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Specific interactions of Mss4 with members of the Rab GTPase subfamily

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Mss4 is a mammalian protein that was identified as a suppressor of a yeast secretory mutant harboring a mutation in the GTPase Sec4 and was found to stimulate GDP release from this protein. We have now performed a biochemical characterization of the Mss4 protein and examined the specificity of its association with mammalian GTPases. Mss4 is primarily a soluble protein with a widespread tissue distribution. Recombinant Mss4 binds GTPases present in tissue extracts, and by a gel overlay assay binds specifically Rab Rab10proteins. We further define the Mss4–GTPase interaction to a subset of Rabs belonging to the same subfamily branch which include Rab1, Rab3, Rab8, Rab10, Sec4 and Ypt1 but not Rab2, Rab4, Rab5, Rab6, Rab9 and Rab11. Accordingly, Mss4 co-precipitates from a brain extract with Rab3a but not Rab5. Mss4 only stimulates GDP release from, and the association of GTPγS with, this Rab subset. Recombinant Mss4 and Rab3a form a stable complex in solution that is dissociated with either GDP or GTPγS. Injection of Mss4 into the squid giant nerve terminal enhances neurotransmitter release. These results suggest that Mss4 behaves as a guanylnucleotide exchange factor (GEF) for a subset of Rabs to influence distinct vesicular transport steps along the secretory pathway.
Key words: GEF/GRF/*ras*/squid/synaptic vesicles

Introduction

Synaptic vesicles are highly specialized secretory organelles of the nerve terminal which contain non-peptide neurotransmitters. These vesicles undergo regulated exocytosis with the pre-synaptic plasmalemma and are continuously reformed by local cycles of exo-endocytosis. Growing evidence suggests that the molecular mechanisms mediating the biogenesis and fusion of synaptic vesicles represents a specialized form of the mechanisms involved in the budding, targeting and fusion of carrier vesicles between intracellular compartments in all cells (Bennett and Scheller, 1993; Burton *et al.*, 1993; Söllner *et al.*, 1993).

The Rab GTPases, members of the Ras GTPase superfamily, are thought to play an important and general role in vesicular traffic (Balch, 1990; Pfeffer, 1992; Ferro-Novick and Novick, 1993; Simons and Zerial, 1993; Nuoffer and Balch, 1994). Numerous Rab proteins have been identified and implicated in specific intracellular transport steps, including synaptic vesicle exocytosis (Simons and Zerial, 1993).

The exact mechanism by which the Rab GTPases participate in intracellular membrane sorting remains unclear. Based on studies of the crystal structure of Ras, it is thought that the Rab proteins exist in two different conformations depending upon whether GDP or GTP is bound, and therefore act as molecular switches to regulate intracellular transport (Bourne, 1988). When in the GTP state, the Rabs are hypothesized to interact with one or more downstream effectors, which makes the transport vesicles competent for docking and fusion (Shirataki *et al.*, 1993; Li *et al.*, 1994; Brennwald *et al.*, 1994). The intrinsic rates of GTP hydrolysis and GDP release for members of the Ras GTPase superfamily are generally quite low, and therefore accessory factors which can modulate the GDP–GTP cycle of these proteins are required. They include the guanine nucleotide exchange factors (GEFs or GRFs), which stimulate the exchange of GDP for GTP, the guanine nucleotide dissociation inhibitors (GDIs), which influence both the nucleotide state and the subcellular localization of the GTPase and the GTPase activating proteins (GAPs), which stimulate the intrinsic GTP hydrolysis rate. The GAP proteins may also function as downstream effectors (Bourne *et al.*, 1990; Boguski and McCormick, 1993; Novick and Brennwald, 1993).

GAP, GEF and effector proteins for the Ras subfamily have been cloned and characterized (Boguski and McCormick, 1993). Less is known about the accessory proteins for the Rab subfamily. The best characterized Rab regulatory protein is Rab GDI, which binds to the GDP form of the Rab protein and promotes its dissociation from the membrane (Sasaki *et al.*, 1990; Soldati *et al.*, 1993; Ullrich *et al.*, 1993). Rab GDI appears to be a general accessory protein for all of the Rab members (Ullrich *et al.*, 1993). Rab GAP activities have been identified biochemically, but only Gyp6, a yeast Rab GAP specific for Ypt6, the yeast Rab6 homolog, has been cloned (Jena *et al.*, 1992; Walworth *et al.*, 1992; Brondyk *et al.*, 1993; Strom *et al.*, 1993). A potential effector protein, rabphilin, has been identified for Rab3a which binds only to the GTP-bound form of this protein (Shirataki *et al.*, 1993; Geppert *et al.*, 1994). A putative Rab3a-specific GEF has also been partially purified from a cytosolic fraction in brain, but remains to be characterized further (Burstein and Macara, 1992). One protein which has been shown to enhance the GDP dissociation from the Rabs is the mammalian protein Mss4 which is 27% identical and 51% similar to Dss4, a yeast protein with similar properties (Burton *et al.*, 1993; Moya *et al.*, 1993).

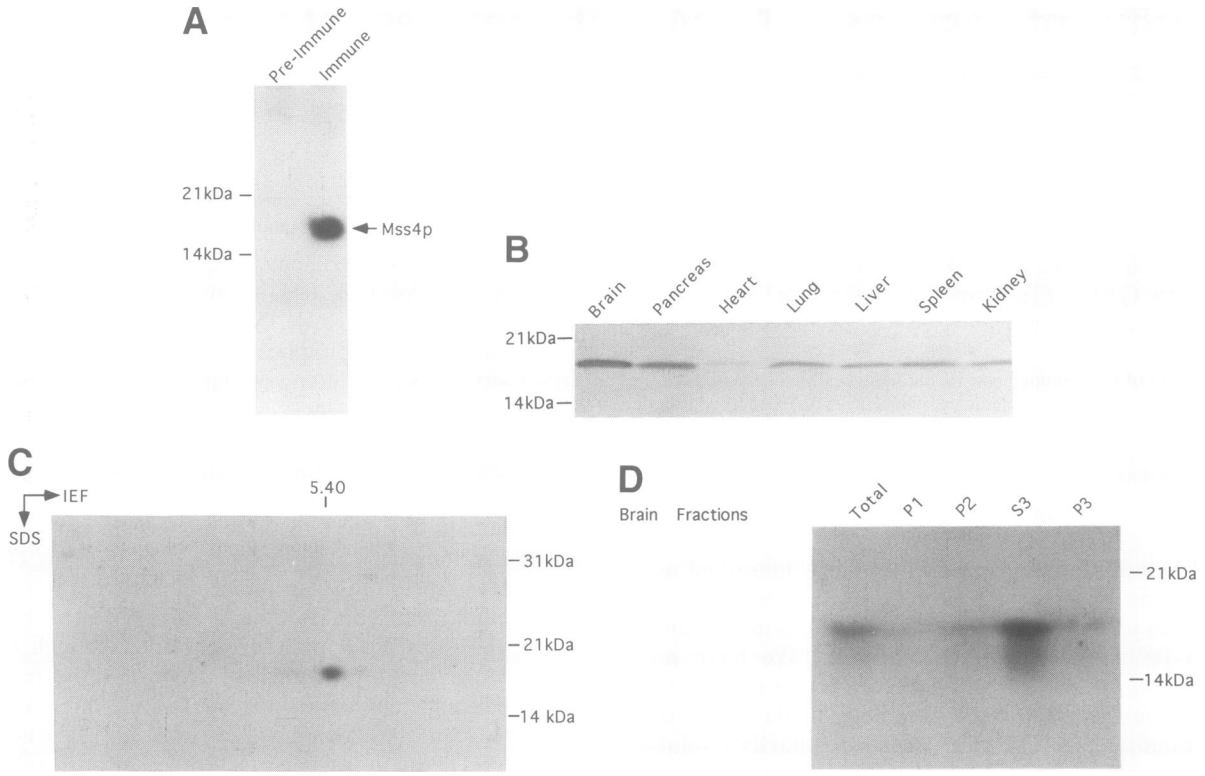

Fig. 1. Mss4 is a soluble protein which migrates as a 17 kDa protein and is present in all tissues. (**A**) Rabbit antibodies raised against recombinant Mss4 recognize a protein of 17 kDa in brain extract. A rat brain soluble extract (see Materials and methods) was immunoprecipitated with anti-Mss4 antibodies (immune) or pre-immune serum. Immunoprecipitates were separated on SDS–PAGE and probed by Western blotting with anti-Mss4 antibodies using [^{125}I]protein A. (**B**) Mss4 is present in all tissues and is most abundantly expressed in brain. Western blots of total tissue homogenates (80 μg total protein/lane). (**C**) Soluble brain proteins were separated by isoelectric point in the first dimension and size in the second dimension. The two-dimensional gel was then probed by Western blotting with anti-Mss4 antibodies. (**D**) A rat brain homogenate was fractionated as described in Materials and methods to yield particulate fractions (P1, P2 and P3) and a soluble fraction (S3). 200 μg protein were loaded in all lanes.

Mss4 was identified using a genetic approach in which rat brain cDNAs were expressed in the *sec4-8* yeast secretory mutant to search for cDNAs which could suppress the defective phenotype (Burton *et al.*, 1993). In the *sec4-8* cells, the Sec4 protein, a member of the Rab subfamily, harbors a point mutation that results in an impairment of its function at the non-permissive temperature of 37°C (Salminen and Novick, 1987). This impairment leads to a block in exocytosis that results in cell growth arrest (Salminen and Novick, 1987). Mss4 was found to suppress both the secretory and growth defect in these cells (Burton *et al.*, 1993). The yeast protein, Dss4, was isolated independently in a suppressor analysis of the *sec4-8* strain (Moya *et al.*, 1993). Both recombinant Mss4 and Dss4 were shown to stimulate GDP release from recombinant Sec4 protein *in vitro* (Burton *et al.*, 1993; Moya *et al.*, 1993). Recombinant Mss4 was also shown to stimulate, albeit to a lesser degree, GDP release from the two other recombinant Rabs that were tested, Rab3a and Ypt1, but had no effect on Ras2 from yeast (Burton *et al.*, 1993). These preliminary results suggested that the Mss4 protein may interact with all Rab proteins.

Here we perform a biochemical characterization of the Mss4 protein and demonstrate a highly specific interaction of Mss4 with mammalian Rabs present in tissue extracts. We show a strikingly selective binding of Mss4 to a subset of the Rabs and demonstrate that Mss4 has properties consistent with those of a GEF. In addition, we show that Mss4 can facilitate neurotransmitter release. Our results suggest that Mss4 is not a general GEF for all Rab proteins, but instead interacts with only a subset of the Rab proteins to influence multiple intracellular transport steps in the secretory pathway including synaptic vesicle exocytosis.

Results

Characterization of the Mss4 protein

Antibodies were generated against the recombinant protein encoded by the *mss4* cDNA. The amino acid sequence of Mss4 predicts a protein that is highly hydrophilic and lacks a membrane-spanning domain, suggesting that Mss4 is a soluble protein (Burton *et al.*, 1993). We therefore tested the antibodies against soluble brain extract by immunoprecipitation and subsequent Western blotting. A 17 kDa protein was specifically immunoprecipitated with immune but not pre-immune sera and this protein has an electrophoretic mobility that is in close agreement with the molecular size of 14 kDa for the Mss4 protein (Figure 1A, Burton *et al.*, 1993). Western blot analysis using affinity-purified antibodies revealed that the 17 kDa protein is expressed in all tissues tested (Figure 1B), in agreement with the expression of *mss4* mRNA (Burton *et al.*, 1993). The protein appears to be most abundantly expressed in brain (Figure 1B). Western blotting of two-dimensional gels containing cytosolic brain proteins demonstrated that

the 17 kDa immunoreactive protein has an isoelectric point of 5.4, very similar to the theoretical isoelectric point of 5.03 for the protein encoded by the *mss4* cDNA (Figure 1C). We therefore conclude that this 17 kDa protein is Mss4.

To localize the Mss4 protein further, total brain extract was fractionated. The Mss4 protein was found to be enriched in a cytosolic fraction of brain (Figure 1D, lane S3). However, a portion of the Mss4 protein was also detected in membrane fractions (Figure 1D, lanes P2 and P3).

Mss4 associates with GTPases in a brain extract

In order to demonstrate the interaction of Mss4 with GTPases in mammalian cells, Mss4−maltose binding protein (MBP) or MBP alone was linked to amylose resin via the MBP moiety, and then incubated with a soluble brain extract. Material that was retained on the beads was run on SDS−PAGE, transferred to nitrocellulose and then incubated with [α-^{32}P]GTP to detect small GTPases. Small GTPases are known to refold and bind [α-^{32}P]GTP in nitrocellulose membranes following denaturation during SDS−PAGE (Lapetina and Reep, 1987; Goud *et al.*, 1988). [α-^{32}P]GTP binding proteins with the expected mobility of the small GTPases were shown to be specifically retained on the Mss4−MBP resin but not on the MBP resin (Figure 2). These data support an interaction between Mss4 and the small GTPases in a protein extract, and are consistent with an interaction between Mss4 and GTPases *in vivo*. This conclusion is further supported by the co-precipitation of Mss4 with Rab3a from brain extracts (see below Figure 7).

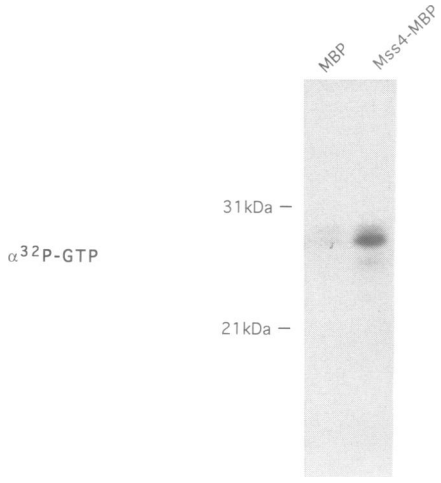

Fig. 2. Mss4 associates with GTPases in solution. Mss4−MBP or MBP alone were bound to amylose resin. The resins were then incubated with a soluble brain extract and washed. Proteins bound to the beads were separated by SDS−PAGE, transferred to nitrocellulose and overlaid with [α-^{32}P]GTP.

Mss4 binds small GTPases immobilized on nitrocellulose

We next investigated the specificity of the interaction of Mss4 with GTPases from mammalian cells. A gel overlay technique has been used successfully in several recent studies to identify protein−protein interactions (Manser *et al.*, 1994; McPherson *et al.*, 1994). We therefore tested the binding of Mss4 to particulate and soluble fractions of rat brain and kidney extracts which had been separated by SDS−PAGE and transferred to nitrocellulose. The nitrocellulose blots were incubated with either Mss4−MBP or MBP alone. The binding of Mss4−MBP or MBP to the proteins on the nitrocellulose was then detected using antibodies against the MBP moiety. The right two panels in Figure 3 show that Mss4−MBP, but not MBP, bound to protein(s) on the nitrocellulose membrane. These Mss4 binding proteins co-migrate with some of the small GTPases as revealed by [α-^{32}P]GTP binding, and migrate at a position corresponding to the Rab GTPases as revealed by immunoblotting for the Rab3a protein (Figure 3, compare first and second panels with the third panel). Mss4 binding proteins and the small GTPases are detected in both a soluble and a particulate fraction in brain and kidney (Figure 3, lanes S and P, data not shown). Furthermore, the Mss4 binding proteins are present in all tissues (data not shown), in agreement with the widespread distribution of the small GTPases and the Mss4 protein.

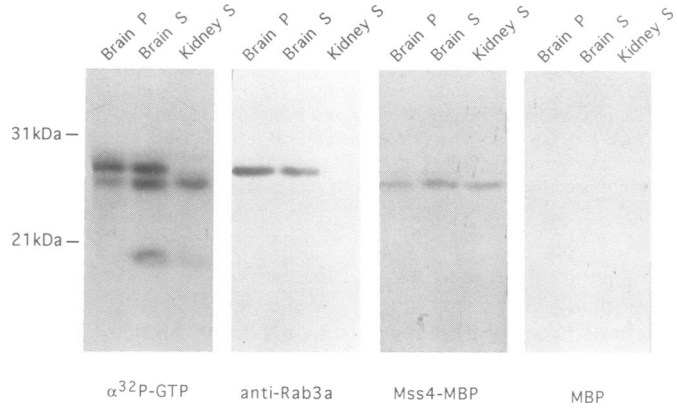

Fig 3. Mss4 specifically binds to proteins with the same electrophoretic mobility as Rab GTPases using an overlay technique. Soluble brain or kidney extracts (S) and a brain particulate fraction (P) were separated by SDS−PAGE and transferred to nitrocellulose. Four identical blots were then processed through either one of the following treatments: [α-^{32}P]GTP overlay; Western blotting for Rab3a; overlay with Mss4−MBP or MBP followed by anti-MBP antibodies and secondary antibodies conjugated to horseradish peroxidase. As expected, Rab3a is only present in brain extracts. Notice the GTPases which migrate below the 21 kDa marker, which probably represent the Arf subfamily, do not bind Mss4.

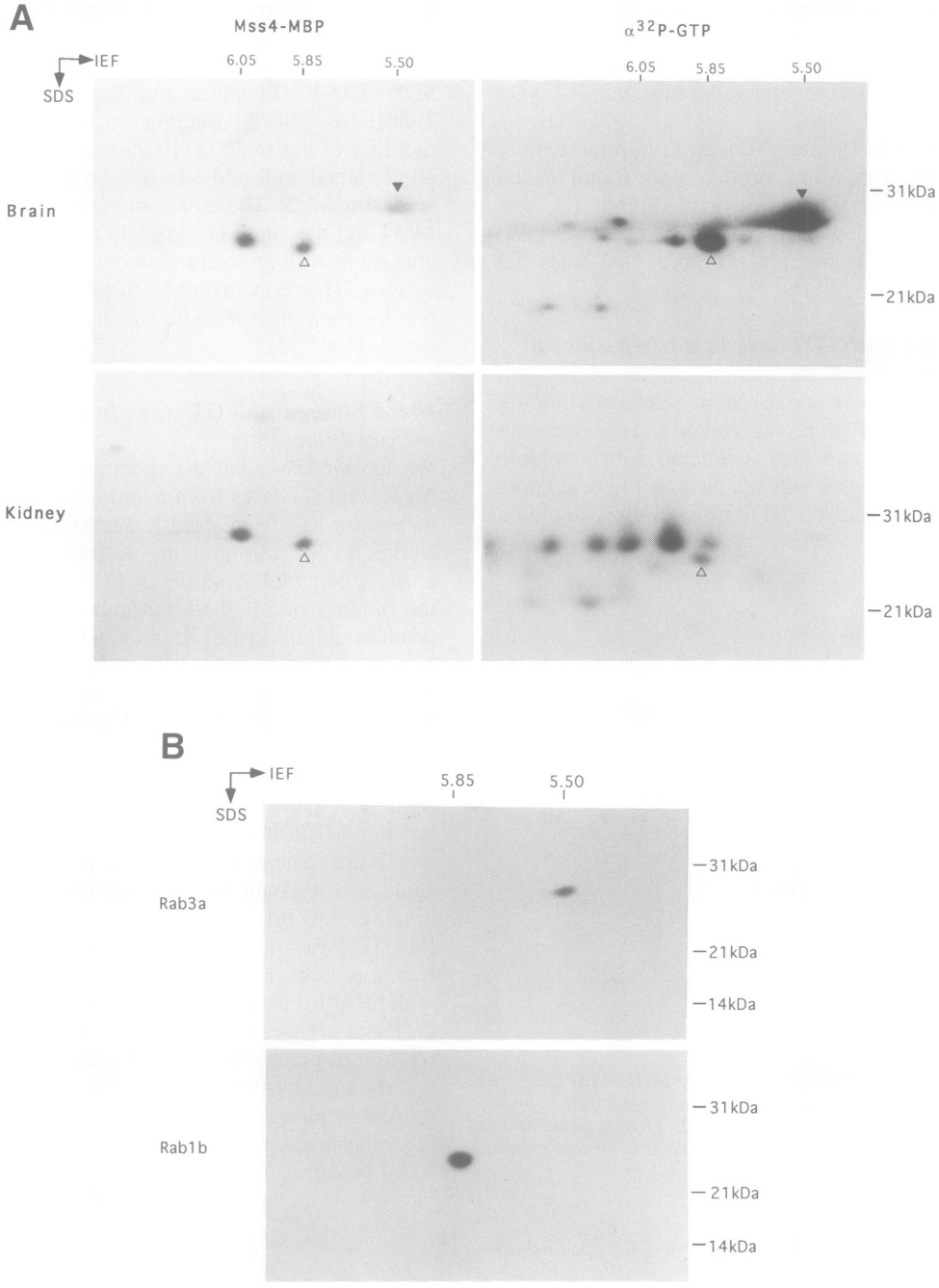

Fig. 4. Mss4 binds only to a subset of small GTPases present in brain and kidney. (**A**) Proteins from a soluble brain extract (top panels) or a soluble kidney extract (bottom two panels) were electrophoresed in two dimensions and transferred to nitrocellulose. The blots were then overlaid with Mss4–MBP (left panels) or [α-^{32}P]GTP (right panels). Bound Mss4 was then revealed by anti-MBP antibodies as described for Figure 3. Two of the Mss4 binding GTPases have the same electrophoretic mobilities as Rab3a and Rab1b (B). (**B**) Two-dimensional gels of soluble brain extracts identical to the ones shown in (A)(top), were treated by Western blotting with antibodies specific for Rab3a and Rab1b. The Rab3a and Rab1b spots have the same electrophoretic mobilities as two of the Mss4 binding proteins (A).

In order to confirm that the Mss4 binding proteins are small GTPases and to resolve how many GTPases bind Mss4, proteins from soluble extracts of brain and kidney were separated by two-dimensional electrophoresis, transferred to nitrocellulose membranes and then incubated with either Mss4–MBP or [α-^{32}P]GTP (Figure 4A). In brain, Mss4 bound primarily to three protein spots, all of which correspond to proteins that also bind [α-^{32}P]GTP and therefore, most likely, represent GTPases (Figure 4A, compare left and right upper panels). These Mss4 binding proteins have isoelectric points of ~6.05, 5.85 and 5.50. Western blot analysis demonstrates that Rab3 corresponds to the Mss4 binding protein with an isoelectric point of 5.50 (closed arrowhead), and Rab1 corresponds to the Mss4 binding protein of 5.85 (open arrowhead) (Figure 4A and 4B). The Mss4 binding GTPase with the isoelectric point of 6.05 has not yet been identified. In kidney, Mss4 bound two GTPases with isoelectric points of 6.05 and 5.85, identical with two of the spots in brain. (Figure 4A, compare upper and lower left panels). The above data

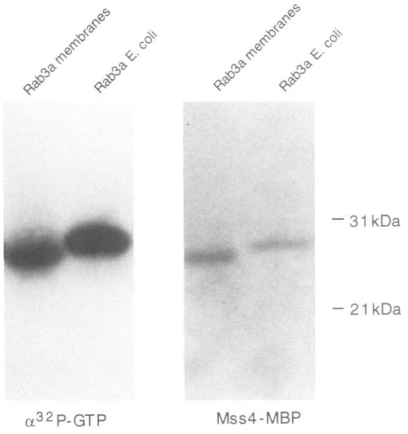

Fig. 5. Mss4 binds to both prenylated and non-prenylated Rab3a. Unmodified Rab3a purified from *E.coli* or modified Rab3a from rat brain membranes were immunoprecipitated with Rab3a-specific antibodies. Precipitates were run on SDS−PAGE and then overlaid with [α-^{32}P]GTP (left panel) or Mss4−MBP (right panel) as described in Figure 3. Modified Rab3a migrates slightly faster in SDS−PAGE.

demonstrate that the Mss4 protein specifically binds to a subset of the small GTPases using the overlay assay, suggesting a selective interaction of Mss4 with a subset of these proteins *in vivo*.

Mss4 can bind both modified and unmodified forms of Rab3a

After translation, the Rab proteins are rapidly prenylated by the enzyme geranylgeranyl transferase II, which adds one or two geranylgeranyl moieties to cysteines (CC or CXC) present at the C-terminus of these proteins (Seabra *et al.*, 1992; Yu *et al.*, 1993). The prenylation of the Rab proteins has been shown to be essential for both their membrane association and function (Novick and Brennwald, 1993; Simons and Zerial, 1993). The ability of Mss4 to bind GTPases from tissue extracts (see Figures 3 and 4A), would suggest that Mss4 is capable of associating with the prenylated form of the Rab proteins. However, to confirm this interaction, we tested whether Mss4 could bind immunoprecipitated, prenylated Rab3a from rat brain membranes or non-prenylated Rab3a from *Escherichia coli*. The immunoprecipitates were run on a SDS−polyacrylamide gel and overlaid either with [α-^{32}P]GTP or Mss4−MBP. Approximately equal amounts of active, unmodified and modified Rab3a were loaded, as assessed by the binding of radiolabeled GTP (Figure 5, left panel). It has been shown previously that the unmodified form of Rab3a migrates more slowly in SDS−PAGE than the prenylated form (Johnston *et al.*, 1991). Accordingly, Rab3a from the brain membrane fraction migrated faster than the recombinant protein and therefore represents geranylgeranylated Rab3a (Figure 5, left panel). In the overlay with Mss4−MBP, Mss4 bound both modified and unmodified Rab3a (Figure 5, right panel).

Mss4 binds selectively to a subset of proteins within the Ras superfamily

To determine more precisely the selectivity of Mss4 binding within the Ras superfamily, and to investigate the possibility that some Mss4 binding GTPases may be present in the tissue extracts at levels too low to be detected (Figure 4A), recombinant GTPases from different subgroups were tested. Recombinant Rab1b, Rab2, Rab3a, Rab4, Rab5, Rab6, Rab8, Rab10, Rab11, Sec4, Ypt1, c-H-ras and RalB proteins were run in SDS−PAGE and transferred to nitrocellulose. The nitrocellulose filters were then incubated with either [α-^{32}P]GTP or with Mss4−MBP (Figure 6A). All of the proteins were able to bind [α-^{32}P]GTP and therefore were refolding in the nitrocellulose membrane (Figure 6A, middle panel and Figure 6B). The Rab1b, Rab8 and Rab10 proteins were less efficient in binding the radiolabeled GTP and longer exposure times were necessary to visualize GTP binding for these proteins (Figure 6B). Strikingly, Mss4 was very selective in its interaction with the GTPases, as seen by its ability to bind only to members of the Rab subclass of the Ras GTPase superfamily (Figure 6A, bottom panel). Furthermore, only some of the Rab proteins exhibited detectable Mss4 binding in this assay, even when processed for longer time periods. Rab1b, Rab3a, Rab8, Rab10, Ypt1 and Sec4 bound Mss4, while Rab2, Rab4, Rab5, Rab6, Rab11, c-H-ras and RalB did not. Mss4 also did not bind Rab9 or RhoC (data not shown). MBP did not bind any of these proteins (data not shown). The lack of detectable Mss4 binding to some of the GTPases was most likely not due to the amount of protein loaded, as can be seen by comparing the Coomassie brilliant blue staining of proteins which do and do not bind Mss4 (Figure 6A, compare Rab11 with Rab1b in bottom and top panels). Furthermore, Mss4 binding activity did not appear to correlate with the protein's ability to bind radiolabeled GTP. For instance, Sec4, Ypt1 and Rab3a efficiently bound both [α-^{32}P]GTP and Mss4, while Rab2, Rab4, Rab5, Rab6 and Rab11 efficiently bound radiolabeled GTP but not Mss4 (Figure 6A). In addition, Rab1b, Rab8 and Rab10 bound Mss4 avidly, yet were not efficient in binding radiolabeled GTP (Figure 6A and B). These data demonstrate that Mss4 selectively binds to a subset of the Rab proteins in the overlay assay.

Mss4 specifically co-precipitates with Rab3a in a brain extract

In order to confirm that Mss4 selectively interacts with this subset of Rabs in the cell, we performed immunoprecipitation experiments using brain extracts. Antibodies directed against Rab3a and Rab5 were used, two representative GTPases which bind and do not bind Mss4 in the overlay assay, respectively. Endogenous, Rab3a or Rab5 was immunoprecipitated from a detergent-solubilized, total brain extract and immunoprecipitates were run on SDS−PAGE and Western blotted for the Mss4 protein. Mss4 was found to co-precipitate specifically with Rab3a but not Rab5 (Figure 7A).

Mss4 and Rab3a form a stable complex in the absence of guanine nucleotides

To investigate further the interaction of Mss4 with Rab3a, we wished to determine the nucleotide dependence of this association. Previous studies had demonstrated that yeast Ras2 and its GEF, Cdc25, could form a stable complex *in vitro*, and that this complex was disrupted by the addition of either GDP or GTP (Lai *et al.*, 1993). We therefore incubated purified recombinant Rab3a with histidine-tagged Mss4 (His−Mss4) in the absence or presence

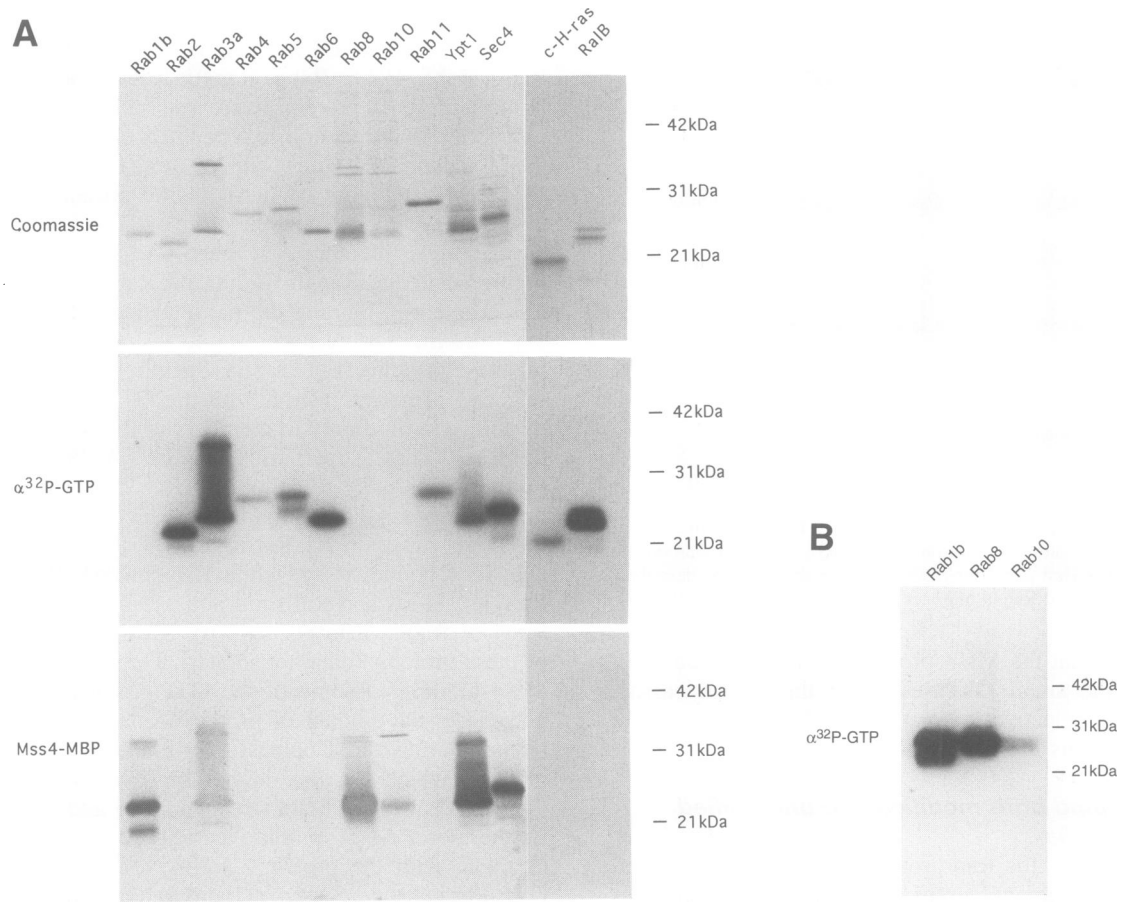

Fig. 6. Mss4 only binds some members of the Rab family of GTPases. (**A**) Recombinant Rabs were separated by SDS−PAGE in three identical gels. One gel was stained for total protein by Coomassie brilliant blue (top panel). The two other gels were transferred to nitrocellulose and overlaid with [α-^{32}P]GTP (middle panel) or with Mss4−MBP (bottom panel) followed by antibodies to detect bound Mss4−MBP and secondary antibodies conjugated to alkaline phosphatase. [α-^{32}P]GTP blot was exposed to film for 15 min. (**B**) [α-^{32}P]GTP binding for Rab1b, Rab8 and Rab10 required a longer exposure time of 18 h with an intensifying screen.

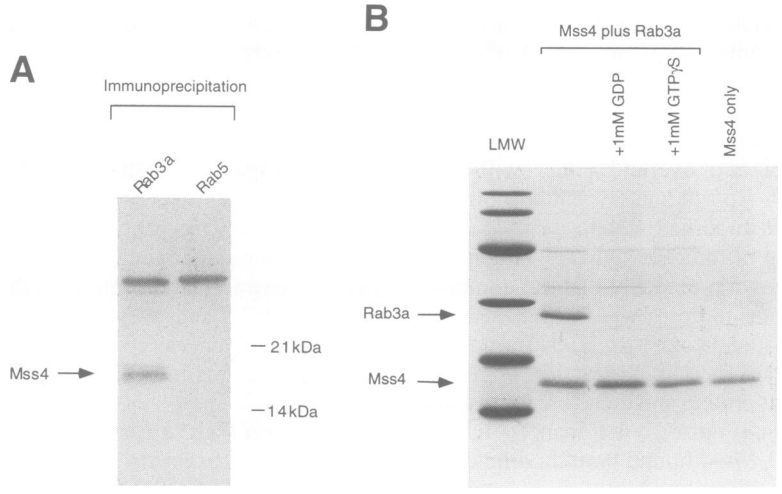

Fig. 7. Mss4 forms a complex with Rab3a. (**A**) Mss4 co-precipitates with Rab3a but not Rab5 from a brain extract. Rab3a or Rab5 were immunoprecipitated from a 1% Triton X-100 soluble total brain extract (see Materials and methods) with Rab3a- or Rab5-specific monoclonal antibodies. Immunoprecipitates were run on SDS−PAGE and Western blotted with antibodies directed against Mss4. Mss4 protein is only detected in the Rab3a immunoprecipitate. The higher immunoreactive band visible in both lanes is the light chain of the IgGs. (**B**) A complex between recombinant Rab3a and Mss4 is disrupted by the addition of guanine nucleotides. Recombinant Rab3a and His−Mss4 were incubated either in the absence of guanine nucleotides (lane 2) or in the presence of 1 mM GDP (lane 3) or 1 mM GTPγS (lane 4). The His−Mss4 protein was then precipitated using Ni^{2+} resin and the bound material was run on SDS−PAGE and stained by Coomassie brilliant blue. The first lane contains low molecular weight markers of 97, 67, 45, 31, 21 and 14 kDa. The last lane is His−Mss4 incubated in the absence of Rab3a.

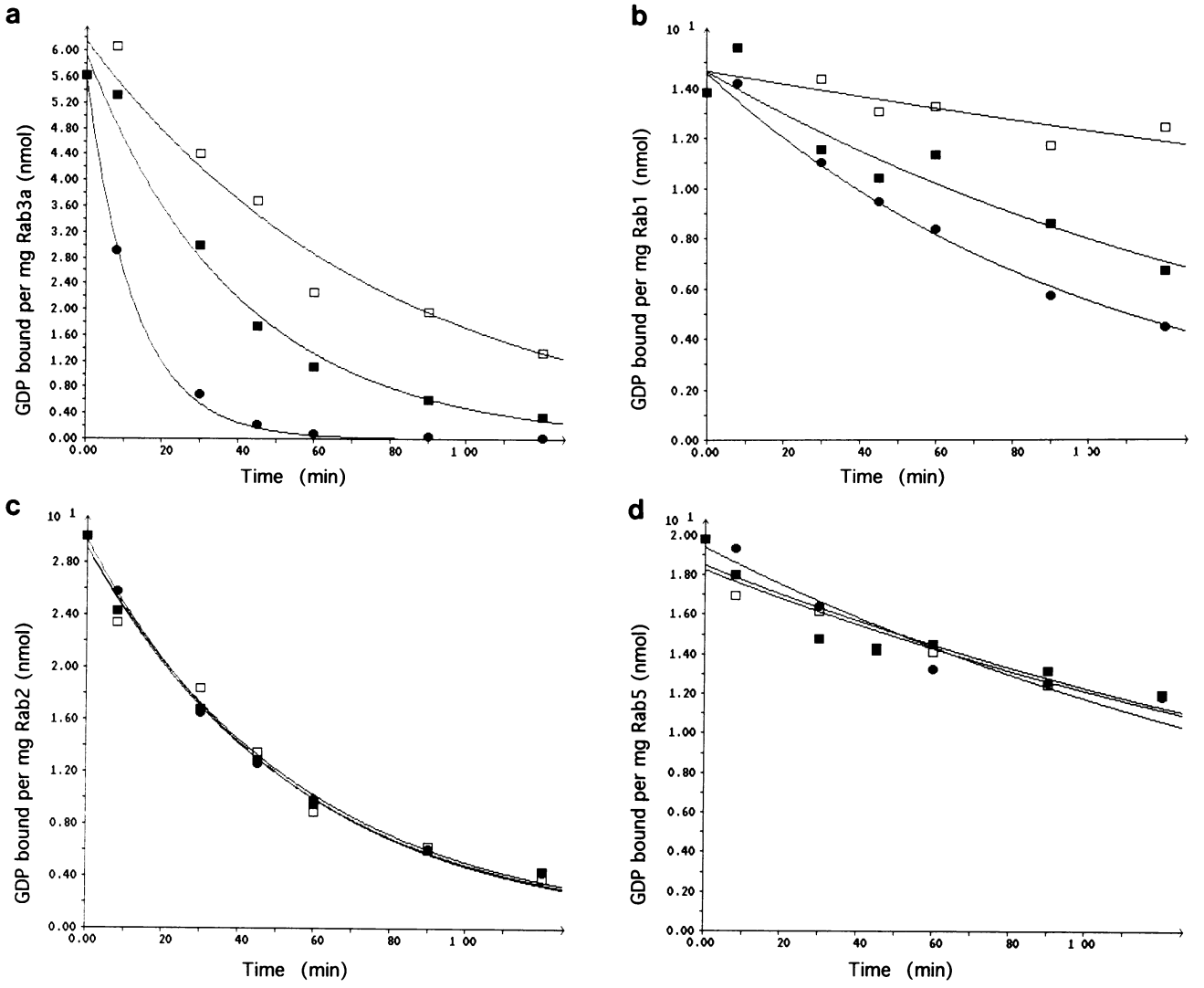

Fig. 8. Mss4 stimulates GDP release only from the Rabs which it binds in the gel overlay. (**a**) Mss4 stimulates release of [^{3}H]GDP from Rab3a in a concentration-dependent manner. At 2- (■) and 8-fold (●) molar excess Mss4 relative to Rab3a (100 nM), a 2- and 6-fold increase, respectively, in GDP release was observed compared with the vector control (□). (**b**) Mss4 used at 12- (■) and 24-fold (●) molar excess relative to Rab1 (25 nM), stimulates ^{3}H-GDP release at 3.5- and 5.5-fold relative to vector control (□). (**c**) and (**d**) Mss4 has no effect on GDP release from Rab2 or Rab5 (25 nM) when used at the same concentrations as in (b). Data points for each curve in (a)–(d) are representative of three independent experiments.

of 1 mM GDP or 1 mM GTPγS. The His–Mss4 protein was then precipitated with Ni^{2+} resin and the associated proteins were visualized by Coomassie brilliant blue staining of a SDS–polyacrylamide gel (Figure 7B). In the absence of added guanyl nucleotides, Rab3a co-precipitates with Mss4 in a complex that appears to have a stoichiometery of 1:1. However, when either GDP or GTPγS is added to the incubation, Rab3a no longer co-precipitates with Mss4 (Figure 7B). These results suggest that Mss4 forms a stable complex with the nucleotide-free Rab protein and are consistent with a role of Mss4 as a GEF.

Mss4 stimulates GDP release only from the Rabs which bind Mss4 in the overlay assay

We next tested whether Mss4 can displace GDP selectively from the Rab proteins which it binds in the overlay assay. Mss4 was incubated for 2 h with either [^{3}H]GDP-labeled Rab1 and Rab3a, which bind Mss4 in the overlay assay, or Rab2 and Rab5 which do not. At specific time points protein-associated counts were determined using a filter binding assay (Kabcenell *et al.*, 1990). Approximately equal amounts of active GTPase were used for each assay based on GTP binding activity in solution. Mss4 was shown to stimulate the intrinsic release rate of [^{3}H]GDP from Rab1 and Rab3a in a concentration-dependent manner (Figure 8a and b). For both Rab3a and Rab1, we observed up to a 6-fold stimulation over the instrinsic rate using an 8-fold molar excess of Mss4 relative to the Rab protein. Mss4 had no effect on the release of GDP from Rab2 or Rab5 using the same concentrations (Figure 8c and d).

Mss4 promotes the association of GTPγS onto recombinant Rab3a

If Mss4 is a GEF, in addition to promoting GDP release, it should also enhance the association of GTP onto the Rabs. The effect of Mss4 on the binding of GTP to Rab proteins was not addressed in our previous study (Burton *et al.*, 1993). We therefore investigated whether Mss4

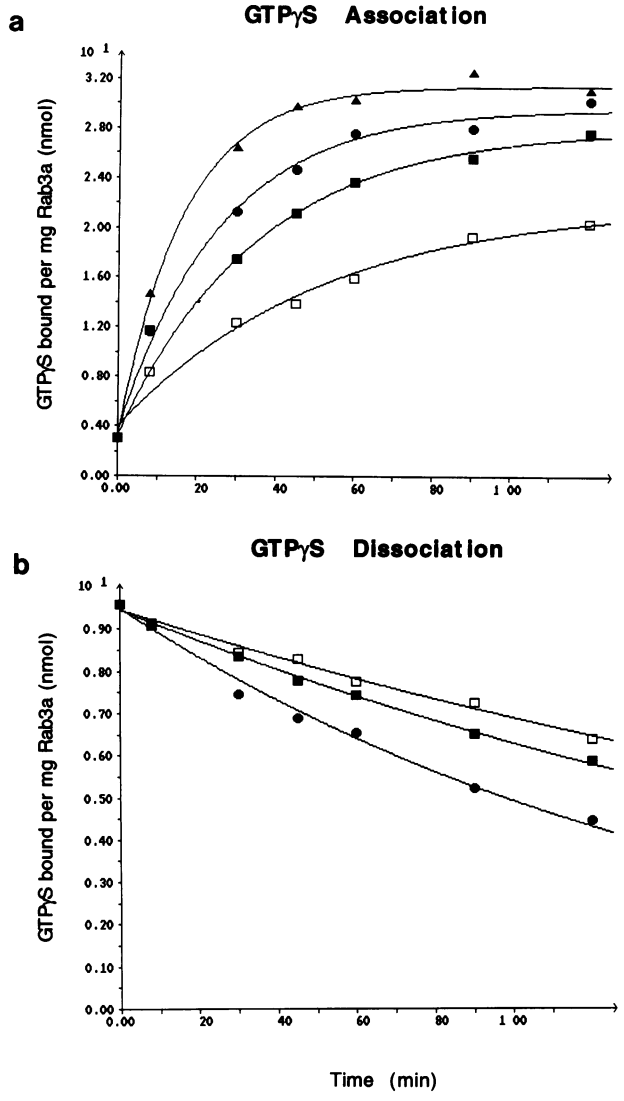

Fig. 9. Mss4 promotes both the association and dissociation of GTPγS on Rab3a. (**a**) Mss4 present at a concentration of 2-(■), 4-(●) and 8-fold (▲) molar excess relative to Rab3a (100 nM) promotes a 1.3-, 2- and 3-fold increase respectively, in the association of GTPγS relative to the vector control (□). (**b**) Mss4 present at a concentration of 2-(■), and 8-fold (●) molar excess stimulates the release of [^{35}S]GTPγS 1.2- and 2-fold, respectively (vector control □). Data points for each curve are representative of three independent experiments.

could also promote the association of GTPγS onto Rabs. In the presence of an 8-fold molar excess of Mss4, we observed a 3-fold increase over the spontaneous association rate of [^{35}S]GTPγS onto Rab3a (Figure 9a). The level of GTPγS bound plateaus at ~0.8 mol/mol of Rab3a after a 1 h incubation. We also observed a stimulation of GTPγS binding onto Rab1 but not Rab2 with Mss4 (data not shown). In addition to promoting GDP release, Mss4 was also capable of promoting GTP-release. With an 8-fold molar excess of Mss4 we observed a 2-fold stimulation in [^{35}S]GTPγS-release compared with the release observed in the absence of Mss4 (Figure 9b). Mss4 is therefore more efficient in promoting the release of GDP than GTPγS (6-fold versus 2-fold) and is consistent with the ability of Mss4 to promote GTPγS association. Together these results would predict that Mss4 enhances the level

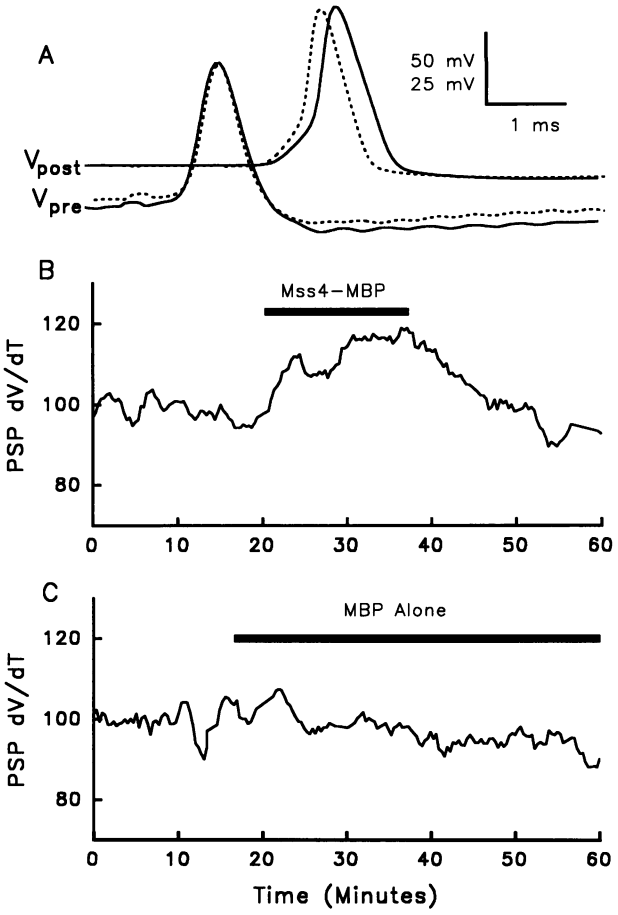

Fig. 10. Injection of Mss4–MBP stimulates release of neurotransmitter at the squid giant synapse. (**A**) Electrophysiological traces of pre-synaptic (V_{pre}) and post-synaptic (V_{post}) potentials before (solid lines) and after (dashed lines) Mss4–MBP injection. (**B**) The slope of the rising phase of the post-synaptic potentials (PSP dV/dT) evoked by pre-synaptic action potentials increased during injection of Mss4–MBP (during bar), indicating an increase in transmitter release. (**C**) Injection of MBP alone (during bar) had no effect on PSP dV/dT.

of GTP-bound form of a subset of Rab proteins. In summary, these data confirm the Mss4 binding results observed in the overlay assay and suggest that Mss4 binding influences the function of these Rab proteins.

Mss4 facilitates neurotransmitter release when injected into the squid giant nerve terminal

The ability of Mss4 to bind selected Rab proteins and to affect their interaction with guanyl nucleotides suggests that Mss4 may influence vesicular traffic governed by these Rab proteins. In particular, we investigated whether Mss4 could affect the exocytosis of synaptic vesicles, a process in which Rab3a is implicated (Fischer von Mollard *et al.*, 1991; Hess *et al.*, 1993). Mss4–MBP was injected into the pre-synaptic terminal of the squid giant synapse while measuring the post-synaptic potential (PSP) evoked by pre-synaptic action potentials as a measure of synaptic transmission. Under conditions of low external calcium concentration (3 mM), injections of Mss4–MBP increased synaptic transmission evoked by pre-synaptic action potentials (Figure 10). This increase was quantified by measuring the intital rate of rise of the PSP (dV/dT), which is correlated with the amount of transmitter released

by exocytosis (Adler *et al.*, 1991). The rate of rise of the evoked PSP was 18.1 ± 3.0% (mean ± SEM, $n = 5$) greater than baseline measurements made prior to injection. As a control, comparable amounts of MBP alone were injected into the terminal and found to have no effect on transmission (−6.3 ± 9.9%, $n = 4$). The difference between responses to Mss4−MBP and MBP injections is statistically significant (t-test, $P<0.05$). Thus, Mss4 increases synaptic vesicle exocytosis in response to presynaptic action potentials. This suggests that Mss4 may also affect other steps of membrane traffic through its selective interaction with specific members of the Rab subfamily.

Discussion

This study provides compelling evidence that Mss4 is an accessory protein for specific members of the Rab subfamily of GTPases and supports its role as a GEF. Previous results had suggested a role for Mss4 in vesicular transport based on its isolation as a suppressor of a yeast secretory mutant (Burton *et al.*, 1993). Here we describe the biochemical and functional properties of Mss4. We show that the interaction of Mss4 with the small GTPases is highly specific, and is selective for a subset of Rab proteins which belong to the same subfamily branch. Furthermore, we demonstrate that Mss4 can influence vesicular traffic in a secretion assay which measures neurotransmitter release mediated by synaptic vesicle exocytosis.

Our previous work suggested that Mss4 is an accessory protein for the Rabs based on two findings: (i) suppression of the secretory defect in yeast *sec4-8* cells, a strain in which Sec4 is mutated and (ii) a direct interaction with Rab3a, Ypt1 and Sec4 but not yeast Ras2 in a GDP release assay (Burton *et al.*, 1993). However, in this intial study, the specificity of the interaction between Mss4 and the Rab GTPases was not addressed. Here, we have complemented these earlier findings by demonstrating a striking specificity in the interaction of Mss4 with Rab proteins in tissue extracts. Closer scrutiny, using a variety of binding assays, revealed that Mss4 bound only a subset of these Rab proteins. The ability to detect these interactions by a gel overlay assay, co-precipitation and a direct binding assay with recombinant Mss4 and Rab3a also suggest they are of high affinity.

Two of the three major Mss4 binding proteins present in a brain extract have been identified as Rab3 and Rab1, in agreement with the binding observed using recombinant proteins. The identity of the third Mss4 binding GTPase, present in both brain and kidney, is currently unknown. We know that this protein is not Rab8 or Rab10 because it migrates differently from these two proteins in a two-dimensional gel system (L.Huber *et al.*, 1994). However, the present data would strongly suggest that this GTPase is also a Rab protein. The number of Mss4 binding Rabs detected using recombinant GTPases is greater than the number of such GTPases detected in tissue extracts. The most probable explanation for this discrepancy is that some of the Mss4 binding Rabs are present in the tissue extracts at levels which are below the sensitivity of this assay.

Figure 11 displays a tree of the GTPases from the Ras superfamily which were tested in this study. The tree was

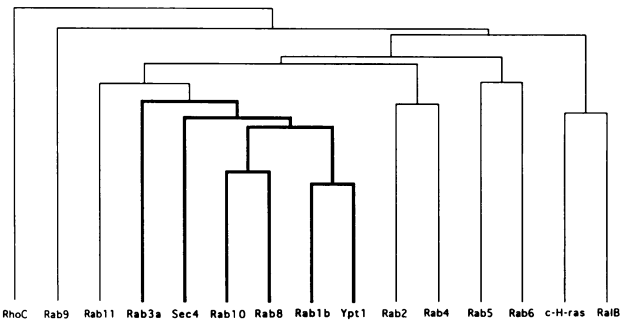

Fig. 11. Dendrogram or tree representation of clustering relationships for the proteins of the Ras superfamily which were tested for Mss4 binding activity. All Mss4 binding Rabs are found on the same branch (bold). The clustering of the GTPases in the tree is based on sequence identity between the proteins. Distances along the vertical axis are proportional to the difference between amino acid sequences for the given proteins. Distances along the horizontal axis are not significant.

constructed based on amino acid sequence homologies between the proteins. Remarkably, the proteins which we have found to bind Mss4 all belong to the same branch of the Rab subfamily (bold), and all are involved in membrane traffic from the endoplasmic reticulum to the plasmalemma (Simons and Zerial, 1993).

We also demonstrate that endogenous Mss4 in a brain extract co-precipitates with at least one member of this branch, Rab3a, further suggesting that Mss4 protein selectively interacts with this subset of Rabs *in vivo*. Taken together, these observations support the conclusion that the Mss4−GTPase interaction is highly specific and functionally relevant.

Evidence that these interactions influence vesicular traffic was previously suggested by the ability of Mss4 to suppress the secretory defect of the *sec4-8* mutation and now by its property to enhance neurotransmitter release when injected into the squid nerve terminal. This effect on release is of special interest, especially in light of the fact that very few proteins have been shown to be stimulatory in this system (Llinas *et al.*, 1985). Although the mechanism of this stimulation is presently unclear, it is conceivable that Mss4 produces this effect by acting on Rab3a.

The selective association of Mss4 with members of one branch of the Rab subfamily, which are grouped according to sequence homology, suggests that Mss4 is recognizing a sequence that is unique to these proteins. At present, we do not know which domain or domains of these Rabs are involved in this interaction. The data, however, suggest that prenylation of these Rabs is not necessary for Mss4 binding because the recombinant proteins used in the overlay and GDP release assays were purified from bacteria and therefore unprenylated. We have also found that a truncated form of Rab3a which is missing the C-terminal domain is still capable of binding Mss4 in the overlay assay (J.L.Burton and P.De Camilli, unpublished results). Likewise, the prenylation of the Rabs does not interfere with Mss4 association as can be seen by its ability to bind to modified Rab3a isolated from brain membranes. We are currently investigating which domain(s) of these Rab proteins may be important for their association with Mss4.

What is the function of Mss4? Our previous findings and the similarity to the yeast protein Dss4, suggested that Mss4 facilitates Rab function. Furthermore, the ability

of Mss4 to stimulate GDP release raised the possibility that Mss4 may assist the Rab protein by acting as a GEF (Burton *et al.*, 1993; Moya *et al.*, 1993). These findings are now corroborated by the demonstration that Mss4 enhances GTP binding onto relevant Rab proteins. In addition, we show that Mss4 has a higher relative affinity for the nucleotide-free state of Rab3a, similar to the interactions observed between the yeast GEF, Cdc25 and Ras2 (Lai *et al.*, 1993) and between RCC1 and Ran (Bischoff and Ponstingl, 1991). This observation predicts that Mss4 stabilizes the nucleotide-free conformation of the Rabs to permit GDP–GTP exchange. Together these properties suggest that Mss4 is a GEF.

Recent studies using either permeabilized or reconstituted cell systems have demonstrated that GDP–GTP exchange onto Rab proteins occurs after these proteins have become associated with their appropriate acceptor membrane (Novick and Garrett, 1994; Soldati *et al.*, 1994; Ullrich *et al.*, 1994). These results have suggested that the specificity of interaction between the Rab and its target membrane may be provided by an as yet to be characterized GDI displacement factor (GDF) (Soldati *et al.*, 1994). GDP–GTP exchange may subsequently be promoted by a more promiscuous protein. Mss4, a partially soluble protein, could be such a promiscuous factor for a variety of Rab proteins which participate in vesicular transport steps from the endoplasmic reticulum to the plasma membrane. Clearly, the validity of such a model requires further experimentation. Future studies using Mss4 in *in vitro* reconstitution assays with relevant Rabs may give insight on precisely how Mss4 facilitates Rab function.

What has become clear from this report, is that Mss4 can function as an accessory protein that is specific for certain members of the Rab subclass of GTPases. Furthermore, all of the properties displayed by Mss4 are consistent with it being a GEF for these Rabs. By assisting in the function of selected Rab proteins, Mss4 may influence the rate of transit through a specific set of vesicular transport steps.

Materials and methods

Recombinant proteins

Mss4–MBP and MBP were produced as described previously (Burton *et al.*, 1993). Mss4 and the vector control used in the filter assays were made using the T7 RNA polymerase system as described previously (Burton and De Camilli, 1994), such that no additional amino acids were added to the Mss4 coding sequence. His–Mss4 was produced using the QIAexpressionist Kit (Qiagen, Chatsworth, CA). The entire coding sequence of Mss4 was subcloned into the pQE-60 vector using the restriction sites *Nco*I and *Bgl*II (New England Biolabs, Beverly, MA). This construct adds six histidine residues to the C-terminus of the Mss4 protein. The His–Mss4 protein was purified using Ni-NTA resin according to manufacturers instructions (Qiagen, Chatsworth, CA). Rab8 cDNA (generous gift from Dr Marino Zerial) was amplified by the polymerase chain reaction (Sambrook *et al.*, 1989) using the 5′-primer CTAGCTAGCATGGCGAAGACCTACGAT and the 3′-primer CGCGATCCGTGTTCCCAGAAGAAC. The Rab10 cDNA (generous gift from Dr Richard Scheller) was amplified in an analogous fashion using the 5′-primer CTAGCTAGCATGGCGAAGAAGACGTAC and the 3′-primer CGGGATCCGAGCGCTCAGCAGCA. These primers add a *Nhe*I and *Bam*HI restriction endonuclease sites onto the 5′ and 3′ end, respectively, of both Rab8 and Rab10 coding sequences and were subcloned into the pET11d vector using these sites (Sambrook *et al.*, 1989; Studier *et al.*, 1990). Both recombinant Rab8 and Rab10 contain three additional amino acids at their N-terminus (Met, Ala and Ser) resulting from coding sequence in the peT11d vector. Rab8 and Rab10 were both found in an insoluble fraction after cell sonication in TSD buffer (20 mM Tris–Cl pH 8.0, 100 mM NaCl, 1 mM DTT plus protease inhibitors). These pellets were washed using TSD buffer containing 5 M urea. The insoluble pellets containing the Rab proteins were used for overlay assays (see below). The following recombinant GTPases were generous gifts: Sec4 and Ypt1 from Dr Peter Novick (Yale University, New Haven, CT), Rab1b and Rab2 from Dr William Balch (Scripps, La Jolla, CA), Rab3a from Dr Thomas Südhof (Texas Southwestern Medical Center, Houston, TX), Rab4 from Dr Ira Mellman (Yale University, New Haven, CT), Rab5 from Dr Marino Zerial (EMBL, Heidelberg, Germany), Rab6 from Dr Bruno Goud (Institute Curie, Paris, France), Rab9 from Dr Suzanne Pfeffer (Stanford University, Stanford, CA), Rab11 from Dr James Goldenring (Medical College of Georgia, Augusta, GA), c-H-ras and RalB from Dr Robert Weinberg (Whitehead Institute, Boston, MA), c-H-ras from Dr Dafna Bar Sagi (Cold Spring Harbor, New York), RhoC from Dr Pierre Chardin (CNRS, Valbonne, France).

Antibodies

Rabbit polyclonal antiserum directed against the Mss4 protein was produced by injection of purified, recombinant Mss4–MBP. Mss4 antibodies were affinity purified using recombinant Mss4–MBP bound to immobilon strips (Sharp *et al.*, 1993). Monoclonal antibodies against Rab3a and Rab5 were generous gifts from Dr Reinhardt Jahn (Yale University, New Haven, CT) and Rab1b monoclonal antibodies were a kind gift from Dr William Balch (Scripps Institute, La Jolla, CA). Affinity-purified polyclonal antibodies against the MBP were purchased from New England Biolabs (Beverly, MA).

Cell fractionation

Total tissue homogenates were obtained by homogenization in buffer A (100 mM NaCl, 10 mM HEPES pH 7.4, 4 μg/ml leupeptin, antipain, aprotonin and pepstatin A, 0.4 mM PMSF and 10 mM benzamidine). Brain and kidney soluble (S) and particulate (P) fractions were prepared by spinning a post-nuclear supernate at 50 000 r.p.m. for 2 h in a Ti70 rotor in a Beckman ultracentrifuge (Fullerton, CA) at 4°C. The soluble fraction (S) is also referred to as cytosolic extract in the text. A membrane fraction was prepared by resuspending the 50 000 r.p.m. pellet in buffer A plus 1% Triton X-100, pelleting again at 50 000 r.p.m. and taking the Triton X-100 soluble material. Total 1% Triton X-100 soluble brain extract was prepared as for the membrane fraction except the first 50 000 r.p.m. spin was replaced by a 2700 r.p.m. spin for 10 min. Subcellular fractionation of rat brain homogenate to yield fractions P1, P2, P3 and S3 was performed as described previously (Huttner *et al.*, 1983).

Electrophoretic techniques

SDS–PAGE and Western blotting were performed essentially as described by Laemmli (1970) and transferred to nitrocellulose according to Towbin *et al.* (1979). Two-dimensional electrophoresis was performed as described by O'Farrell (1975). For overlay with Mss4–MBP, 500 μg of protein was loaded per isoelectric focusing (IEF) gel, in all other cases, 250 μg of protein was used per IEF gel. The first dimension solution consisted of 1.6% Biolyte 5/7 ampholyte (Bio-Rad, Hercules, CA), 0.4% 3/10 ampholyte (Pharmacia, Piscataway, NJ), 4% acrlyamide, 2% NP-40 (Calbiochem, La Jolla, CA), 9.2 M urea (American Bioanalytical, Natick, MA), for a pH gradient from 4 to 7 pH units. Proteins were separated in the second dimension using SDS–PAGE (12.5%).

Overlay assays

For overlay with [α^{32}P]GTP, proteins were transferred to nitrocellulose using $NaCO_3$ buffer (10 mM $NaHCO_3$ and 3 mM NaH_2CO_3). [α-^{32}P]GTP overlay was performed as a modification of Lapetina and Reep (1987) essentially as described (Huber *et al.*, 1993). To detect Mss4 binding proteins, nitrocellulose blots were incubated for 1 h in blocking buffer (20 mM Tris–Cl pH 7.5, 150 mM NaCl, 5% milk, 0.1% Tween-20 and 0.02% sodium azide) and then incubated with blocking buffer containing either Mss4–MBP or MBP alone at a concentration of 10 μg/ml for 2 h at 4°C. Unbound protein was removed by washing with blocking buffer. Bound protein was detected using MBP antibodies. Blots were then processed as standard Western blots (see above). Secondary antibodies coupled to alkaline phosphatase (Boeringher Mannheim, Indianapolis, IN) or horseradish peroxidase (Sigma, St Louis, MO) were used and processed according to manufacturer's instructions.

Affinity purification and co-precipitation procedures

Mss4–MBP or MBP were incubated with amylose resin for 2 h at 4°C in 150 mM NaCl and 10 mM HEPES pH 7.4. Resin was then washed

in the same buffer and incubated with soluble brain extract (S) as prepared above for 2 h at 4°C. Resin was washed several times with the same buffer and resuspended in Laemmli sample buffer for SDS–PAGE and [α-^{32}P]GTP overlay as described above. Immunoprecipitation was performed as cited (Reetz *et al.*, 1991). Experiments addressing the nucleotide dependence of the Rab–Mss4 complex were done as follows: 15 μg of His–Mss4 was incubated with 15 μg of purified Rab3a for 2 h at 4°C in 140 mM KCl, 10 mM HEPES pH 8 and 1% Triton X-100 without the addition of nucleotides or with the addition of 1 mM GDP or 1 mM GTPγS (Boehringer Mannheim, Indianapolis, IN). A negative control of His–Mss4 without Rab3a was also performed. Then 50 μl of Ni-NTA resin (Qiagen, Chatsworth, CA) in the same buffer was added and incubated for an additional hour at 4°C. Resin was washed four times with the same buffer and then boiled in 1× Laemmlli buffer loaded on a 15% SDS–polyacrylamide gel and stained with Coomassie brilliant blue.

Filter binding assays

The assay for measuring Mss4 effects on [^{3}H]GDP from the recombinant Rab proteins was performed as described previously with the exception that a 100 nM rather than 25 nM concentration of the active GTPase was used for Rab3a (Burton *et al.*, 1993; Burton and De Camilli, 1994). [^{35}S]GTPγS release from the recombinant Rab3a was done in an analogous fashion but the loading was done using 0.02 μM [^{35}S]GTPγS and 0.18 μM GTPγS instead of 0.2 μM [^{3}H]GDP and the release assay was done in the presence of 0.5 mM cold GTPγS rather than GDP. For [^{35}S]GTPγS association, a 100 nM concentration of active GTPase in the presence of 14 nM [^{35}S]GTPγS and 0.4 μM cold GTPγS was incubated for 2 h in the presence or absence of Mss4. Aliquots were removed at specific time points, rapidly filtered and protein associated counts were determined as in the case of the release assay.

Electrophysiology

Pressure injection of proteins into the pre-synaptic terminal of squid (*Loligo pealei*) stellate ganglia was performed as described by Bommert *et al.* (1993). Briefly, two microelectrodes were inserted into the pre-synaptic neuron; one in the axon to deliver current pulses to evoke action potentials, and the second directly into the nerve terminal to record the pre-synaptic action potential and to microinject the protein solutions by pressure injection (Picospritzer, General Valve Co., NJ). A third microelectrode, in the post-synaptic cell, recorded the post-synaptic potential (PSP) resulting from neurotransmitter released in response to the pre-synaptic action potential. The initial rate of rise of the PSP was measured as an indication of the amount of neurotransmitter released (Adler *et al.*, 1991). Electrophysiological traces were stored and analyzed by a program, Tomahacq, written by T.A.Goldthorpe (University of Toronto). External saline contained 466 mM NaCl, 3 mM $CaCl_2$, 61 mM $MgCl_2$, 10 mM KCl, 3 mM $NaHCO_3$, 10 mM HEPES.

The pre-synaptic stimulating electrode and the post-synaptic electrode were filled with 3 M KCl. The pre-synaptic recording electrode was filled with a carrier solution containing 250 mM potassium isethionate, 100 mM taurine, 50 mM HEPES and 100 mM KCl with 1.2 or 1.4 mg/ml Mss4–MBP or 2.1 mg/ml MBP. Coinjected with this solution was 100 μM dextran conjugated to fluorescein isothiocyanate (FITC-dextran M_r 20 000, Molecular Probes, Eugene, OR) as an indicator of injection. The intensity of the fluorescence of the FITC–dextran was monitored with a Nikon Optiphot Noran Odyssey real-time confocal microscope and processed with Image-1 software. Based on our estimates of terminal path length, these intensity values could be roughly converted into dye concentration to estimate the final concentration of intracellular injected protein ranging from 140 to 1240 nM in different experiments.

Generation of GTPase tree

A tree (dendrogram) of the GTPases which were tested in the Mss4 overlay assay was generated using the PileUp program from the Genetic Computer Group (Madison, WI). This program generates a multiple sequence alignment using the progressive alignment of Feng and Doolittle (1987). The clustering strategy represented by the dendrogram is generated using the UPGMA (unweighted pair group method using arithmetic averages) algorithm (Sneath and Sokal, 1973).

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References

Adler,E.M., Augustine,G.J., Duffy,S.N. and Charlton,M.P. (1991) *J. Neurosci.*, **11**, 1496–1507.
Balch,W.E. (1990) *Trends Biochem. Sci.*, **15**, 473–477.
Bennett,M.K. and Scheller,R.H. (1993) *Proc. Natl Acad. Sci. USA*, **90**, 2559–2563.
Bischoff,F.R. and Ponstingl,H. (1991) *Proc. Natl Acad. Sci. USA*, **267**, 10830–10834.
Boguski,M.S. and McCormick,F. (1993) *Nature*, **366**, 643–654.
Bommert,K., Charlton,M.P., DeBello,W.M., Chin,G.J., Betz,H. and Augustine,G.J. (1993) *Nature*, **363**, 163–165.
Bourne,H.R. (1988) *Cell*, **53**, 669–671.
Bourne,H.R., Sanders,D.A. and McCormick,F. (1990) *Nature*, **348**, 125–132.
Brennwald,P., Kearns,B., Champion,K., Keränen,S., Vytas,B. and Novick,P. (1994) *Cell*, in press.
Brondyk,W.H., McKiernan,C.J., Burstein,E.S. and Macara,I.G. (1993) *J. Biol. Chem.*, **268**, 9410–9415.
Burstein,E.S. and Macara,I.G. (1992) *Proc. Natl Acad. Sci. USA*, **89**, 1154–1158.
Burton,J.L. and De Camilli,P. (1994) *Methods Enzymol.*, in press.
Burton,J., Roberts,D., Montaldi,M., Novick,P. and De Camilli,P. (1993) *Nature*, **361**, 464–467.
Feng,D.F. and Doolittle,R.F. (1987) *J. Mol. Evol.*, **25**, 351–360.
Ferro-Novick,S. and Novick,P. (1993) *Annu. Rev. Cell Biol.*, **9**, 575–599.
Fischer von Mollard,G., Südhof,T.C. and Jahn,R. (1991) *Nature*, **349**, 79–81.
Geppert,M., Bolshakov,V.Y., Siegelbaum,S.A., Takei,K., De Camilli,P., Hammer,R.E. and Südhof,T. (1994) *Nature*, **369**, 493–497.
Goud,B., Salminen,A., Walworth,N.C. and Novick,P.J. (1988) *Cell*, **53**, 753–768.
Hess,S.D., Doroshenko,P.A. and Augustine,G.J. (1993) *Science*, **259**, 1169–1172.
Huber,L.A., de Hoop,M.J., Dupree,P., Zerial,M., Simons,K. and Dotti,C. (1993) *J. Cell Biol.*, **123**, 47–55.
Huber,L.A. *et al.* (1994) *Proc. Natl Acad. Sci. USA*, **91**, 7874–7878.
Huttner,W.B., Schiebler,W., Greengard,P. and De Camilli,P. (1983) *J. Cell Biol.*, **96**, 1374–1388.
Jena,B.P., Brennwald,P., Garrett,M.D., Novick,P. and Jamieson,J.D. (1992) *FEBS Lett.*, **309**, 5–9.
Johnston,P.A., Archer,B.T., Robinson,K., Mignery,G.A., Jahn,R. and Südhof,T.C. (1991) *Neuron*, **7**, 101–109.
Kabcenell,A.K., Goud,B., Northup,J.K. and Novick,P.J. (1990) *J. Biol. Chem.*, **265**, 9366–9372.
Laemmli,U.K. (1970) *Nature*, **227**, 680–685.
Lai,C.C., Boguski,M., Broek,D. and Powers,S. (1993) *Mol. Cell. Biol.*, **13**, 1345–1352.
Lapetina,E. and Reep,B. (1987) *Proc. Natl Acad. Sci. USA*, **84**, 2261–2265.
Li,C., Takei,K., Geppert,M., Daniell,L., Stenius,K., Chapman,E.R., Jahn,R., De Camilli,P. and Südhoff,T.C. (1994) *Neuron*, **13**, 1–20.
Llinas,R., McGuinness,T.L., Leonard,C.S., Sugimori,M. and Greengard,P. (1985) *Proc. Natl Acad. Sci. USA*, **82**, 3035–3039.
Manser,E., Leung,T., Salihuddin,H., Zhao,Z.S. and Lim,L. (1994) *Nature*, **367**, 40–46.
McPherson,P.S., Czernik,A.J., Chilcote,T.J., Onofri,F., Benfenati,F., Greengard,P., Schlessinger,J. and De Camilli,P. (1994) *Proc. Natl Acad. Sci. USA*, **91**, 6486–6490.
Moya,M., Roberts,D. and Novick,P. (1993) *Nature*, **361**, 460–463.

Novick,P. and Brennwald,P. (1993) *Cell*, **75**, 597–601.
Novick,P. and Garrett,M.D. (1994) *Nature*, **369**, 18–19.
Nuoffer,C. and Balch,W.E. (1994) *Annu. Rev. Biochem.*, **63**, 949–990.
O'Farrell,P.H. (1975) *J. Biol. Chem.*, **250**, 4007–4021.
Pfeffer,S.R. (1992) *Trends Cell Biol.*, **2**, 41–46.
Reetz,A., Solimena,M., Matteoli,M., Folli,F., Takei,K. and De Camilli,P. (1991) *EMBO J.*, **10**, 1275–1284.
Salminen,A. and Novick,P. (1987) *Cell*, **49**, 527–538.
Sambrook,J., Fritsch,E.F. and Maniatis,T. (1989) *Molecular Cloning: A Laboratory Manual.* Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
Sasaki,T., Kikuchi,A., Araki,S., Hata,Y., Isomura,M., Kuroda,S. and Takai,Y. (1990) *J. Biol. Chem.*, **265**, 2333–2337.
Seabra,M.C., Goldstein,J.L., Südhof,T.C. and Brown,M.S. (1992) *J. Biol. Chem.*, **267**, 14497–14503.
Sharp,A.H., McPhearson,P.S., Dawson,T.M., Aoki,C., Campbell,K.P. and Synder,S.H. (1993) *J. Neurosci.*, **13** , 3051–3063.
Shirataki,H., Kaibuchi,K., Sakoda,T., Kishida,S., Yamaguchi,T., Wada,K., Miyazaki,M. and Takai,Y. (1993) *Mol. Cell. Biol.*, **13**, 2061–2068.
Simons,K. and Zerial,M. (1993) *Neuron*, **11**, 789–799.
Sneath,P.H.A. and Sokal,R.R. (1973) *Numerical Taxonomy.* W.H.Freeman and Company, San Francisco, CA.
Soldati,T., Riederer,M.A. and Pfeffer,S.R. (1993) *Mol. Biol. Cell*, **4**, 425–434.
Soldati,T., Shapiro,A.D., Svejstrup,A.B.D. and Pfeffer,S.R. (1994) *Nature*, **369**, 76–78.
Strom,M., Vollmer,P., Tan,T.J. and Gallwitz,D. (1993) *Nature*, **361**, 736–739.
Studier,W.F., Rosenberg,A.H., Dunn,J.J. and Dubendorff,J.W. (1990) *Methods Enzymol.*, **185**, 61–89.
Söllner,T., Bennett,M.K., Whiteheart,S.W., Scheller,R. and Rothman,J.E. (1993) *Cell*, **75**, 409–418.
Towbin,H., Staehelin,T. and Gorden,J. (1979) *Proc. Natl Acad. Sci. USA*, **76**, 4350–4354.
Ullrich,O., Stenmark,H., Alexandrov,K., Huber,L.A., Kaibuchi,K., Sasaki,T., Takai,Y. and Zerial,M. (1993) *J. Biol. Chem.*, **268**, 18143–18150.
Ullrich,O., Horiuchi,H., Bucci,C. and Zerial,M. (1994) *Nature*, **368**, 157–160.
Walworth,N.C., Brennwald,P., Kabcenell,A.K., Garrett,M. and Novick,P. (1992) *Mol. Cell. Biol.*, **12**, 2017–2028.
Yu,J., Rossi,G. and Ferro-Novick,S. (1993) *Nature*, **366**, 84–86.

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