

Comparison of kinetic properties between MSS4 and *Rab3A* GRF GDP/GTP exchange proteins

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Abstract

The kinetic properties of MSS4 are studied in comparison with those of *Rab3A* GRF. MSS4 stimulates the dissociation of [³H]GDP from the lipid-modified and lipid-unmodified forms of *Rab3A* to the same extent, although *Rab3A* GRF is more effective on the lipid-modified form than on the lipid-unmodified form. Both MSS4 and *Rab3A* GRF are inactive on other *Rab/Sec/Ypt* family members including at least *Rab2*, *Rab5*, and *Rab11*. *Rab* GDI inhibits the MSS4 and *Rab3A* GRF effects on the lipid-modified form of *Rab3A*, but the doses of *Rab* GDI necessary for this inhibitory effect on *Rab3A* GRF are lower than those on MSS4. Moreover, *Rab* GDI slightly inhibits the *Rab3A* GRF effect on the lipid-unmodified form of *Rab3A*, but does not affect the MSS4 effect on the lipid-unmodified form of *Rab3A*. These results suggest that MSS4 and *Rab3A* GRF are different GDP/GTP exchange proteins for *Rab3A*.

Key words: Small G protein; *Rab3A*; MSS4; *Rab3A* GRF; *Rab* GDI

1. Introduction

The *Rab/Sec/Ypt* small G protein³ family regulates intracellular vesicle transport such as exocytosis, endocytosis, and transcytosis (for reviews, see [1–6]). Most of the *Rab/Sec/Ypt* family members terminate in either of two sequences: Cys-Cys or Cys-X-Cys (where X is alanine, serine, or glycine). These cysteine residues are geranylgeranylated and the C-terminal cysteine of the Cys-X-Cys structure is carboxylmethylated.

These small G proteins have two interconvertible forms: GDP-bound inactive and GTP-bound active forms [3]. The GDP-bound form is converted to the GTP-bound form by the GDP/GTP exchange reaction which is regulated by GEPs [3]. MSS4 has been isolated as a mammalian counterpart of yeast DSS4 [7], which has been isolated as a GEP for *Sec4* [8]. Both recombinant DSS4 and MSS4 stimulate the GDP/GTP exchange reaction of *Sec4*, but their detailed substrate specificities have not been studied. *Rab3A* GRF has been partially

purified from rat brain as a GEP for *Rab3A* [9], which is involved in regulated secretion, particularly in neurotransmitter release [10–13]. *Rab3A* GRF is active on both the lipid-modified and lipid-unmodified forms of *Rab3A*, but is far more active on the former form than on the latter form [14]. However, requirement of MSS4 for the lipid modifications of its substrate small G protein has not been studied.

In contrast to MSS4 and *Rab3A* GRF, *Rab* GDI is an inhibitory GEP for all the *Rab/Sec/Ypt* family members thus far tested [15–19]. Moreover, *Rab* GDI regulates the cyclical translocation of its substrate small G proteins between the membrane and the cytosol [20]. *Rab* GDI is active only on the lipid-modified form of its substrate small G proteins [21]. *Rab* GDI inhibits the *Rab3A* GRF-stimulated GDP/GTP exchange reaction of *Rab3A*, but the sensitivity of MSS4 to *Rab* GDI has not been studied.

In the present study, we have examined here the substrate specificity, lipid-requirement, and sensitivity to *Rab* GDI of recombinant MSS4 in comparison with those of partially purified *Rab3A* GRF.

2. Materials and methods

2.1. Materials and chemicals

Human *Rab2* and canine *Rab5* were kindly provided by Dr. S. Orita, Shionogi Institute for Medical Science, Settsu, Japan, and Dr. M. Zerial, European Molecular Biology Laboratory, Heidelberg, Germany, respectively. *Rab3A*, *RhoA*, *Rac1*, and c-Ha-Ras were expressed in *Spodoptera frugiperda* cells (Sf9 cells) and the lipid-modified and lipid-unmodified forms were purified from the membrane and soluble fractions, respectively, of the cells overexpressing each small G protein

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Abbreviations: G protein, GTP-binding protein; GEP, GDP/GTP exchange protein; GRF, guanine nucleotide releasing factor; GDI, GDP dissociation inhibitor; *E. coli*, *Escherichia coli*; GST, glutathione-S-transferase; GTPγS, guanosine 5'-(3-*O*-thio)triphosphate; GDS, GDP dissociation stimulator.

[22]. *Rab2*, *Rab3A*, *Rab5*, and *Rab11* were purified from *E. coli* which overexpressed each small G protein as a fusion protein with N-terminal GST [23]. *Rab3A* GRF was partially purified from rat brain as described [9]. *Rab* GDI was purified from bovine brain as described [15]. [35 S]GTP γ S (44.4 TBq/mmol) was purchased from Du Pont-New England Nuclear. [3 H]GDP (518 GBq/mmol) was purchased from Amersham Corp. Nitrocellulose filters (BA-85, 0.45 μ m pore size) were obtained from Schleicher and Schuell.

2.2. Expression and purification of MSS4 in *E. coli*

The expression plasmid pGEX-2T-MSS4 was constructed as follows. The 1.7-kilobase fragment coding for the complete MSS4 cDNA with the *Bam*HI and *Kpn*I sites upstream of the initiator methionine codon and downstream of the termination codon was obtained by a polymerase chain reaction from the rat brain cDNA library previously used [24]. This fragment was digested by *Bam*HI and inserted into the *Bam*HI site of pGEX-2T vector (Pharmacia Biotech Inc.) and expressed as a fusion protein with N-terminal GST. *E. coli* MC1061 transformed with pGEX-2T-MSS4 was cultured and treated with 1 mM isopropyl- β -D-thiogalactopyranoside to induce production of GST-MSS4. GST-MSS4 was purified by glutathione-Sepharose 4B column chromatography as described [23]. The GST carrier was cleaved off from MSS4 by digestion with thrombin as described [23]. Purified MSS4 was dialyzed against 50 mM HEPES/NaOH at pH 8.0 containing 100 mM NaCl, 5 mM $MgCl_2$, and 1 mM EDTA, and stored at -80°C until use.

2.3. Assays

The dissociation of [3 H]GDP from the small G protein to be tested was assayed by measuring the radioactivity of [3 H]GDP bound to the small G protein after incubation with MSS4, *Rab3A* GRF, or *Rab* GDI by the filtration method using a nitrocellulose filter as described previously [14]. The activity of MSS4 to stimulate the binding of [35 S]GTP γ S to the small G protein to be tested was similarly assayed.

3. Results

MSS4 stimulated the dissociation of [3 H]GDP from and the binding of [35 S]GTP γ S to the lipid-modified form of *Rab3A* in a time-dependent manner (Fig. 1). The time courses for these two reactions were similar. These activities of MSS4 were dependent on its doses and the doses necessary for these activities were similar (Fig. 2). The

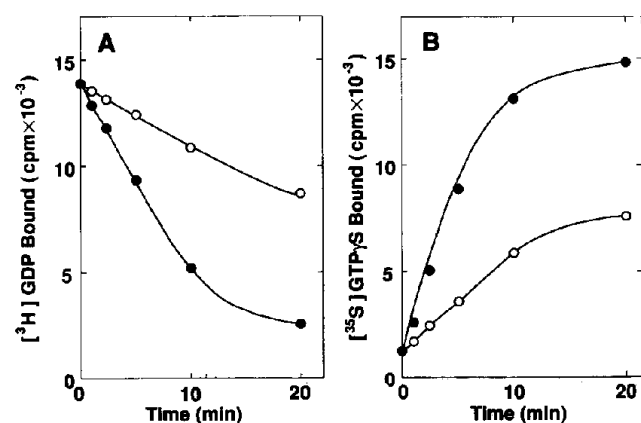


Fig. 1. Effect of MSS4 on the dissociation of [3 H]GDP from and the binding of [35 S]GTP γ S to *Rab3A*. The velocities of the dissociation of [3 H]GDP from and the binding of [35 S]GTP γ S to *Rab3A* (2 pmol) were assayed in the presence or absence of MSS4 (20 pmol). (A) The dissociation of [3 H]GDP from *Rab3A*. (B) The binding of [35 S]GTP γ S to *Rab3A*. (●) In the presence of MSS4; (○) in the absence of MSS4. The results shown are representative of three independent experiments.

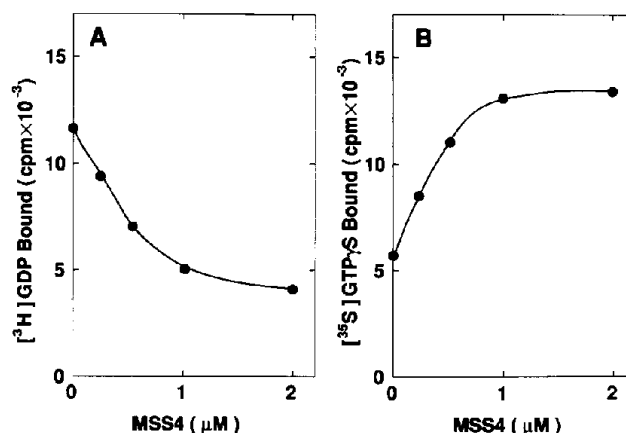


Fig. 2. Dose-dependent effect of MSS4 on *Rab3A*. The dissociation of [3 H]GDP from and the binding of [35 S]GTP γ S to *Rab3A* (2 pmol) were assayed for 10 min at 30°C in the presence of various doses of MSS4. (A) The dissociation of [3 H]GDP from *Rab3A*. (B) The binding of [35 S]GTP γ S to *Rab3A*. The results shown are representative of three independent experiments.

dose of MSS4 giving half maximum dissociation of [3 H]GDP from *Rab3A* was about 0.4 μ M and the dose of MSS4 giving half maximum binding of [35 S]GTP γ S to *Rab3A* was also about 0.4 μ M.

We have previously shown that *Rab3A* GRF is active on both the lipid-modified and lipid-unmodified forms of *Rab3A* but is more active on the lipid-modified form than on the lipid-unmodified form [14]. Consistently, *Rab3A* GRF was active on the both forms but was more active on the lipid-modified form than on the lipid-un-

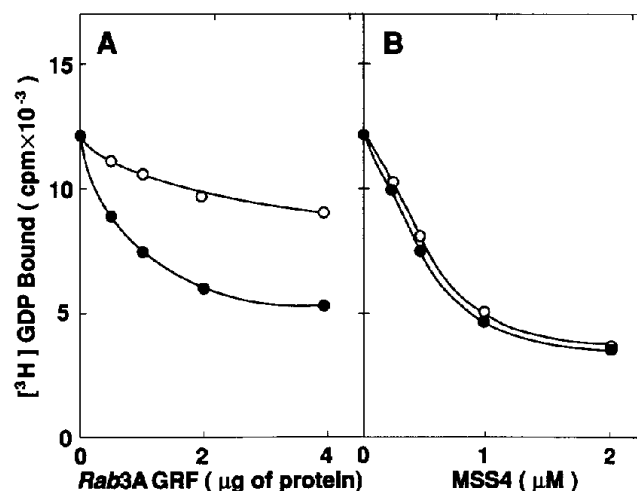


Fig. 3. Sensitivity of MSS4 and *Rab3A* GRF to the lipid-modified and lipid-unmodified forms of *Rab3A*. The dissociation of [3 H]GDP from the lipid-modified form or lipid-unmodified form of *Rab3A* (2 pmol each) was assayed for 10 min at 30°C in the presence of various doses of MSS4 or *Rab3A* GRF. (A) With *Rab3A* GRF. (B) With MSS4. (●) With the lipid-modified form; (○) with the lipid-unmodified form. The results shown are representative of three independent experiments.

modified form (Fig. 3A). In contrast, MSS4 was equally active on both the lipid-modified and lipid-unmodified forms (Fig. 3B).

We have previously shown that *Rab* GDI inhibits the *Rab*3A GRF-induced GDP/GTP exchange reaction of the lipid-modified form of *Rab*3A but shows far less effect on the lipid-unmodified form [14]. *Rab* GDI inhibited not only the effect of *Rab*3A GRF on the lipid-modified form of *Rab*3A but also that of MSS4, but the doses of *Rab* GDI necessary for this inhibitory effect on *Rab*3A GRF were lower than those on MSS4 (Fig. 4A). Moreover, *Rab* GDI slightly inhibited the *Rab*3A GRF effect on the lipid-unmodified form of *Rab*3A, but did not affect the MSS4 effect on the same form of *Rab*3A (Fig. 4B).

It has previously been shown that MSS4 enhances the dissociation of [3 H]GDP from the lipid-unmodified form of *Rab*3A but is inactive on the lipid-unmodified form of yeast *Ras*2 [7]. Consistently, MSS4 was active on the lipid-unmodified form of *Rab*3A (Fig. 5) and was inactive on the same form of c-Ha-*Ras*, *Rho*A, and *Rac*1 (data not shown). Moreover, MSS4 was inactive on the same form of other *Rab* family members including *Rab*2, *Rab*5, and *Rab*11 (Fig. 5). *Rab*3A GRF showed similar substrate specificity (data not shown).

4. Discussion

We have previously shown that one group of GEPs, such as *Sos* for the *Ras* family members [25], *Cdc25* for the *Ras* family members [24], *Dbl* for the *Rho* family

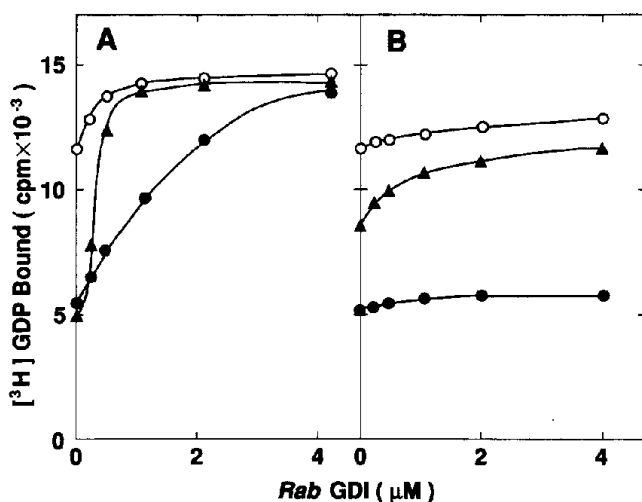


Fig. 4. Effect of *Rab* GDI on the MSS4 and *Rab*3A GRF actions. The activity of *Rab* GDI to inhibit the dissociation of [3 H]GDP from the lipid-modified form or lipid-unmodified form of *Rab*3A (2 pmol each) was assayed in the presence or absence of MSS4 (20 pmol) or *Rab*3A GRF (4 μ g of protein). (A) With the lipid-modified form. (B) With the lipid-unmodified form. (○) Without MSS4 and *Rab*3A GRF; (●) with MSS4; (▲) with *Rab*3A GRF. The results shown are representative of three independent experiments.

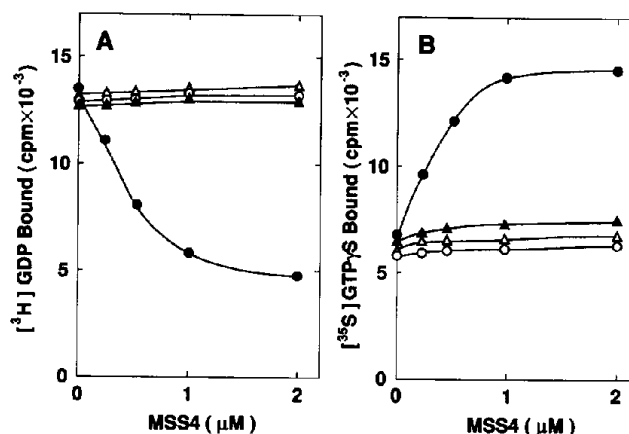


Fig. 5. Substrate specificity of MSS4. The dissociation of [3 H]GDP from and the binding of [35 S]GTP γ S to each small G protein (2 pmol) were assayed in the presence of various doses of MSS4. (A) The dissociation of [3 H]GDP. (B) The binding of [35 S]GTP γ S. (○) With *Rab*2; (●) with *Rab*3A; (▲) with *Rab*5; (△) with *Rab*11. The results shown are representative of three independent experiments.

members, the *Rac* family members, and *Cdc42* [26], and *Rab*3A GRF for *Rab*3A [14], are active on both the lipid-modified and lipid-unmodified forms of their respective substrate small G proteins but prefer the lipid-modified form to the lipid-unmodified form, whereas another group of GEPs, such as *Smg* GDS for *Ki-Ras*, *Rap1*, the *Rho* family members, the *Rac* family members, and *Cdc42* [22,26,27], *Rho* GDI for the *Rho* family members, the *Rac* family members, and *Cdc42* [27,28], and *Rab* GDI for the *Rab* family members [21], absolutely require the lipid-modifications of their respective substrate small G proteins and are totally inactive on the lipid-unmodified form. In contrast to these two groups of GEPs, we have shown here that MSS4 is equally active on both the lipid-modified and lipid-unmodified forms of *Rab*3A. It is likely that MSS4 may belong to the third group of GEPs in terms of the requirement of the lipid-modifications.

As to the relationship between MSS4 and *Rab*3A GRF, the definitive conclusion could not be made here because *Rab*3A GRF has only partially been purified and its primary structure has not been determined. However, we have shown here that MSS4 and *Rab*3A GRF show different properties including the requirement of the lipid-modifications of *Rab*3A and the sensitivity to *Rab* GDI. These results suggest that MSS4 is a different molecule from *Rab*3A GRF.

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