Yrb1p Interaction with the Gsp1p C Terminus Blocks Mog1p Stimulation of GTP Release from Gsp1p*

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Mog1p, a multicopy suppressor of gsp1, the temperature-sensitive mutant of the Saccharomyces cerevisiae Ran homologue, binds to GTP-Gsp1p but not to GDP-Gsp1p. The function of Mog1p in the Ran cycle is as yet unknown. This study found that Mog1p releases a nucleotide from GTP-Gsp1p but not from GDP-Gsp1p. Yrb1p, the S. cerevisiae homologue of RanBP1, which is a strong inhibitor of RCC1-stimulated nucleotide release, also inhibited the Mog1p-stimulated nucleotide release from GTP-Gsp1p. At a concentration corresponding to the molar concentration of GTP-Gsp1p, Yrb1p completely inhibited the Mog1p-stimulated nucleotide release. Consistently, the Yrb1p-GTP-Gsp1p complex was more stable than the Mog1p-GTP-Gsp1p complex. Yrb1p did not inhibit the Mog1p-stimulated nucleotide release from GTP-Gsp1pΔC. The Gsp1pΔC protein lacks the final eight amino acids of the C terminus, and for this reason, the interaction between GTP-Gsp1pΔC and Yrb1p was strongly reduced. On the other hand, Mog1p binds to GTP-Gsp1pΔC more efficiently than to GTP-Gsp1p.

Saccharomyces cerevisiae Gsp1p is a homologue of a mammalian Ras-like nuclear GTPase, Ran (1). Unlike Ras, Ran does not possess a lipid modification site at the C terminus. Instead, it possesses the DEDDDL sequence conserved in the Ran family (2). RCC1, a guanine nucleotide exchange factor of Ran, is localized on the chromatin (3), whereas RanGAP1, a Ran GTPase-activating protein, is localized within the cytoplasm (4, 5). GTP-Ran produced in the nucleus enters the cytoplasm where the GTP of Ran is hydrolyzed through the aid of RanGAP1. The resulting GDP-Ran enters the nucleus through the aid of p10/NTF2, which specifically binds to GDP-Ran (6).

Consistent with the fact that Ran is required for nucleocytoplasmic transport, the majority of proteins binding to GTP-Ran belong to the family of proteins possessing RanBD (Ran-binding domain) which are RanBP1/Yrb1p, RanBP2, RanBP3/Yrb2p, and the importin β family. All of them are involved in the nucleus/cytosol exchange of macromolecules (7, 8). In addition, Dis3p (9), RanBPM (10), and Mog1p (11) have been reported to bind to GTP-Ran. Dis3p is one of the subunits comprising the exosome (12), and RanBPM demonstrated the finding that Ran is also involved in microtubule assembly (13, 14). Mog1p is a novel protein binding to GTP-Gsp1p. It was isolated as a multicopy suppressor of gsp1, the temperature-sensitive (ts) mutant of the S. cerevisiae Ran homologue (11). The disruptant of MOG1,Δ mog1, is temperature-sensitive for growth. In this mutant, both nuclear localization signal, nuclear localization signal-dependent and -independent nuclear protein pathways are abolished, but mRNA export seems to be normal (11). Interestingly, the overproduction of Ntf2p confers the ts+ phenotype to Δ mog1 cells. Ntf2p is also essential for nuclear protein import (15, 16).

In order to clarify the function of Mog1p in the Ran GTPase cycle, recombinant Mog1p was produced in Escherichia coli and purified. Interestingly, recombinant Mog1p releases a nucleotide from GTP-Gsp1p but not from GDP-Gsp1p. It did not interfere with Prp20p, the S. cerevisiae RCC1 homologue, or with Rna1p, the S. cerevisiae RanGAP1 homologue. Yrb1p, the S. cerevisiae RanBP1 homologue which inhibits the RCC1-stimulated nucleotide release, also inhibited the Mog1p-stimulated nucleotide release from GTP-Gsp1p but not from GTP-Gsp1pΔC. Gsp1pΔC lacks the final eight amino acids of the C terminus, and for this reason, the interaction between Yrb1p and Gsp1pΔC was strongly reduced. However, Gsp1pΔC still binds to Mog1p, with an efficiency higher than when wild-type (wt) Gsp1p binds to Mog1p. The strong interaction between Mog1p and Gsp1pΔC should be a clue toward clarifying Mog1p function in the Ran GTPase cycle, which is as yet unknown.

EXPERIMENTAL PROCEDURES

Strains and Medium—S. cerevisiae wild-type strain, YPH499, and E. coli strains, BL21(DE3) and XL1-blue, were used for producing recombinant proteins and for plasmid engineering, as described previously (17). The media used for yeast and bacteria have also been described previously (18).

Construction and Purification of Recombinant Proteins—Plasmids carrying the glutathione S-transferase (GST)-fused DNAs (pGEX-MOG1 (11), pGEX-RNA1 (19), pGEX-GSP1 (19), pGEX-YRB1Δ1–9 (19), and pGEX-PRP20 (19)) have been described previously. The deletion of the C terminus of Gsp1p was accomplished using polymerase chain reaction mutagenesis to introduce a stop codon and remove the final 8 amino acid residues of the open reading frame. The coding region of the GSP1 gene was amplified using as primers 5′-CAT TTA TAT TTA TCC ATG GCT GCC CCA G-3′ and 5′-CCC TTA TAA ATC AAA GCA GAC G-3′. The resulting GSP1 DNA was digested with the restriction enzymes NcoI and HindIII and then inserted into the NcoI/HindIII sites of pGEX-KG.

The E. coli strains, BL21 (DE3)/pGEX-MOG1, BL21(DE3)/pGEX-PRP20, BL21 (DE3)/pGEX-YRB1Δ1–9, and BL21(DE3)/pGEX-RNA1, were each cultured individually in 750 ml of 5B medium at 30 °C up to an OD600 of 0.4, treated with isopropyl-1-thio-β-D-galactopyranoside (final concentration 0.2 mM) for 4 h, and then collected by centrifugation at 3,200 rpm for 15 min. Cells were lysed, and GST-fused proteins were purified using glutathione-Sepharose 4B beads (Amersham Pharmacia

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1 The abbreviations used are: wt, wild type; GAP, GTPase-activating protein; Pipes, 1,4-piperazinediethanesulfonic acid; CHAPS, 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonic acid; DTT, dithiothreitol; GST, glutathione S-transferase; PAGE, polyacrylamide gel electrophoresis.

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Biotech) as described (9). GST-fused proteins were eluted by reduced glutathione as described (20) or else treated with thrombin as described (9). After digestion with thrombin, GST beads were spun down. The resulting supernatant was adjusted to the MonoQ column buffer (20 mM Hepes (pH 7.5), 10 mM KF, (pH 7.4), and 1 mM DTT) and then charged onto the column. Both Mog1p and Prp20p were eluted between 300 and 400 mM NaCl, whereas Yrb1p and Rna1p were eluted between 100 and 200 mM and between 400 and 500 mM NaCl, respectively. The E. coli strains, BL21 (DE3)pGEX-wtGSP1 or GSP1ΔC, were cultured in 7.5 liters of SB medium at 30 °C up to an OD600 of 0.4, treated with isopropyl-1-thio-β-D-galactopyranoside (final concentration 0.1 mM) for 4 h, and then collected by centrifugation at 3,200 rpm for 15 min. After cell lysis, GST-fused Gsp1p was purified in the presence of 1 mM GTP or GDP, using glutathione-Sepharose 4B beads (Amersham Pharmacia Biotech) as described (20). After digestion with thrombin, GST beads were spun down. The resulting supernatant containing Gsp1 proteins was incubated with 1 mM GTP or GDP, adjusted to 25 mM Pipes (pH 6.5), 10 mM KPi (pH 6.5), 1 mM MgCl2, and 1 mM DTT, and charged onto the SO4 column. GTP-Gsp1p and GDP-Gsp1p were eluted at between 500 and 600 mM and between 150 and 250 mM NaCl, respectively.

Gsp1pGTPase-activating Assay—Recombinant wtGsp1p and Gsp1ΔC were loaded with [γ-32P]GTP (30 Ci/mmol nucleotide; 2.0 mCi/ml; usually in a 40-μl volume) or [α-32P]GTP (3000 Ci/mmol nucleotide; 10 mCi/ml; usually in a 15-μl volume) in loading buffer (500 μl) as described (19). Uncharged free nucleotides were removed with NAP Sephadex G-25 DNA Grade (Amersham Pharmacia Biotech). 10 pmol of [γ-32P] or [α-32P]GTP-wtGsp1p and Gsp1ΔC were suspended in GAP buffer (25 mM Tris-HCl (pH 7.5), 50 mM NaCl, 20 mM MgCl2, 1 mM DTT, and 1 mM CHAPS) (total 200 μl) incubated at 30 °C for 5 min, and then given Rna1p and Mog1p. After incubation at 30 °C for the indicated time, the reaction was stopped by the addition of the ice-cold stopping buffer (20 mM Tris-HCl (pH 7.5), 25 mM MgCl2, and 100 mM NaCl), and the mixture was then filtered through a nitrocellulose filter (0.45 μm, BA85, Schleicher & Schuell). The filters were dried, and the radioactivity remaining with proteins on the filter was counted in a liquid scintillation counter. The amount of [α-32P] bound at t = 0 was defined as 100%, then being 12,000–42,000 dpm ([α-32P]GTP-wtGsp1p, 300,000–500,000 dpm ([γ-32P]GTP-Gsp1ΔC, 670,000–710,000 dpm ([α-32P]GTP-wtGsp1p), and 910,000–1,020,000 dpm ([γ-32P]GTP-Gsp1ΔC) in different experiments. The radioactivity remaining on Gsp1p was estimated as described (19). Reactions were performed in triplicate, unless otherwise indicated. The mean value and the error bar calculated were shown.

Five pmol of the [α-32P]GTP-Gsp1p mixture were boiled, spotted on PEI-cellulose F (Merck) to be separated in TLC buffer (1 mM LiCl and 1 mM formic acid) for 90 min, and then analyzed by autoradiography (21).

Guanine Nucleotide Release Assay—Recombinant wtGsp1p and Gsp1ΔC were loaded with [3H]GDP or GTP (25–50 Ci/mmol nucleotide, 1.0 mCi/ml; usually in a 25-μl volume) in loading buffer (500 μl) as described (19). Ten pmol of [3H]GDP- or [3H]GTP-wtGsp1p and Gsp1ΔC were suspended in GAP buffer (25 mM Tris-HCl (pH 7.5), 50 mM NaCl, 20 mM MgCl2, 1 mM DTT, and 1 mM CHAPS, total 200 μl) incubated at 30 °C for 5 min, and then given Rna1p and Mog1p. After incubation at 30 °C for the indicated time, the reaction was stopped by the addition of the ice-cold stopping buffer (20 mM Tris-HCl (pH 7.5), 25 mM MgCl2, and 100 mM NaCl), and the mixture was then filtered through a nitrocellulose filter (0.45 μm, BA85, Schleicher & Schuell). The filters were dried, and the radioactivity remaining with proteins on the filter was counted in a liquid scintillation counter. The amount of [3H] bound at t = 0 was defined as 100%, then being 12,000–42,000 cpm ([3H]GTP-wtGsp1p, 300,000–500,000 cpm ([γ-32P]GTP-Gsp1ΔC, 670,000–710,000 cpm ([α-32P]GTP-wtGsp1p), and 910,000–1,020,000 cpm ([γ-32P]GTP-Gsp1ΔC) in different experiments. The radioactivity remaining on Gsp1p was estimated as described (19). Reactions were performed in triplicate, unless otherwise indicated. The mean value and the error bar calculated were shown.

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RESULTS

Mog1p Stimulates GTP Release from GTP-Gsp1p—Since Mog1p specifically binds to GTP-Gsp1p (11), we first examined whether Mog1p stimulates the activity of RanGAP. In order to address this issue, recombinant GST fusion proteins of Mog1p and Rna1p were produced in E. coli, and highly purified by glutathione-Sepharose columns, before being cleaved to remove the GST through incubation with thrombin. The resulting Mog1p and Rna1p were further purified over MonoQ columns to become a single band stained with Coomassie Brilliant Blue (data not shown).

Ten pmol of [γ-32P]GTP-wtGsp1p were mixed with both Rna1p and Mog1p and, as a control, Rna1p, Mog1p, or buffer alone. After incubation at 30 °C for the indicated time, the radioactivity remaining on Gsp1p was quantified using a liquid scintillation counter. As shown in Fig. 1A, the RanGAP activity of Rna1p was enhanced by the addition of Mog1p. After incubation for 10 min, about 80% of the radioactivity of [γ-32P]GTP-Gsp1p was released, compared with 40% in the case of Rna1p alone. Surprisingly, the radioactivity of [γ-32P]GTP-wtGsp1p was significantly reduced by the addition of only Mog1p, although [γ-32P]GTP-wtGsp1p was stable following incubation in buffer alone. In order to confirm further the effect of Mog1p on GTP-Gsp1p, we used another substrate, Gsp1ΔC, that lacks the final eight amino acid residues of the C terminus, instead of wtGsp1p. When 10 pmol of [γ-32P]GTP-Gsp1ΔC were incubated with the same dose of Rna1p and Mog1p, the radioactivity was strongly reduced. For this reason, the doses of Rna1p and Mog1p, which reduced the radioactivity of [γ-32P]GTP-Gsp1ΔC by around 40–60% after incubation for 10 min, were used for a comparison. As shown in Fig. 1B, the RanGAP activity of Rna1p was enhanced by the addition of Mog1p. Furthermore, the radioactivity of [γ-32P]GTP-Gsp1ΔC was reduced by the addition of Mog1p alone at a concentration 3-fold less than in the case of wtGsp1p.

In order to confirm the effect of Mog1p on Gsp1p, 10 pmol of [γ-32P]GTP-wtGsp1p or Gsp1ΔC were mixed with the increasing doses of Mog1p and then incubated at 30 °C for 10 min. At the doses of Mog1p higher than 10 pmol corresponding to the concentration of Gsp1p, the radioactivity of both wtGsp1p and Gsp1ΔC was significantly reduced (Fig. 1C). Similar to the case of Rna1p, Gsp1ΔC is more sensitive to the action of Mog1p, compared with wtGsp1p. The radioactivity of [γ-32P]GTP-wtGsp1p and Gsp1ΔC was not reduced by the degradation of wtGsp1p and Gsp1ΔC, since the amount of wtGsp1p and Gsp1ΔC, which was estimated by immunoblotting analysis, was not changed by the addition of Mog1p during the incubation (Fig. 1D).

The above results indicated that Mog1p has either the ability to activate the GTPase of Gsp1p or to stimulate the nucleotide release from GTP-Gsp1p. In order to address this issue, we used [α-32P]GTP instead of [γ-32P]GTP. Ten pmol of [α-32P]GTP-wtGsp1p or Gsp1ΔC were mixed with increasing doses of Rna1p and, as a control, Mog1p. After incubation at 30 °C for 10 min, the radioactivity remaining on wtGsp1p or Gsp1ΔC was quantified using a liquid scintillation counter. As expected from the function of RanGAP that activates the hy-
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Fig. 1. Effect of Mog1p on the activity of Rna1p. A and B, 10 pmol of [γ-32P]GTP-wtGsp1p (A) (open symbols) or Gsp1ΔC (B) (closed symbols) were mixed with Rna1p (3 fmol) (○), Mog1p (60 pmol) (△), or the mixture of Rna1p and Mog1p (○), and as a control with buffer alone (△). After incubation at 30 °C for the indicated time, the reactions were stopped, and the radioactivity remaining on Gsp1p was counted. The vertical axis shows the ratio of radioactivity remaining after incubation, compared with the value at the commencement of incubation (time 0). C and D, 10 pmol of [γ-32P]GTP-wtGsp1p (open circle) or Gsp1ΔC (closed circle) were mixed with increasing doses of Mog1p (0, 1, 10, 30, 100, and 300 pmol) in GAP buffer. After incubation at 30 °C for 10 min, the reactions were stopped, and the radioactivity remaining on Gsp1p was counted (C). The vertical axis shows the ratio of radioactivity after incubation, compared with the value in the absence of Mog1p (0 mol). The reaction mixtures were boiled after incubation, separated by 11.25% SDS-PAGE, transferred to polyvinylidene difluoride membrane, and assayed for the presence of Gsp1p by immunoblotting analysis using anti-Gsp1p antibodies as a probe (D). Lanes 1–6, wtGsp1p; lanes 7–12, Gsp1ΔC. The doses of Mog1p were as follows: lanes 1 and 7, 0 pmol; lanes 2 and 8, 1 pmol; lanes 3 and 9, 10 pmol; lanes 4 and 10, 30 pmol; lanes 5 and 11, 100 pmol; lanes 6 and 12, 300 pmol.

The addition of Mog1p to GTP-wtGsp1p or Gsp1ΔC was not reduced by the addition of Rna1p (Fig. 2A). To the contrary, it was significantly reduced by the addition of Mog1p at doses higher than 10 pmol (Fig. 2B), as observed in Fig. 1C. These results indicate that Mog1p has the activity to release a nucleotide from GTP-Gsp1p, rather than to activate Gsp1pGTPase. We further confirmed this finding with TLC analysis. Ten pmol of [α-32P]GTP-wtGsp1p and Gsp1ΔC were mixed with 100 fmol of Rna1p, at which dose the radioactivity of [α-32P]GTP was not reduced (Fig. 2A), and with 300 pmol of Mog1p, at which dose the radioactivity remaining on [α-32P]GTP-wtGsp1p and Gsp1ΔC was reduced to less than 20% of the original value (Fig. 2B). After incubation for 10 min at 30 °C, the reaction mixtures were boiled, and the remaining nucleotides were separated by thin layer chromatography. As a control, the original mixtures without incubation were also boiled in order to analyze the nucleotides bound to wtGsp1p and Gsp1ΔC. The radioactivity of [α-32P]GTP and [α-32P]GDP was analyzed by autoradiography. As shown in Fig. 2C, all of the [α-32P]GTP bound to wtGsp1p or Gsp1ΔC was changed to [α-32P]GDP upon incubation with Rna1p (Fig. 2C, wtGsp1p, lanes 3 and 4; Gsp1ΔC, lanes 9 and 10). In contrast, the amount of GDP was not increased by incubation with Mog1p, indicating that Mog1p has no ability to activate Gsp1pGTPase.

Mog1p Does Not Interfere with the Nucleotide Exchange Activity of Prp20p—Thus far, only RCC1 has been known to have the ability to exchange the nucleotide on Ran (6, 11). RCC1 stimulates a nucleotide release from GTP- or GDP-Ran (25). In this regard, we addressed the question of whether or not Mog1p also stimulates a nucleotide release from GDP-Gsp1p. Ten pmol of [3H]GDP-wtGsp1p or Gsp1ΔC and, as a control, [3H]GTP-wtGsp1p or Gsp1ΔC were mixed with increasing doses of Mog1p. After incubation at 30 °C for 10 min, the radioactivity remaining on Gsp1p was quantified using a liquid scintillation counter. The radioactivity of [3H]GTP-wtGsp1p did not decrease, whereas the radioactivity of [3H]GDP-Gsp1ΔC was reduced at the doses of Mog1p greater than 100 pmol, although the reduction in the radioactivity of [3H]GDP-Gsp1ΔC was far less than in the case of [3H]GTP-Gsp1ΔC. These findings taken together led to the conclusion that Mog1p efficiently releases a nucleotide from GTP-Gsp1p but not from GDP-Gsp1p. The result of Gsp1ΔC may indicate that Mog1p can release a nucleotide from GDP-Gsp1p under certain conditions.

We examined the possible functional interaction between...
prior to incubation (mixtures were incubated at 30 °C for 10 min. The reaction mixtures with GAP buffer alone (B, bation, compared with the value in the absence of Rna1p (0 mol). The number of lanes 4, 9, and 10, and 100 pmol). After incubation at 30 °C for 10 min, the remaining radioactivity on Gsp1p was counted by a liquid scintillation counter. The vertical axis shows the ratio of radioactivity remaining on Gsp1p after incubation, compared with the value in the absence of Mog1p (0 mol).

Fig. 2. Mog1p has no activity for activating the Gsp1pGTPase. A. 10 pmol of [γ-32P]GTP-wtGsp1p (open circles) or Gsp1ΔC (closed circles) were mixed with increasing amounts of Rna1p (0.0, 0.01, 0.1, 1.0, and 100 pmol). After incubation at 30 °C for 10 min, the remaining radioactivity on Gsp1p was quantified using a liquid scintillation counter. The vertical axis shows the ratio of radioactivity remaining after incubation, compared with the value in the absence of Mog1p (0 mol). B, 10 pmol of [γ-32P]GTP-wtGsp1p (open circles) or Gsp1ΔC (closed circles) were mixed with increasing amounts of Mog1p (0.0, 1.0, 10, 30, 100, and 300 pmol). After incubation at 30 °C for 10 min, the remaining radioactivity on Gsp1p was quantified using a liquid scintillation counter. The vertical axis shows the ratio of radioactivity remaining after incubation, compared with the value in the absence of Mog1p (0 mol). C, 10 pmol of [γ-32P]GTP-wtGsp1p (lanes 1–6) or Gsp1ΔC (lanes 7–12) were mixed with GAP buffer alone (lanes 1, 2, 7, and 8), 100 pmol of Rna1p (lanes 3, 4, 9, and 10), and 300 pmol of Mog1p (lanes 5, 6, 11, and 12). The mixtures were incubated at 30 °C for 10 min. The reaction mixtures prior to incubation (odd-numbered lanes) and after incubation (even-numbered lanes) were boiled, separated by thin layer chromatography, and analyzed by autoradiography.

Prp20p and Mog1p by kinetic analysis. Prp20p is the S. cerevisiae RCC1 homologue. Ten pmol of [3H]GDP-wtGsp1p were mixed with both Prp20p (0.1 pmol) and Mog1p (60 pmol) and, as a control, with Prp20, Mog1p, or buffer alone. After incubation at 30 °C for the indicated time, the radioactivity remaining on Gsp1p was quantified using a liquid scintillation counter (Fig. 4). As reported (19), the radioactivity of [3H]GDP-Gsp1p was reduced by the addition of Prp20p (Fig. 4A). The remaining radioactivity of [3H]GTP-wtGsp1p was almost identical between the mixture containing both Prp20p and Mog1p and the mixture containing Prp20p alone. This was not due to the inactivation of Mog1p, since the same preparation of Mog1p released nucleotide from [3H]GTP-wtGsp1p (Fig. 4B). Upon the addition of Prp20p, the Mog1p-stimulated nucleotide release from [3H]GTP-wtGsp1p was further enhanced. The identical results were obtained by using [3H]GDP and GTP-Gsp1ΔC as substrates (Fig. 4, C and D). In the case of Gsp1ΔC, we used 20 pmol as the dose of Mog1p for comparison, as described in Fig. 1. We thus concluded that Mog1p does not interfere with the Prp20p-stimulated nucleotide release from GDP-Gsp1p or GTP-Gsp1p. Both Mog1p and Prp20p additively enhanced a nucleotide release from GTP-Gsp1p.

Mog1p-stimulated GTP Release from GTP-Gsp1p was Blocked by Yrb1p—RanBP1 has been known to inhibit RCC1-stimulated nucleotide release (26). In addition, the S. cerevisiae RanBP1 homologue, Yrb1p, also inhibits the Prp20p-stimulated nucleotide release (19). Based on these previous reports, we examined the functional relationship between Yrb1p and Mog1p. Ten pmol of [α-32P]GTP-wtGsp1p were mixed with 100 pmol of Mog1p and increasing doses of Yrb1p. After incubation at 30 °C for 10 min, the radioactivity remaining on Gsp1p was quantified using a liquid scintillation counter. At the doses of Yrb1p, higher than 10 pmol corresponding to the concentration of [α-32P]GTP-wtGsp1p, Yrb1p completely inhibited the Mog1p-stimulated nucleotide release from GTP-Gsp1p (Fig. 5, open circles). This finding indicates that the interaction between Yrb1p and GTP-wtGsp1p is important for Yrb1p to inhibit Mog1p-stimulated GTP release.

Ran lacking the C-terminal amino acids conserved in the Ran family is unable to bind tightly to RanBP1 (17, 27, 28). If this is the case regarding the interaction between Yrb1p and Gsp1p, then the C-terminal amino acids of Gsp1p may be essential for Yrb1p to inhibit the Mog1p-stimulated nucleotide release. Indeed, when GTP-Gsp1ΔC was used as a substrate instead of GTP-wtGsp1p, the activity of Yrb1p for inhibiting the Mog1p-stimulated nucleotide release was almost com-
Yrb1p inhibits the Mog1p-stimulated nucleotide release from GTP-Gsp1p. 10 pmol of [γ-32P]GTP-wtGsp1p (open circles) were mixed with 100 pmol of Mog1p, and 10 pmol of [γ-32P]GTP-Gsp1pΔC (closed circles) were mixed with 30 pmol of Mog1p in guanine nucleotide exchange factor buffer. The mixtures were incubated with increasing amounts of Yrb1p (0.0, 1.0, 10, and 30 pmol) at 30 °C for 10 min. The reactions were stopped by the addition of stopping buffer, and the radioactivity remaining on Gsp1p was counted by a liquid scintillation counter. The vertical axis shows the ratio of radioactivity remaining after incubation, compared with the value in the absence of Mog1p and Yrb1p (0 mol). Mog1p-stimulated nucleotide release from Gsp1p by directly binding to Gsp1p.

Since RanBP1 makes a complex consisting of RCC1, Ran,
and RanBP1 (26), another possibility is that Yrb1p may inhibit the Mog1p-stimulated nucleotide release by making the Yrb1p-Gsp1p-Mog1p complex. In order to address this question, Mog1p and Yrb1p were mixed with GTP-Gsp1p. As controls, Mog1p was mixed with Yrb1p or GTP-Gsp1p, and Yrb1p was mixed with GTP-Gsp1p as shown in Fig. 6. Those mixtures and the solution containing a single component were subjected to gel filtration analysis. Proteins contained in the resulting fraction were detected by immunoblotting analysis. Gsp1p, molecular mass of 25 kDa, eluted with a peak in fraction 57 (molecular mass of 27 kDa), and Mog1p, molecular mass of 24 kDa, eluted with a peak in fraction 55 (molecular mass of 32 kDa) (Fig. 6, A and B). In contrast, Yrb1p, molecular mass of 23 kDa, eluted with a peak in fraction 50 (molecular mass of 48 kDa) (Fig. 6, C), indicating that Yrb1p may behave as a dimer in solution, as reported for RanBP1 (26). When mixed, the complex comprising Mog1p, Yrb1p, and Gsp1p was not detected. Instead, the Mog1-Gsp1p complex eluted with a peak in fraction 43 (molecular mass of 84 kDa) (Fig. 6, A and B), the stoichiometry of which is either 2:1 or 1:2. The Yrb1p-Gsp1p complex eluted with a peak in fraction 46 (molecular mass of 66 kDa) (Fig. 6C). The stoichiometry of Yrb1p-Gsp1p complexes appears to be 2:1. It is noticeable that most of the Yrb1p bound to Gsp1p, when Yrb1p was mixed with Gsp1p alone or with Gsp1p plus Mog1p (Fig. 6, A and C). This is consistent with the fact that the formation of the Mog1p-Gsp1p complex was strongly reduced by the addition of Yrb1p (Fig. 6B). These results suggested that the Yrb1p-GTP-Gsp1p complex is more stable than the Mog1p-GTP-Gsp1p complex, and thereby Yrb1p inhibited the interaction between Mog1p and GTP-Gsp1p at doses higher than that corresponding to the concentration of GTP-Gsp1p.

**Mog1p Binds to GTP-Gsp1pΔC**—The above results indicated that Mog1p was able to bind to GTP-Gsp1ΔC, whereas the same deletion of mammalian Ran is reported to reduce the affinity between GTP-Ran and RanBP1 by approximately 8,000-fold (17). In order to confirm the interaction between Mog1p and GTP-Gsp1ΔC, we performed real time protein-protein interaction analysis using BIAcore (Fig. 7). The monoclonal antibody (mAb) to glutathione S-transferase was immobilized on the sensor chip of the BIAcore to trap GST-fused Mog1p and, as a control, GST-fused Yrb1p. The interaction of Mog1p or Yrb1p with GTP-Gsp1ΔC and, as a control, GTP-wtGsp1p, was then determined by injecting increasing doses of GTP-Gsp1ΔC (curve 1 (magenta), 2.0 μM; curve 2 (green), 0.5 μM; curve 3 (blue), 0.2 μM; curve 4 (red), 0.05 μM; curve 5 (cyan), 0.02 μM) was injected, and the binding was calculated as described under “Experimental Procedures.” GST-Mog1p was mixed with GTP-wtGsp1p (A) or GTP-Gsp1ΔC (B), and GST-Yrb1p was mixed with GTP-wtGsp1p (C) or GTP-Gsp1ΔC (D).
wtGsp1p (Fig. 7, A and B). The calculated association constants ($k_a$, $\text{m}^{-1}$ $\text{s}^{-1}$) of the interaction of Mog1p and Yrb1p with wtGsp1p or Gsp1ΔC are as follows: Mog1p/GTP-wtGsp1p, $0.97 + 0.47 \times 10^4$; Mog1p/GTP-Gsp1ΔC, $1.40 + 0.90 \times 10^4$; Yrb1p/ GTP-wtGsp1p, $1.33 + 0.70 \times 10^4$.

**DISCUSSION**

Mog1p is a novel protein binding to GTP. It is required for nuclear protein import but not for mRNA export (11). This study found that Mog1p can release a nucleotide from GTP- Gsp1p, but not from GDP-Gsp1p, in a dose-dependent manner. In this context, Mog1p differs from a guanine nucleotide exchange factor of Gsp1p, RCC1 which releases a nucleotide from both GTP-Gsp1p, and GDP-Gsp1p (29). During the preparation of this manuscript, Steggerda and Paschal (30) reported that murine Mog1p has no nucleotide exchange activity on Ran, whereas it releases GTP from GTP-Ran similar to *S. cerevisiae* Mog1p. Mog1p may have a role to play in the nucleus. One of the possibilities is that the nuclear Yrb1p inhibits the Mog1p-stimulated nucleotide release from the Gsp1p. Yrb1p more stably than to Mog1p (Fig. 7). Thus, Yrb1p may inhibit the interaction between Mog1p and GTP-Gsp1p by depleting the GTP-Gsp1p. This is consistent with the finding that the Mog1p-stimulated nucleotide release from the Gsp1ΔC was not inhibited by the addition of Yrb1p. It is possible that Yrb1p inhibits Mog1p-stimulated nucleotide release by making a complex of Mog1p-Gsp1p-Yrb1p, since Mog1p was coprecipitated with overexpressed GST-Yrb1p in vivo (data not shown), and murine Mog1 is reported to make a trimeric complex of Mog1-Ran-RanBP1 (30).

Both Yrb1p and Mog1p bind to GTP-Gsp1p. However, upon the C-terminal deletion of Gsp1p, the ability of Yrb1p to bind to GTP-Gsp1ΔC was profoundly reduced. In contrast, the interaction between Mog1p and Gsp1ΔC was somewhat strengthened, compared with that between Mog1p and wtGsp1p. These results indicate that Mog1p binds to GTP-Gsp1p in a manner different from that of Yrb1p. The method by which Mog1p binds to GTP-Gsp1p may reflect the biological function of Mog1p that still remains to be investigated. Mog1p is conserved in *S. pombe* (DDBJ/EMBL/GenBank™ accession number AL031179) and probably in humans (DDBJ/EMBL/GenBank™ accession number N48015), indicating that Mog1p plays some important role in the Ran GTPase cycle. The present results provide us with the first clue toward clarifying the function of Mog1p.

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