-Ran interaction. These observations support the hypothesis that the Mog1-Ran interaction is required for efficient nuclear protein import.

Lys136 in Gsp1p corresponds to Lys134 in vertebrate Ran, which is located on helix α4 in the Ran-GDP structure (39). Because the COOH-terminal helix α6 is juxtaposed against helix α4 (Fig. 4B), Mog1 would likely have to displace the COOH-terminal helix to bind and release nucleotide from Ran-GDP. This might explain the higher affinity of Mog1 for Ran-GTP compared with Ran-GDP, since the COOH-terminal helix is already displaced from this region in Ran-GTP (40–42). Consistent with this hypothesis, deletion of the COOH-terminal DEDDDL motif, which stabilizes the conformation of helix α6 in Ran-GDP (40), increases the affinity of Mog1 for Ran (13). Deletion of the Ran-DEDDDL motif also stimulates the formation of complexes between importin-β and Ran (43, 44) and between RCC1 and Ran (45). Since both importin-β and RCC1 make contacts with Lys134 and other residues in the helix α4 region of Ran (41, 42, 46), the binding sites for importin-β and RCC1 on Ran partially overlap with the putative Mog1-binding site. Overlapping binding sites for Mog1 and importin-β on Ran would explain our inability to detect a trimeric complex between Mog1, Ran, and importin-β. Although the Mog1- and NTF2-binding sites on Ran do not appear to overlap, simultaneous binding is likely prevented because the two molecules bind different conformational states of Ran. NTF2 binds Ran in the GDP-bound state in which the COOH-terminal helix is stabilized against the core of the molecule, whereas NTF2 is prevented from binding Ran-GTP by a steric clash with the COOH-terminal linker in its displaced conformation (40). Conversely, our model suggests that Mog1 binding to Ran requires displacement of the COOH terminus, which may explain why Mog1 and NTF2 binding to Ran is mutually exclusive.

The putative Mog1-binding site in the Lys123 region is adjacent to a loop containing residues Lys122 and Asp125 which are part of the highly conserved NKxD nucleotide-binding motif (Fig. 4B). These residues interact with the guanine base in both nucleotide states of Ran (39, 40). Therefore, Mog1 binding to helix α4 may induce a conformational change in this loop that disrupts binding of Lys123 and Asp125 to the guanine base promoting nucleotide release from Ran-GDP or Ran-GTP. Like Mog1, RCC1 also interacts with Ran residues in helix α4 and large conformational changes are observed for helix α4 and the NKxD base-binding motif of Ran in the RCC1-Ran structure (46).

In summary, we have defined residues on Mog1 and Ran important for their interaction in vitro and in vivo and have demonstrated the importance of the Mog1-Ran interaction in nuclear protein import. We have also shown that Mog1 binds and stimulates nucleotide release from both Ran-GDP and Ran-GTP in vitro, suggesting that the function of Mog1 in nuclear protein import may involve its modulation of the nucleotide state of Ran. The genetic interaction we detected between MOG1 and PRP20, the yeast RanGGEF, raises the possibility that these two proteins may act synergistically to generate Ran-GTP in the nucleus.

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