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MSS4 Does Not Function as an Exchange Factor for Rab in Endoplasmic Reticulum to Golgi Transport

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Mss4 and its yeast homologue, Dss4, have been proposed to function as guanine nucleotide exchange factors (GEFs) for a subset of Rab proteins in the secretory pathway. We have previously shown that Rab1A mutants defective in GTP-binding potently inhibit endoplasmic reticulum to Golgi transport, presumably by sequestering an unknown GEF regulating its function. We now demonstrate that these mutants stably associate with Mss4 both in vivo and in vitro and that Mss4 effectively neutralizes the inhibitory activity of the Rab1A mutants. An equivalent Rab3A mutant (Rab3A[N135I]), a Rab protein specifically involved in regulated secretion at the cell surface, associates with Mss4 as efficiently as the Rab1A[N124I] mutant. Although Rab3A[N135I] prevents the ability of Mss4 to neutralize the inhibitory effects of Rab1A mutants on transport, it has no effect on Rab1 function or endoplasmic reticulum to Golgi transport. Furthermore, quantitative immunodepletion of Mss4 fails to inhibit transport in vitro. We conclude that Mss4 and its yeast homologue, Dss4, are not GEFs mediating activation of Rab, but rather, they interact with the transient guanine nucleotide-free state, defining a new class of Ras-superfamily GTPase effectors that function as guanine nucleotide-free chaperones (GFCs).

INTRODUCTION

Rab proteins are small GTPases that are thought to function as molecular switches to control protein interactions mediating vesicular transport through the endocytic and exocytic pathways (reviewed by Nuoffer and Balch, 1994). To date, a large number of Rab proteins have been identified and localized to distinct intracellular compartments (reviewed by Simons and Zerial, 1993). Rab1, which is associated with pre-Golgi and Golgi compartments, is essential for transport from the endoplasmic reticulum (ER) through early Golgi compartments (Plutner *et al.*, 1991). In contrast, Rab3 isoforms are localized to regulated exocytic vesicles found in endocrine and neuroendocrine cells and to synaptic vesicles present in the synapse (Fischer von Mollard *et al.*, 1994). Given their unique subcellular distributions, Rab proteins have been proposed to play a role in directing the docking and/or fusion of carrier vesicles to target membranes (Bourne, 1988). Recent studies have revealed that the GTPase cycle of Rab proteins may actually serve as a molecular timer controlling the interactions between membranes (Rybin *et al.*, 1996). According to this model, the lifetime of the GTP-bound state, which is dependent on the rate of guanine nucleotide exchange and GTP hydrolysis, determines the ability of membranes to engage in docking and/or fusion events (Aridor and Balch, 1996).

The GTPase cycle of Rab proteins, like that observed for other Ras superfamily GTPases, is expected to be controlled by various types of accessory factors. These include proteins that stabilize Rab in the GDP-bound form (GDP-dissociation inhibitors, GDIs), promote guanine nucleotide exchange (guanine nucleotide exchange factors, GEFs), and accelerate GTP hydrolysis (GTPase-activating proteins). Numerous studies (reviewed by Pfeffer *et al.*, 1995; Wu *et al.*, 1996) have focused on the role of Rab-GDI, which forms a cytosolic complex with the GDP-bound form of Rab proteins and serves as an escort that shuttles the GTPases to and from their target membranes. The structure of GDI has recently been solved at 1.8-Å resolution, and the region responsible for interaction with Rab pro-

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teins has been defined (Schalk *et al.*, 1996). In contrast, little is known about the events after the delivery of Rab proteins to the membranes, which results in their release from GDI and coincides with activation of these GTPases through guanine nucleotide exchange (Soldati *et al.*, 1994; Ullrich *et al.*, 1994).

A candidate Rab GEF, designated Mss4 in mammalian cells, and its yeast homologue, Dss4, were identified by using a genetic screen in yeast (Burton *et al.*, 1993; Moya *et al.*, 1993). Both proteins are small hydrophilic molecules that reside principally as monomers in the cytosol. In vitro, Mss4 and Dss4 accelerate guanine nucleotide exchange by several members of the Rab family involved in anterograde transport through the exocytic pathway, including Rab1 and Rab3, but are inactive toward Rab proteins operating in the endocytic pathway (Burton *et al.*, 1994). The stimulatory activity of Mss4 and Dss4 is weaker than that of Ras-specific GEFs, raising a concern regarding the function of these proteins as GEFs in vivo. Moreover, the cytosolic distribution and broad substrate specificity of Mss4 and Dss4 are unexpected. Given that guanine nucleotide exchange occurs upon delivery of Rab GTPases to their target membranes (Soldati *et al.*, 1994; Ullrich *et al.*, 1994), one would anticipate that GEFs for Rab proteins would be membrane-associated and specific for individual members of the Rab family. However, the promiscuous properties of Mss4 and Dss4 do raise an alternative possibility. Similar to GDI, these proteins may promote exchange by transiently associating with Rabs during their recruitment by specific membrane-associated receptors.

To test the hypothesis that Mss4 may act as a functional GEF in vivo, we have established the kinetic parameters for the interaction of Mss4 with Rab1A and Rab3A and have directly examined the role of Mss4 in transport through the early secretory pathway. We have previously shown that Rab1A[N124I], a mutant defective in guanine nucleotide binding, is a dominant inhibitor of transport of the vesicular stomatitis virus glycoprotein (VSV-G) between the ER and the Golgi compartments in vivo and in vitro (Tisdale *et al.*, 1992; Nuoffer *et al.*, 1994; Pind *et al.*, 1994). By analogy to the properties of the equivalent $p21^{ras}$ mutant (Hwang *et al.*, 1993), exogenous Rab1A[N124I] is likely to inhibit transport by sequestering a putative Rab1-specific GEF into a catalytically inactive complex, thereby preventing activation of the endogenous Rab1. Consistent with this model, we find that Rab1A[N124I] interacts with Mss4 to form a stable 1:1 complex that is resistant to high concentrations of guanine nucleotides. Moreover, increasing the level of Mss4 efficiently neutralizes the inhibitory potential of Rab1A[N124I] and other Rab1A mutants predicted to be defective in guanine nucleotide exchange. However, we also show that Rab3A[N135I], a mutant analogous to Rab1A[N124I], sequesters Mss4 as efficiently as the Rab1A mutant yet does not inhibit ER to Golgi transport in vivo or in vitro. Thus, it seems unlikely that Mss4 represents the sole target of the Rab1A[N124I] mutant. Moreover, immunodepletion of the putative GEF does not affect the ability of cytosol to support ER to Golgi transport in vitro, indicating that Mss4 is not critical for this process. The combined results lead us to conclude that Mss4 and Dss4 are not directly responsible for Rab activation but, rather, function as chaperone-like factors to stabilize Rab proteins in their transient nucleotide-free state.

MATERIALS AND METHODS

Materials

[^{35}S]Methionine (specific activity > 1000 Ci/mmol) was purchased from DuPont-New England Nuclear Research (Boston, MA). Other reagents were obtained from Sigma (St. Louis, MO) or Boehringer Mannheim (Indianapolis, IN), unless otherwise indicated. The fluorescent GDP analogue 2′(3′)-bis-*O*-(*N*-methylanthraniloyl)-GDP (mGDP) was kindly provided by A. Wittinghofer (Max Planck Institute, Heidelberg, Germany). Anti-hemagglutinin (HA) monoclonal antibodies were a gift from I. Wilson (The Scripps Research Institute, La Jolla, CA).

Cloning, Expression, and Purification of Mss4

Total poly(A)$^+$ mRNA was isolated from NRK cells (FastTrack, Invitrogen, San Diego, CA) and reverse transcribed (SuperScript, Life Technologies, Gaithersburg, MD) according to the manufacturers' instructions. The Mss4 sequence was then amplified from the first-strand cDNA by polymerase chain reaction using specific primers with flanking *Nde*I and *Bam*HI sites. The identity of the polymerase chain reaction product with the published Mss4 sequence was confirmed by DNA sequencing. The Mss4 cDNA was then cloned as an *Nde*I-*Bam*HI fragment into pET-His_6, which was constructed for the expression of proteins with an N-terminal His_6 tag by insertion of an appropriate cassette into the *Nde*I site of pET11d (Novagen, Madison, WI).

Recombinant His_6-Mss4 was expressed in *Escherichia coli* strain BL21(DE3), and the soluble fraction was purified to homogeneity by metal-chelate affinity chromatography on Ni^{2+}-saturated nitrilotriacetic acid-agarose (Qiagen, Chatsworth, CA) followed by gel filtration through a Sephacryl S-100 (Pharmacia, Piscataway, NJ) column as described previously (Nuoffer *et al.*, 1995).

Production and Affinity Purification of Anti-Mss4 Antibodies

Recombinant His_6-Mss4 was used to immunize rabbits as described elsewhere (Harlow and Lane, 1988) and specific antibodies were affinity purified by using an AminoLink column (Pierce, Rockford, IL) containing covalently coupled recombinant His_6-Mss4 according to the manufacturer's instructions.

Preparation of Recombinant Rab1A Proteins

His_6-tagged wild-type or mutant forms of Rab1A and Rab3A were expressed and purified from *E. coli* as described earlier (Nuoffer *et al.*, 1995).

Guanine Nucleotide Exchange Assays

Recombinant His_6-Rab1A and 3A were loaded with the fluorescent GDP analogue mGDP by incubation with 25-fold excess of mGDP in

25 mM HEPES-KOH (pH 7.2), 125 mM potassium acetate (KOAc), 5 mM EDTA, and 0.1 M dithiothreitol (DTT) for 30 min at 32°C. The reaction was transferred to ice, supplemented with magnesium acetate (MgOAc) to a final concentration of 10 mM, and the free mGDP was removed by desalting through a PD-10 column (Pierce) equilibrated with 25 mM HEPES-KOH (pH 7.2), 125 mM KOAc, 10 mM MgOAc, and 0.1 M DTT (equilibration buffer). The dissociation of mGDP was assessed by measuring the decrease in relative fluorescence ($\lambda_{excitation}$, 360 nm; $\lambda_{emission}$, 440 nm) that accompanies the release of mGDP from the GTPase (Herrmann *et al.*, 1995). The dissociation was measured by using 10–25 nM Rab-mGDP in equilibration buffer supplemented with 2.5 mM GTP and various amounts of recombinant His_6-Mss4. Under these conditions, Rab cannot be detected in a stable complex with Mss4 (see RESULTS). Although mant-nucleotide exchange rates are reported for a final concentration of 10 mM Mg^{2+} in the buffer, identical exchange rates were observed at concentrations of Mg^{2+} greater than 1 mM, the approximately physiological concentration of free Mg^{2+} in the cell.

Determination of Binding Constant

To determine the k_d for Mss4-catalyzed dissociation of GDP from Rab, a nonlinear regression fit was made to the data by using the derived equation $k_{obs} = k_2[Mss4]/(k_3 + [Mss4])$ (Eq. *1*) based on the scheme

$$\text{Rab} + \text{GDP} \underset{k_{-1}}{\rightleftharpoons} \text{Rab·GDP} + \text{Mss4} \overset{k_2}{\underset{k_{-2}}{\rightleftharpoons}}$$

$$\text{Rab·GDP·Mss4} \overset{k_3}{\underset{k_{-3}}{\rightleftharpoons}} \text{Rab-Mss4} + \text{GDP},$$

where k_2 and k_3 are fitted rate constants. k_{-1} (the dissociation of GDP from Rab in the absence of Mss4) is small (Figure 1A) and can be neglected under the current experimental conditions. k_2 is the rate constant for the association of Rab·GDP with Mss4 to form the Rab·GDP·Mss4 ternary complex and k_3 is the rate constant for the dissociation of the ternary complex to yield free GDP, Rab, and Mss4. k_{-2} and k_{-3} are assumed to be negligible under the current experimental conditions. In the presence of excess guanine nucleotide, the ternary complex is transient and $d[GDP]/dt = k_3[Rab·GDP·Mss4]$. k_{obs} (Eq. *1*) is the observed rate constant for the dissociation of mGDP from Rab in the presence of a given concentration of Mss4. k_3 (or k_{cat}) can be determined directly when Mss4 is at saturation) ($k_{obs} = k_3$). k_2 can be determined using Eq. *1*, the concentration of Mss4 in which 50% of the maximum dissociation from Rab·GDP is observed (k_d).

Gel Filtration Analysis of Interactions between Rab Proteins and Mss4

Wild-type and mutant forms of His_6-tagged Rab proteins were mixed with an equimolar amount or a 10-fold molar excess of His_6-Mss4 in 25 mM HEPES-KOH (pH 7.2), 125 mM KOAc, 1 mM MgOAc, 5 mM $MgCl_2$, and 1 mM DTT. The mixtures were kept on ice or incubated at 32°C for 30 min in the presence or absence of 1 mM GTP. The samples were then processed by gel filtration through a Superdex 75 FPLC column (Pharmacia) equilibrated in 25 mM Tris-HCl (pH 7.5), 0.3 M NaCl, and 5 mM $MgCl_2$. The column was developed with equilibration buffer, fractions were collected, and proteins were precipitated. Elution profiles were obtained by monitoring the absorbance at 280 nm. The Rab1A, Rab3A, and Mss4 proteins were visualized and identified by analyzing the fractions by SDS-PAGE and staining of the gels with Coomassie blue.

Transient Expression and Analysis of Transport In Vivo

Proteins were expressed in HeLa cells by using a recombinant vaccinia virus-dependent transient cotransfection protocol and transport of VSV-G measured as described earlier (Tisdale *et al.*, 1992). The expression levels of the various proteins were monitored by quantitative immunoblotting as described previously (Dascher *et al.*, 1994).

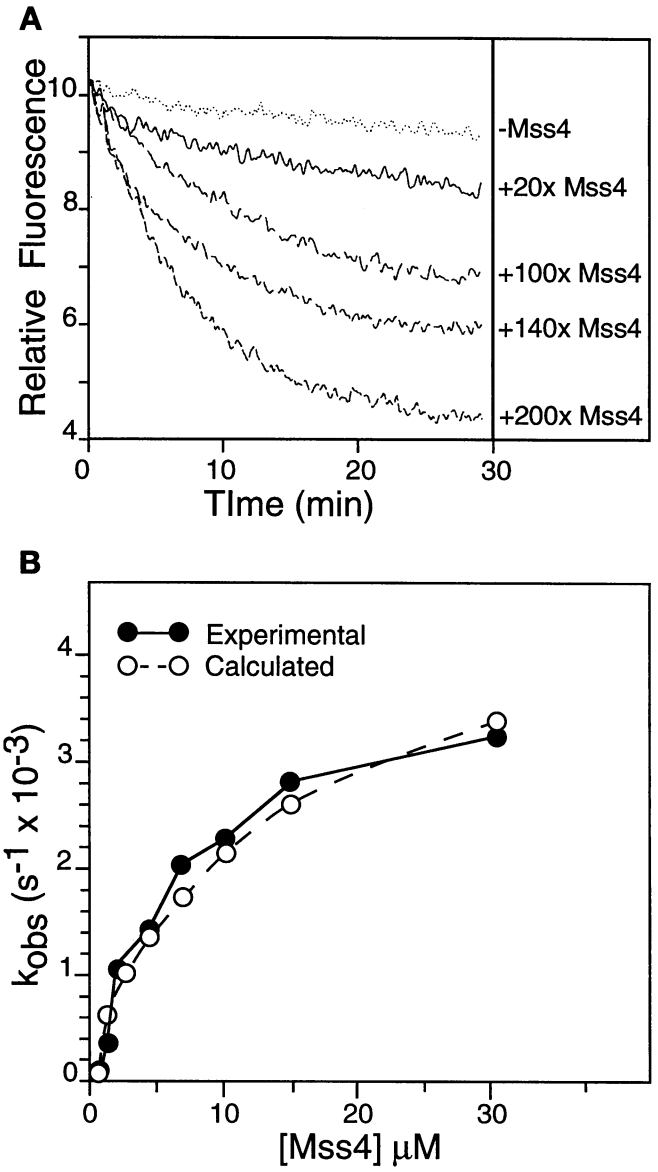

Figure 1. Mss4 stimulates the dissociation of GDP from Rab1A *in vitro*. (A) Recombinant Rab1A loaded with the fluorescent GDP analogue mGDP was incubated with recombinant Mss4 at the indicated molar ratios in the presence of excess GTP, and the dissociation of mGDP was monitored by measuring the decrease in relative fluorescence ($\lambda_{excitation}$, 360 nm; $\lambda_{emission}$, 440 nm) that accompanies the release of mGDP from the GTPase. A representative experiment is shown. (B) The experimental data (k_{obs}) was fitted to Eq. *1* (see MATERIALS AND METHODS) to derive k_d and k_{cat} as reported in RESULTS.

Construction, Expression, and Immunoprecipitation of HA-tagged Rab Proteins

The relevant Rab sequences were excised from the corresponding pET-His_6 constructs by digestion with *Nde*I and *Bam*HI, and the

fragments were introduced into pET-HA, which was constructed to permit the expression of proteins with an N-terminal HA tag by insertion of an appropriate cassette into the *Nco*I site of pET11d. HA-tagged Rab proteins were transiently expressed in HeLa cells and immunoprecipitated from detergent extracts essentially as previously described (Tisdale *et al.*, 1992).

Analysis of Transport In Vitro and Immunodepletion of Mss4

The procedures for reconstituting transport of VSV-G in vitro using semi-intact NRK and rat liver cytosol have been described previously (Plutner *et al.*, 1992; Davidson and Balch, 1993; Nuoffer *et al.*, 1994).

For immunodepletion of the cytosolic Mss4, protein A-Sepharose (Pharmacia) was treated for 10 min on ice with rat liver cytosol to block unspecific binding sites. The beads were recovered and incubated with a solution of affinity-purified anti-Mss4 antibodies for 1 h at 4°C under continuous agitation. After washing with 25 mM HEPES-KOH (pH 7.2) and 125 mM KOAc, the beads were added to a fresh aliquot of rat liver cytosol, and the sample was kept in the cold for 2 to 3 h with constant agitation. The supernatant was separated from the beads and immediately tested for transport activity. Mock-depleted cytosol was prepared in parallel by replacing the antibody solution with buffer. Depletion of Mss4 was confirmed by analyzing aliquots of the supernatant and pellet fractions by quantitative immunoblotting.

RESULTS

Mss4 Stimulates the Dissociation of GDP from Rab1A In Vitro

To determine whether Mss4 promotes guanine nucleotide exchange by Rab1A, a Rab essential for ER to Golgi transport (Plutner *et al.*, 1991; Tisdale *et al.*, 1992; Nuoffer *et al.*, 1994; Peter *et al.*, 1994; Pind *et al.*, 1994), we measured the effect of the putative GEF on the dissociation of the fluorescent GDP analogue mGDP from Rab1A in vitro. mGDP has been extensively characterized as a substrate for GTPases of the Ras superfamily (Herrmann *et al.*, 1995; Nomanbhoy and Cerione, 1996).

Recombinant Rab1A prepared from *E. coli* was loaded with mGDP in the absence of Mg^{2+}, stabilized by the addition of excess free Mg^{2+} (see MATERIALS AND METHODS), and incubated with increasing levels of recombinant Mss4 in the presence of excess Mg^{2+} and GTP. Guanine nucleotide exchange was assessed by measuring the decrease in relative fluorescence ($\lambda_{excitation}$, 360 nm; $\lambda_{emission}$, 440 nm) that accompanies the release of mGDP from the GTPase. As shown in Figure 1A, Mss4 stimulated the dissociation of mGDP from Rab1A in a dose-dependent manner. Kinetic analyses (see MATERIALS AND METHODS) revealed a k_d of 1.1×10^{-5} M and a k_{cat} of 4.7×10^{-3} s^{-1} (Figure 1B). By using an identical approach, the k_d for the association of Rab3A with Mss4 was determined to be 1.5×10^{-5} with a k_{cat} of 1.6×10^{-2} s^{-1}. The stimulatory activity of Mss4 toward Rab1A is several orders of magnitude weaker than GEFs specific for other members of the Ras superfamily (Klebe *et al.*, 1995). These data are consistent with previous results based on assays involving dissociation of radiolabeled guanine nucleotides from Mss4/Dss4 (Burton *et al.*, 1993, 1994; Moya *et al.*, 1993) and demonstrates that the GEF activities of these proteins toward members of the Rab family are low.

Interaction of Mss4 with Rab1A In Vitro

GEFs appear to exert their function by stabilizing the nucleotide-free form of Ras-like GTPases (Boguski and McCormick, 1993). Therefore, it might be possible to detect a stable association between Mss4 and Rab1A under appropriate conditions.

To explore this possibility, we combined equimolar amounts of recombinant Rab1A and Mss4 in physiological buffer, incubated the mixture, and subsequently analyzed the sample by gel filtration using a Superdex 75 FPLC column. Pure Rab1A and Mss4 elute from the column as single peaks in the range of 24 kDa and 14 kDa, respectively (Figure 2A, arrows). However, upon incubation of an equimolar mixture of the two proteins at 32°C for 30 min in the absence of free guanine nucleotides, a prominent new peak was detected, representing ~25% of the total protein (Figure 2A). This peak corresponded to a molecular weight of ~36 kDa, which closely matches the size expected for a 1:1 complex of Rab1A and Mss4. Analysis of the fractions by SDS-PAGE and Coomassie blue staining showed that the new peak indeed contained equimolar levels of both Mss4 and Rab1A. No complex was detected when the mixture was retained on ice, indicating that conditions supporting guanine nucleotide exchange are essential for association of Mss4 with wild-type Rab1A. Complex formation was greatly reduced in the presence of excess free GTP (Figure 2B). This became particularly evident when Mss4 was incubated with Rab1A at a 10-fold excess (Figure 2, C and D). In the absence of GTP, increasing the level of Mss4 enhanced recovery of complex, whereas only residual amounts of the complex were recovered in its presence.

We conclude that Mss4 is capable of associating with wild-type Rab1A to form a binary complex, which is readily disrupted by the presence of physiological levels of GTP. Therefore, it seems likely that Mss4 facilitates guanine nucleotide exchange by transiently interacting with, and thereby stabilizing, the nucleotide-free form of Rab1A, a property characteristic of other GEFs.

Mss4 and the Rab1A[N124I] Mutant Form a Stable Complex In Vitro

We have previously demonstrated that Rab1A[N124I], a mutant that fails to stably bind guanine nucleotides in vitro, potently inhibits transport of the type 1 membrane protein VSV-G between the ER and Golgi com-

partments (Tisdale *et al.*, 1992; Nuoffer *et al.*, 1994; Pind *et al.*, 1994). By analogy to the equivalent $p21^{ras}$ mutant (Hwang *et al.*, 1993), exogenous Rab1A[N124I] is likely to exert its dominant inhibitory effect by sequestering a Rab1A-specific GEF into a catalytically inactive form, thereby interfering with activation of the endogenous Rab1A.

To test this hypothesis, we used the gel filtration approach outlined above to analyze the capacity of the Rab1A[N124I] to interact with Mss4 in vitro. As shown in Figure 3, the two proteins were quantitatively recovered in the form of a stable 1:1 complex. In contrast to wild-type Rab1A, the mutant readily associated with Mss4 even when the mixture was kept on ice. Moreover, the interaction between Rab1A[N124I] and Mss4 proved totally insensitive to the addition of up to 10 mM GTP, consistent with the extremely low affinity of the Rab1A[N124I] mutant for guanine nucleotides. Identical results were obtained with Rab3A[N135I], the Rab1A[N124I] equivalent of wild-type Rab3A. Rab3A shows less than 30% identity to Rab1 and the function of members of the Rab3 subfamily have been specifically implicated in the fusion of exocytic vesicles with the cell surface in regulated secretory pathways (Fischer von Mollard *et al.*, 1994). The combined results support the notion that Mss4 facilitates guanine nucleotide exchange by associating with, and thereby stabilizing, the transient nucleotide-free intermediate and emphasize the promiscuous nature of the putative GEF.

Overexpression of Mss4 Antagonizes Inhibition of ER to Golgi Transport by Rab1A Mutants Defective in GTP Binding

The above results raise the possibility that Rab1A[N124I] and other mutants defective in guanine nucleotide exchange block ER to Golgi transport by sequestering Mss4. To address this point, we first explored whether the inhibitory effects of transiently overexpressing dominant negative Rab1A mutants might be reversible by simultaneously overexpressing Mss4. As shown in Figure 4, column b, overexpressing Mss4 (~20-fold more than endogenous levels) alone did not affect transport of VSV-G from the ER to the Golgi complex, as measured by the acquisition of endoglycosidase H (endo H)-resistant carbohydrate modifications, a hallmark for arrival of VSV-G in the cis/medial Golgi compartments (Schwaninger *et al.*, 1991; Davidson and Balch, 1993). Overexpression of Rab1A[N124I] (two- to eightfold more than endogenous levels) completely inhibits processing (Figure 4, column c), in agreement with previous results (Pind *et al.*, 1994). However, cooverexpressing Mss4 with the Rab1A mutant restored transport of VSV-G to the Golgi stack to control levels (Figure 4, column d). Similar results were obtained for the Rab1A[S25N]

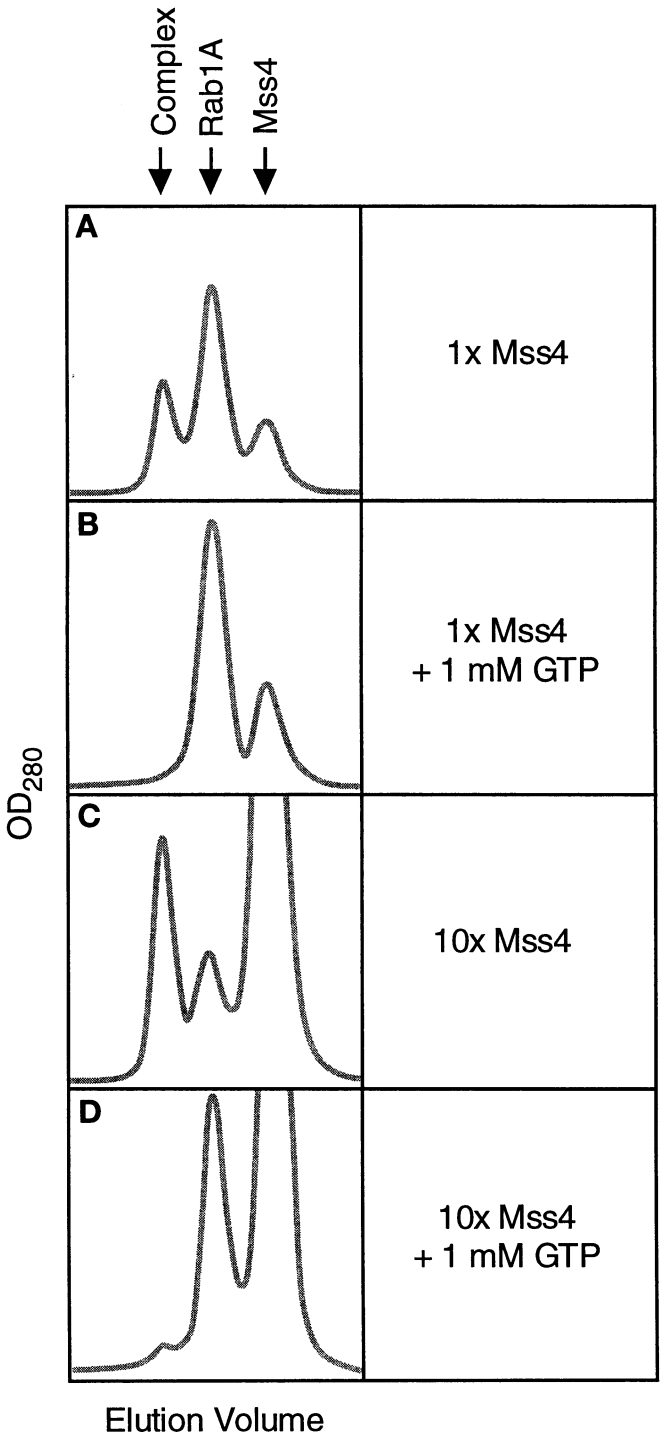

Figure 2. Interaction of Mss4 with wild-type and mutant Rab1A in vitro. A 1:1 (A and B) or 10:1 (C and D) mixture of Rab1A and Mss4 were incubated for 30 min at 32°C in physiological buffer in the absence (A and C) or presence (B and D) of 1 mM GTP. The samples were then processed by gel filtration through a Superdex 75 FPLC column. Proteins eluting from the column were detected by monitoring the absorbance at 280 nm (OD_{280}). Arrows, elution peaks for Mss4 and Rab1A when run separately (our unpublished results).

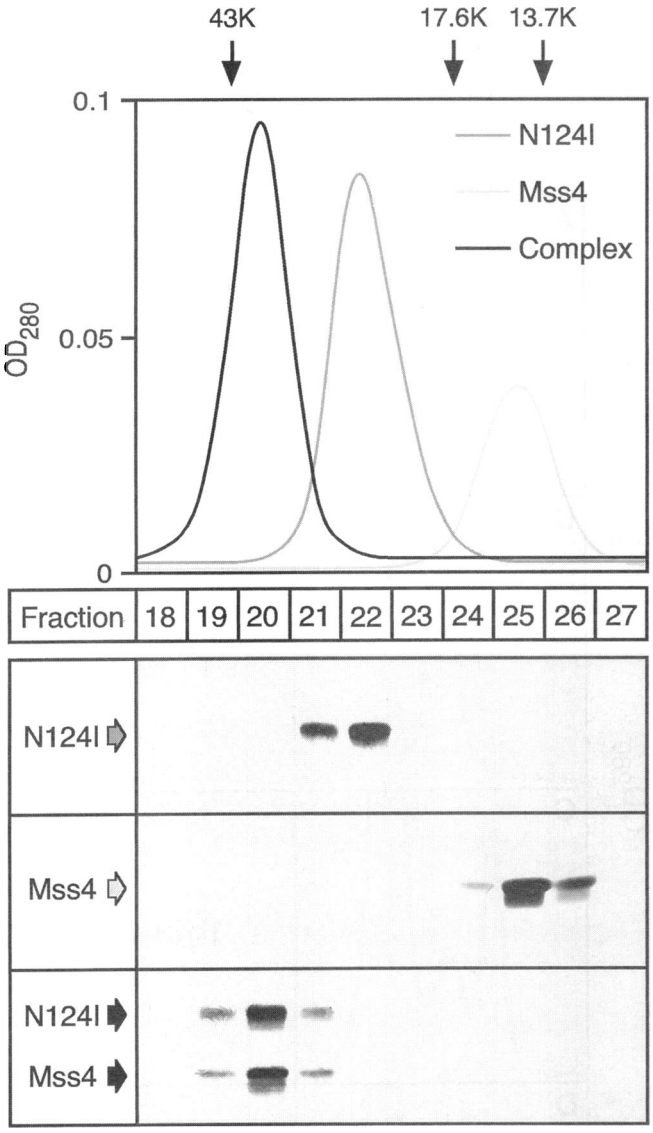

Figure 3. Mss4 forms a stable complex with the Rab1A[N124I] mutant. Samples containing Rab1A[N124I], Mss4, or an equimolar mixture of the two proteins were incubated and analyzed by gel filtration. Fractions were collected, and the proteins were precipitated and resolved by SDS-PAGE and Coomassie blue staining. Top, distinct elution profiles; bottom, distribution of Rab1A[N124I] and Mss4 across the fractions. The elution peaks of molecular markers are indicated at the top.

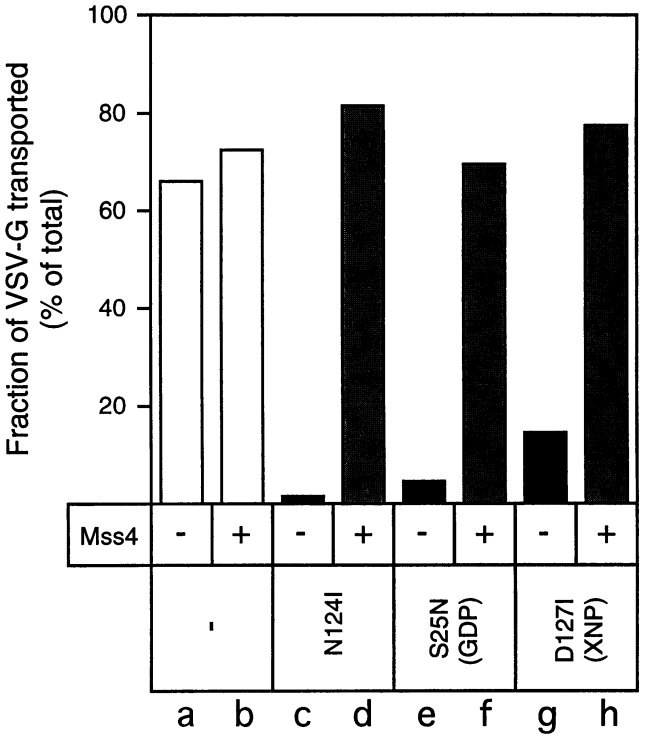

Figure 4. Overexpression of Mss4 antagonizes inhibition of ER to Golgi transport by Rab1A mutants defective in GTP binding. VSV-G was transiently expressed in HeLa cells alone (column a) or along with Mss4 (column b), with Rab1A[N124I] (columns c and d), Rab1A[S25N] (columns e and f), or Rab1A[D127N] (columns g and h), alone (columns c, e, and g) or with Mss4 (columns d, f, and h). Transport from the ER to the Golgi complex was measured by determining the fraction of pulse-labeled VSV-G processed to endo H-resistant forms during a 1-h chase (see MATERIALS AND METHODS).

mutant (Figure 4, columns e and f), which is defective in guanine nucleotide exchange due to a reduced affinity for GTP (Nuoffer *et al.*, 1994) and the Rab1A[D127N] mutant predicted to specifically interact with xanthine rather than guanine nucleotides (Jones *et al.*, 1995; Rybin *et al.*, 1996; Figure 4, columns g and h). The latter mutant is likely to be present in the nucleotide-free state because cells contain only very low levels of free xanthine nucleotide. Thus, the overexpression of Mss4 antagonizes the inhibitory effects of three Rab1A mutants that are defective in nucleotide binding.

Mss4 Suppresses the Inhibitory Activity of the Rab1A[N124I] Mutant In Vitro

To further explore the relationship between dominant negative Rab1A mutants and Mss4, we took advantage of a well-characterized semi-intact cell system that has been used extensively to study vesicular traffic between the ER and the Golgi stack in vitro (Plutner *et al.*, 1992; Davidson and Balch, 1993). As shown previously, supplementing this assay with recombinant Rab1 mutants faithfully reconstitutes in vitro the inhibitory phenotypes observed in vivo (Tisdale *et al.*, 1992; Davidson and Balch, 1993; Nuoffer *et al.*, 1994; Pind *et al.*, 1994). Semi-intact cells normally transport 70–90% of the total VSV-G from the ER to the cis/medial Golgi compartments with a t of ~30 min, as judged by processing of the protein to endo H-resis-

tant forms (Davidson and Balch, 1993). In the presence of 1 μg of recombinant Rab1A[N124], only 15–25% of the total VSV-G present in the ER at the beginning of the reaction is typically delivered to Golgi compartments (Pind *et al.*, 1994; Figure 5A, filled circles). However, supplementing the reaction with up to stoichiometric levels of recombinant Mss4 completely suppressed the inhibitory activity of the Rab1A mutant (Figure 5A, filled circles).

To establish that the neutralization by Mss4 was specific for the guanine nucleotide-free Rab1[N124I] mutant, we supplemented the assay with Rab3A[N135I]. The Rab3A[N135I was shown previously to have no effect on ER to Golgi transport when overexpressed in vivo (Tisdale *et al.*, 1992). As expected, addition of Rab3A[N135I] did not inhibit ER to Golgi transport in vitro (Figure 5A, columns a and b). An assay containing a twofold molar excess of Rab3A over that of Rab1[N124I] also remained fully inhibited (Figure 5A, open circles), demonstrating that the Rab3A[N135I] mutant alone cannot neutralize the effects of the Rab1A[N124I] mutant. Furthermore, the amount of Mss4 required for neutralization of Rab1A[N124I] inhibition in the presence of the Rab3A[N135I] mutant was nearly threefold that observed in its absence (Figure 5A, compare filled circles, minus Rab3A[N135I], to open circles, plus Rab3A[N135I]). These results demonstrate that Mss4 specifically relieves inhibition of the Rab1A[N124] mutant in the context of its unique function in ER to Golgi transport.

We have previously reported that VSV-G transport becomes insensitive to the effects of the Rab1A[N124I] mutant well before its processing to endo H-resistant forms is complete (Pind *et al.*, 1994). For example, whereas only ~20% of the total VSV-G is delivered to the Golgi stack during a 20-min incubation in the absence of Rab1A[N124I] (Figure 5B, squares, 20 min), addition of the mutant to the reaction at the 20-min point does not abruptly block transport. The fraction of endo H-resistant VSV-G increased to more than 50% over the remaining 70-min incubation (Figure 5B, open circles, 20 min). Given this observation, it was important to determine whether the suppressing effect of Mss4 was limited to the Rab1A[N124I]-sensitive interval or whether Mss4 might also be able to reverse the inhibition after prior exposure of the reaction to the mutant. For this purpose, samples were incubated in the presence of Rab1A[N124I] for increasing periods. The reactions were then supplemented with a twofold molar excess of Mss4 and further incubated for a total of 90 min (Figure 5B, filled circles). As shown above (Figure 4), supplying Mss4 with Rab1A[N124I] at the beginning of the incubation completely abolished the inhibitory phenotype of the mutant (Figure 5B, 0 min). However, upon prior exposure to Rab1A[N124I], the reactions rapidly became resistant to the suppressing effect of Mss4. These results show that the inhibitory effects of the Rab1A[N124I] mutant, once established, cannot be reversed by excess Mss4.

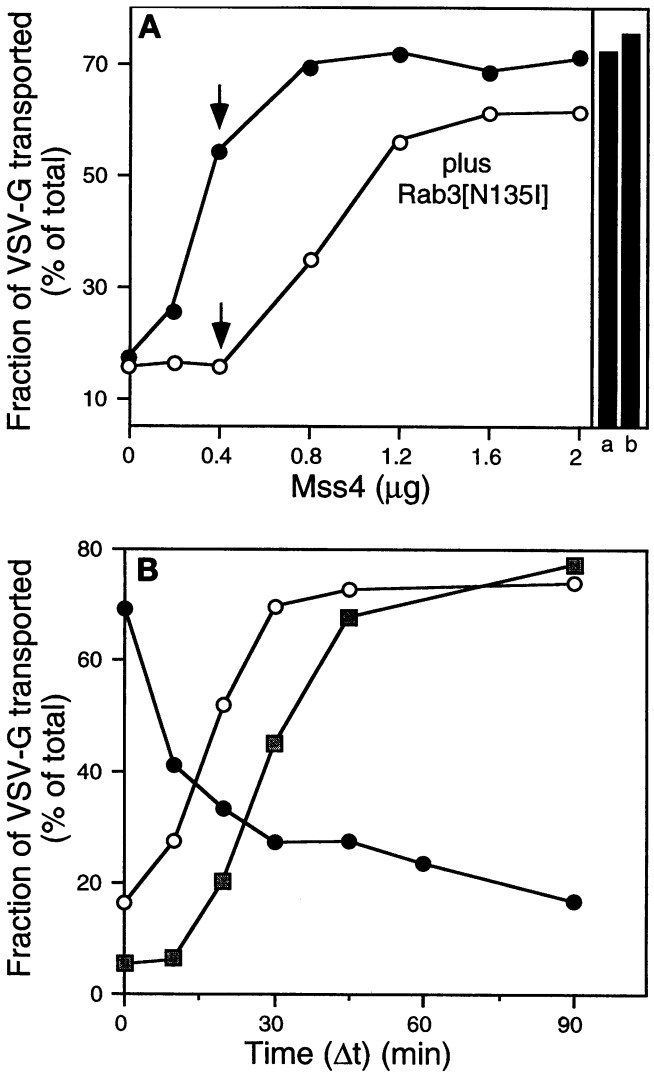

Figure 5. Mss4 suppresses the inhibitory phenotype of the Rab1A[N124I] mutant in vitro. (A) NRK cells expressing VSV-G were radiolabeled, permeabilized, and washed as described in MATERIALS AND METHODS. Transport reactions containing semi-intact cells, rat liver cytosol, and ATP, as well as 1 μg of recombinant Rab1A[N124I] (● and ○) or, in addition, 2 μg of Rab3A[N135I] (○), were supplemented with the indicated amounts of recombinant Mss4 before incubation for 90 min. In the inset, the reaction was either not supplemented (column a) or supplemented with 2 μg of Rab3A[N135I] (column b). Transport from the ER to the Golgi complex was assessed by determining the fraction of VSV-G processed to endo H-resistant forms. (B) (Squares) Transport reactions were incubated for the specified time (Δt) before terminating the reaction by transferring the samples to ice. (Open circles) Transport reactions were incubated for the indicated time (Δt). The samples were then supplemented with 1 μg of the Rab1A[N124I] mutant and the incubation was continued for a total time of 90 min—Δt. (Closed circles) Transport reactions containing 1 μg of Rab1A[N124I] were supplemented with a twofold molar excess of Mss4 at the time indicated (Δt), and the samples incubated for a total time 90 min—Δt.

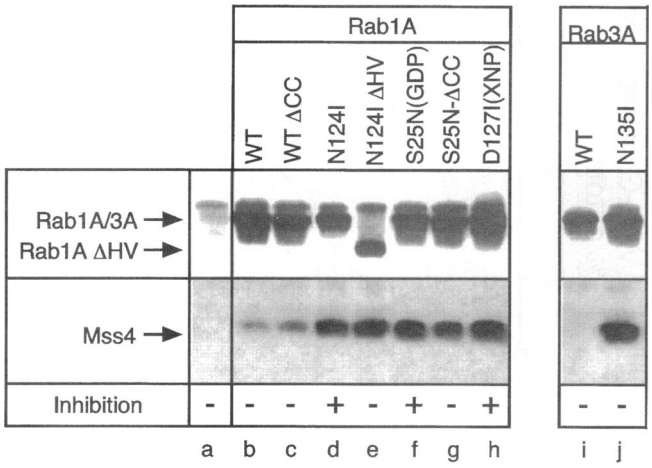

Figure 6. Mss4 associates with both inhibitory and noninhibitory Rab mutants in vivo. HA-tagged wild-type (WT) and mutant forms of Rab1A and Rab3A were transiently expressed in HeLa cells as described in MATERIALS AND METHODS. The proteins were immunoprecipitated from detergent lysates with anti-HA antibodies, and the precipitates were analyzed by SDS-PAGE and quantitative Western blotting for the presence of the Rab proteins (top) and Mss4 (bottom) with anti-HA and anti-Mss4 antibodies, respectively. ΔCC (lanes c and g) refers to mutants lacking the C-terminal cysteine residues essential for posttranslational prenylation. ΔHV (lane e) denotes a deletion encompassing the entire C-terminal hypervariable domain. The heavy band above the signals corresponding to the individual Rab proteins represents the light chain of the anti-HA antibodies, as illustrated by control immunoprecipitation from mock-transfected cells (lane a).

Mss4 Associates with Both Inhibitory and Noninhibitory Rab Mutants In Vivo

To provide more direct evidence that Mss4 is the target for Rab1A mutants in vivo, experiments were conducted to determine whether it was possible to detect an interaction between these mutants and the endogenous pool of Mss4. For this purpose, we overexpressed variants of Rab1A bearing an N-terminal HA epitope tag. Association between the Rab1A constructs and the endogenous Mss4 was then assessed by immunoprecipitation with an anti-HA antibody and probing the immune complexes for the presence of Mss4 by quantitative Western blotting.

Relatively low levels of endogenous Mss4 were coimmunoprecipitated with wild-type HA-Rab1A (Figure 6, lane b). This is consistent with our previous data showing that only a small fraction of wild-type Rab1A stably associates with Mss4 in vitro in the presence of GTP (Figure 1). It is interesting to note that similar amounts of Mss4 were recovered with a truncated form of Rab1A lacking the pair of C-terminal cysteine residues required for posttranslational prenylation (Rab1A[ΔCC]) (Figure 6, lane c). Thus, it appears that unlike binding to GDI (Peter *et al.*, 1995), interaction of Rab1A with Mss4 is independent of geranylgeranylation both in vivo and in vitro, even though this posttranslational modification is essential for Rab1A function (Tisdale *et al.*, 1992; Nuoffer *et al.*, 1994).

Significantly higher levels of Mss4 (5–10 fold) were coimmunoprecipitated with the three Rab1A mutants that are defective in nucleotide exchange, including Rab1A[N124I] (Figure 6, lane d), Rab1A[S25N] (Figure 6, lane f), and Rab1A[D127N] (Figure 6, lane h). These data are consistent with the observation that Mss4 associates with the nucleotide-free form of Rab proteins in vitro. While the inhibitory activity of Rab1A[S25N] requires posttranslational prenylation of the protein (Nuoffer *et al.*, 1994), interaction of the mutant with Mss4 was independent of the C-terminal CC motif (Rab1A[S25NΔCC]; Figure 6, lane g). As shown previously, geranylgeranylation is not essential for inhibition by the Rab1A[N124I] mutant (Tisdale *et al.*, 1992; Pind *et al.*, 1994), but a more severe truncation, including the C-terminal hypervariable domain (Rab1A[N124I-ΔHV]) abolishes the inhibitory activity (Pind *et al.*, 1994). In contrast, our present data show that deletion of the hypervariable domain from Rab1A[N124I] had no effect on interaction of the mutant with Mss4 (Figure 6, lane e). Given that the hypervariable domain represents the most divergent portion of Rab proteins and is thought to play an important role in their specific targeting to distinct subcellular compartments (Chavrier *et al.*, 1990), these results are consistent with the promiscuous nature of Mss4 in recognition of a number of Rab GTPases (Burton *et al.*, 1994). However, the data also demonstrate the absence of an absolute correlation between the capacity of Rab1A mutants to associate with Mss4 and their ability to inhibit transport, raising a doubt concerning the possibility that endogenous Mss4 may represent the target of the mutant proteins.

To further explore this point, we extended our studies to include the Rab3A[N135I] mutant, which also showed a strong interaction with Mss4 (Figure 6, lanes i–k). In fact, we observed that Rab3A[N135I] was able to bind Mss4 with roughly the same efficiency as the equivalent Rab1A mutant (Figure 6, compare lane e to j). This is supported by our observation that wild-type Rab1A and Rab3A have similar affinities for Mss4 in vitro. Because Rab3A[N135I] has no effect on transport between the compartments of the early secretory pathway, yet efficiently neutralizes the ability of Mss4 to blocked Rab1A[N1124I] inhibition (Figure 5A), these data provide added support against the hypothesis that Rab1A[N124I] and other mutants defective in guanine nucleotide exchange exert their inhibitory effects by sequestering endogenous Mss4.

Depletion of Cytosolic Mss4 Has No Effect on ER to Golgi Transport In Vitro

The promiscuous nature of Mss4 stands in sharp contrast to the specific function of distinct Rab proteins.

Therefore, although it appears that the Rab1A mutants are able to sequester Mss4, it seems unlikely that this interaction is responsible for their inhibitory activity. Indeed, the observation that Rab3A mutants strongly associate with Mss4 without interfering with transport through the early secretory pathway suggests that Mss4 may not be essential for this process.

Mss4 predominantly behaves as a cytosolic protein, although it has been reported that a minor fraction of the protein may sediment during high-speed centrifugation of brain tissue homogenates (Burton *et al.*, 1994). As judged by Western blotting or immunoprecipitation, there is no Mss4 in the semi-intact cells membranes that we use to reconstitute ER to Golgi transport in vitro (our unpublished results). It follows that rat liver cytosol, which provides the soluble factors essential for the reaction, must serve as the sole source of Mss4. Therefore, we sought to eliminate Mss4 from the cytosol through immunodepletion to directly examine the role of the putative GEF in transport. By using affinity-purified anti-Mss4 antibodies linked to protein A-Sepharose beads, we were able to generate cytosolic fraction lacking Mss4, as judged by our inability to detect any remaining antigen by Western blotting (Figure 7A). We then determined whether the absence of Mss4 might impair the ability of the cytosol to drive the transport of VSV-G from the ER to the Golgi in vitro. As shown in Figure 7B, the activity of the Mss4-depleted fraction was essentially indistinguishable from that of mock-treated cytosol.

Further attempts to unveil a dependence of the process on the putative GEF by including Mss4-specific antibodies directly into the reaction mixture were unsuccessful (Figure 7C), which argues against the possibility that low levels of residual Mss4 might be responsible for the activity of the depleted cytosol. Moreover, reactions conducted in the presence of Mss4-deficient cytosol proved as sensitive to the inhibitory effects of the Rab1A[N124I] as those containing mock-depleted cytosol and the inhibition was insensitive to the addition of Rab3A[N135I] mutant (Figure 7C). These results allow us to conclude that Mss4 is not the target responsible for the dominant negative phenotype of the Rab1[N124I] mutant.

DISCUSSION

Previous studies raised the important possibility that Mss4 may operate as a GEF to regulate the function of Rab1A and possibly other Rabs functioning in the exocytic pathway (Burton *et al.*, 1993, 1994; Moya *et al.*, 1993). In keeping with this hypothesis, Mss4 promotes the dissociation of GDP from Rab1A in vitro. However, the stimulatory activity is very weak and the putative GEF is not specific for Rab1A because several other Rab proteins operating at different stages along the exocytic pathway have also been shown to be poor substrates of Mss4 (Burton *et al.*, 1994). GEFs for Ras-like GTPases are thought to facilitate guanine nucleotide exchange by interacting with, and thereby stabilizing, the transient nucleotide-free intermediate, which follows the release of GDP and precedes the binding of GTP (Boguski and McCormick, 1993). Consistent with such a mechanism, our binding studies indicate that Mss4 interacts with Rab1A to form a binary complex that is easily disrupted by GTP. Similar results have been obtained by studies examining the relationship between Mss4 and Rab3A (Burton *et al.*, 1994). In addition, we have established that mutations known to decrease the affinity of Rab1A and Rab3A for GTP dramatically stabilize the association between Mss4 and the GTPase. Thus, Rab1A[N124I] and Rab1A[N135I], which fail to bind guanine nucleotides in vitro (Pind *et al.*, 1994), readily form a complex with Mss4 that is entirely resistant even to high concentrations of GTP.

These data not only support the previous view that Mss4 specifically interacts with the nucleotide-free form of Rab proteins (Burton *et al.*, 1993, 1994; Moya *et al.*, 1993) but also suggest that Rab1A[N124I] and other mutants defective in GTP binding might inhibit ER to Golgi transport by sequestering the putative GEF into a catalytically inactive complex. Indeed, we discovered that supplementary Mss4 was able to antagonize the inhibitory phenotypes of dominant negative Rab1A mutants both in vivo and in vitro. However, we began to question the role of Mss4 as a putative GEF when our coimmunoprecipitation studies revealed that the capacity of various Rab1A constructs to associate with Mss4 did not correlate with their phenotype. For example, the interaction of Mss4 with Rab proteins is independent of their posttranslational prenylation (Burton *et al.*, 1994). In fact, although this modification is critical for inhibition by the Rab1A[S25N] mutant (Nuoffer *et al.*, 1994), an inactive truncated version of the mutant was capable of binding Mss4 as efficiently as the full-length protein. By taking advantage of Rab1A[N124I], a mutant that does not require prenylation for inhibition (Pind *et al.*, 1994), we were also able to show that the C-terminal variable domain, which plays an important role in the specific localization of individual Rab proteins to distinct subcellular compartments (Chavrier *et al.*, 1990), is not essential for association of Rab1A[N124I] with Mss4, even though it is critical for the inhibitory activity of the mutant protein. Further experiments revealed that Mss4 interacts with Rab proteins mainly through regions found proximal to hypervariable C termini. Mss4, therefore, differs significantly from Rab-GDI, a Rab-binding protein that only associates with full-length Rab proteins bearing the C-terminal geranylgeranyl modifications (Pfeffer *et al.*, 1995; Wu *et al.*, 1996). However, GDI and Mss4 may interact with partially overlapping regions, because both com-

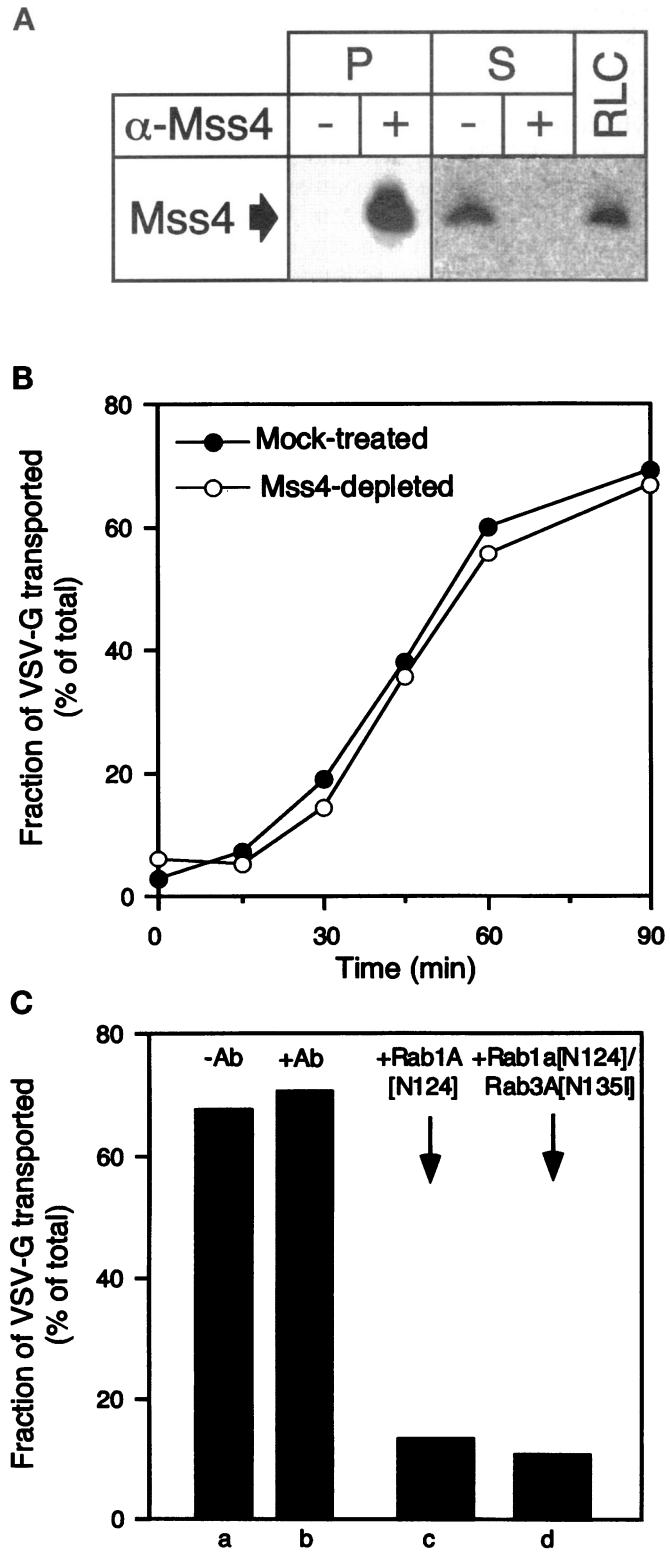

Figure 7. Depletion of cytosolic Mss4 has no effect on ER to Golgi transport in vitro. (A) Rat liver cytosol (RLC) was treated with affinity-purified anti-Mss4 antibodies linked to protein A-Sepharose beads (+) as described in MATERIALS AND METHODS. Controls were performed with beads lacking the antibodies (−). Pellet (P) and supernatant (S) fractions were separated, and the distribution of Mss4 was determined by SDS-PAGE and immunoblotting with the entire pellet but only 5% of the supernatant. (B) Transport reactions containing equivalent concentrations of mock-treated or Mss4-deficient cytosol were incubated for the indicated time, the samples were transferred to ice, and transport was measured by determining the fraction of VSV-G processed to endo H-resistant forms as described in MATERIALS AND METHODS. (C) Cytosol was either not supplemented (column a) or supplemented with 5 μg of an immunopurified polyclonal antibody (Ab) specific for Mss4 (column b). Mss4-deficient cytosol was supplemented with 1 μg of Rab1A[N124I] (a concentration just sufficient to elicit maximum inhibition; column c) or, in addition, 2 μg of Rab3A[N135I] (column d) and transport was quantitated as described above.

pete with one another for Rab3A binding in vitro (Miyazaki *et al.*, 1994).

Other results also support the conclusion that Mss4 is not the target for dominant negative Rab1A mutants. Specifically, we have shown that Rab3A[N135I] does not perturb transport through the early secretory pathway, even though it binds Mss4 as efficiently as the analogous Rab1A[N124I] mutant. In this regard, although it was able to prevent the ability of Mss4 to neutralize Rab1A[N124I] inhibition, it was unable to antagonize Rab1A[N124I] effects, thereby providing direct evidence that the target for Rab1A[N124] is not Mss4. Additional compelling evidence stemmed from our studies involving immunodepletion of the putative GEF from rat liver cytosol, which serves as the sole source of Mss4 in our assay reconstituting ER to Golgi transport in vitro. Reactions performed in the presence of Mss4-deficient cytosol remained fully susceptible to inhibition by Rab1A[N124I] and remained insensitive to Rab3A[N135I]. This result rules out a contribution of the putative GEF in the dominant negative phenotype. The striking ability of exogenous Mss4 to suppress the inhibitory activity of Rab1A mutants defective in GTP binding most likely reflects an irreversible association between Mss4 and the nucleotide-free forms of the mutant proteins. This effectively neutralizes the Rab1A mutants by preventing them from interacting with their true target, which remains to be identified. This interpretation is entirely consistent with the finding that Mss4 was unable to reverse the inhibition after prior exposure of the transport machinery to the Rab1A[N124I] mutant.

Because the removal of Mss4 from rat liver cytosol did not affect the rate or extent of VSV-G transport from the ER to the Golgi stack in vitro, our results raise doubts concerning the proposed role of Mss4 as a GEF for Rab1A and other Rab proteins (Burton *et al.*, 1993, 1994). It also seems unlikely that guanine nucleotide exchange solely depends on the intrinsic low rate of GDP dissociation from Rab proteins. In keeping with this point of view, disruption of the GEFs regu-

lating Sar1 and ARF1 exchange, two Ras-like GTPases that regulate the assembly/disassembly of COPII and COPI coats, respectively, is lethal in yeast (Barlowe and Schekman, 1993; Peyroche *et al.*, 1996). Not only is Mss4 not required for ER to Golgi transport in vitro, but Dss4 (the yeast Mss4 homologue) is not essential for cell viability (Burton *et al.*, 1993). Although we cannot exclude the possibility that our results reflect the existence of multiple, functionally redundant isoforms of Mss4 that do not cross-react with our anti-Mss4 antibodies, this explanation seems unlikely given the effects of the Rab3A[N135I] that efficiently binds Mss4 like the Rab1A[N124I] mutant yet fails to block transport or prevent Rab1A[N124I] inhibition of transport. Moreover, a survey of the complete yeast genome does not reveal the presence of any new gene products structurally related to Mss4/Dss4.

Evidence for the putative GEF function of Mss4 in vesicular transport came from experiments involving microinjection of the protein into the synapse of the giant squid axon (Burton *et al.*, 1994). This approach revealed that excess Mss4 caused a modest but statistically significant increase of neurotransmitter release, a process that is believed to be subject to regulation by Rab3 isoforms. Although these results may reflect a response of synaptic vesicle fusion to the GEF activity of Mss4 for Rab3, our current data now raise questions concerning the physiological relevance of such observations in attempting to provide evidence for a role of Mss4 as a functional GEF in the brain. Indeed, another potential GEF for Rab3 family members has been recently purified and cloned (Miyazaki *et al.*, 1994; Wada *et al.*, 1997). In contrast to Mss4, this ~180-kDa protein exhibits a marked preference for the fully processed prenylated form of the GTPase, is specific for the Rab3 subfamily, and is enriched in brain tissue. This is more likely to be the GEF involved in Rab3 function in mammalian tissues.

The ability of Mss4 to associate with the nucleotide-free form of Rab proteins is unlikely to be purely coincidental. Mss4 was originally identified in a genetic screen for mammalian suppressors of the temperature-sensitive lethal phenotype of a yeast *sec4–8* strain (Burton *et al.*, 1993). Due to a point mutation in the Sec4 protein, a member of the Rab family that governs transport from the Golgi complex to the cell surface in yeast (Ferro-Novick and Novick, 1993), these cells are inviable at the restrictive temperature. Overexpression of Mss4, however, efficiently suppresses both the secretory and the growth defect of *sec4–8* cells. Independently, a dominant mutation in *dss4* (*DSS4–1*), which encodes a yeast protein that is 27% identical (51% similar) to Mss4, was identified as a suppressor of the *sec4–8* phenotype (Moya *et al.*, 1993). The disruption of *dss4* is not lethal but results in synthetic lethality with mutant alleles of *sec2*, *sec8*, and *sec15*, which are thought to facilitate Sec4 function, since these mutations can be suppressed by duplication of *SEC4*. These genetic data were presented as evidence for the physiological role of Dss4 and Mss4 as GEFs.

The activity of Dss4–1 and Mss4 to suppress the phenotype of *sec4–8* cells is remarkably similar to the ability of Mss4 to neutralize inhibitory Rab1A mutants. Whereas our studies involve dominant negative Rab1A mutants that operate in the context of an endogenous pool of functional wild-type Rab1A that will be able to support transport upon sequestration of the mutants by overexpression of Mss4, *sec4–8* cells entirely depend on the Sec4–8 mutant for Sec4 function. Because the Dss4–1 mutant and Mss4 do not render the cells independent of *SEC4* (Burton *et al.*, 1993; Moya *et al.*, 1993), it now appears that these suppressors operate by simply allowing the mutant Sec4 protein to function at the nonpermissive temperature. On the basis of our results, we now reinterpret these yeast experiments to suggest that Dss4–1 and Mss4 suppress the *sec4–8* cells indirectly by facilitating transition of the GTPase through the nucleotide-free state. Thus, Dss4, like Mss4, is not the physiological GEF mediating Sec4 function, but rather, it may serve as a chaperone, assisting Sec4 and other Rab proteins that fail to undergo productive interactions with components of the transport machinery during guanine nucleotide exchange. This raises the new and unanticipated possibility that optimal Rab function occurs when the nucleotide-free state of Rab GTPases is protected from either misfolding or from having a dominant negative effect on transport. As such, Mss4 and Dss4 may define a new class of Ras-superfamily GTPase effectors that function as guanine nucleotide-free chaperones (GFCs). Although the extent of the role of GFCs in vivo remains to be experimentally addressed, it may be related to the timer function of Rab in mediating interactions between membranes (Aridor and Balch, 1996; Rybin *et al.*, 1996).

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