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Two New Members of a Family of Ypt/Rab GTPase Activating Proteins

PROMISCUITY OF SUBSTRATE RECOGNITION*

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Monomeric GTPases of the Ras superfamily have a very slow intrinsic GTPase activity which is accelerated by specific GTPase-activating proteins. In contrast to Ras- and Rho-specific GTPase-activating proteins (GAPs) that have been studied in great detail, little is known about the functioning of GAPs specific for Ypt/Rab transport GTPases. We have identified two novel Ypt/Rab-GAPs because of their sequence relatedness to the three known GAPs Gyp1p, Gyp6p, and Gyp7p. Mdr1/Gyp2p is an efficient GAP for Ypt6p and Sec4p, whereas Msb3/Gyp3p is a potent GAP for Sec4p, Ypt6p, Ypt51p, Ypt31/Ypt32p, and Ypt1p. Although the affinity of Msb3/Gyp3p for its preferred substrate Sec4p is low (K_m = 154 μM), it accelerates the intrinsic GTPase activity of Sec4p 5 × 10^5-fold. Msb3/Gyp3p appears to be functionally linked to Cdc42p-regulated pathway(s). The results demonstrate that in yeast there is a large family of Ypt/Rab-GAPs, members of which discriminate poorly between GTPases involved in regulating different steps of exo- and endocytic transport routes.

In eukaryotic cells, monomeric GTPases of the Ras superfamily serve as regulators of a wide variety of vital activities, among them the vesicular protein and membrane transport. In the budding yeast *Saccharomyces cerevisiae*, the full complement of Ypt/Rab-GTPases is known, and a critical functional role of most of the eleven members of the family could be assigned to specific steps of the exocytic and endocytic transport routes (1). Ypt/Rab proteins share with other GTPases of the Ras superfamily a high affinity for guanine nucleotides and a rather slow intrinsic GTP hydrolysis rate. As the GTP-bound conformation of these proteins is the active one, the switch to the inactive conformation requires an acceleration of their inbuilt slow GTPase activity. This is achieved by GTPase-activating proteins (GAPs)[1] first discovered for Ras proteins (2). A large number of GAPs with specificity for Ras and Rho proteins has been identified from divergent species and analyzed in great detail (for review, see Refs. 3–5). The first GAP specific for a Ypt/Rab-GTPase, Gyp6p, was isolated from yeast by high expression cloning (6). With the same experimental strategy, two structurally related GAPs, Gyp1p and Gyp7p, were cloned and shown to significantly accelerate the intrinsic GTPase activity of several Ypt proteins (7–10). From mammalian cells, the structure of only two Ypt/Rab-GAPs has been described. One of them, GAPCenA, prefers Rab6 as substrate and shares sequence similarity with the yeast Gyp proteins (11). The other, Rab3-GAP (12), is not related at all to yeast Ypt/Rab-GAPs.

Ras- and Rho-GAPs, although not homologous in primary structure, are topologically related and fulfill their catalytic activity in a similar way by inserting a conserved arginine residue ("arginine finger") into the active site of their substrate GTPases (4, 5, 13, 14). A recent study in which we identified the catalytically active domains of Gyp1p and Gyp7p, and within them a conserved arginine required for catalytic activity (10), suggests that Ypt/Rab-GAPs, despite their sequence disparity from Ras- and Rho-GAPs, might act by the same basic mechanism. In a data base search using a multiple alignment program, Gyp6p and Gyp7p were reported to have sequence segments related to a number of proteins from different species, among them several yeast proteins with unknown function (15). Interestingly, the related sequences are confined to what we have identified as the catalytic domain of Gyp1p and Gyp7p, and they invariably contain the conserved arginine residue that we have found to be essential for catalytic activity (10). As the three known yeast Ypt/Rab-GAPs accept several of the Ypt proteins as substrate, but the essential GTPases Ypt1p and Ypt31/32p are not preferred substrate of either of the GAPs, we set out to investigate whether some of the candidate yeast Ypt/Rab-GAPs are indeed GTPase-activating proteins and, if so, which of the transport GTPases would serve as their prime substrate. We here report that Msb3p (product of ORF YNL293w), thought to be involved in Cdc42p signaling pathways (16), and Mdr1p (product of ORF YGR100w), thought to interact with the transcriptional activator Mac1p,[2] are potent GAPs for several Ypt/Rab-GTPases.

EXPERIMENTAL PROCEDURES

Materials—Restriction endonucleases and DNA polymerase with proofreading activity, Deep Vent, were purchased from New England Biolabs, a monoclonal antibody against the His_6 tag from Invitrogen and [γ-^{32}P]GTP, specific activity 6000 Ci/mmol, from NEN Life Science Products.

Cloning of MSB3 and MDR1 Genes—Both genes were cloned by polymerase chain reaction. 5′-Primers were designed to contain the *Bam*HI recognition sequence in front of the ATG initiation codons, and 3′-primers contained six histidine codons preceding the translation stop codons and recognition sequences for *Nhe*I and *Xho*I following the stop codons: MSB3 primer 1, 5′-CATATGGATCCAGCATGCAGAACGATCAAC-3′; MSB3 primer 2, 5′-ACGCTCGAGGCTAGCTTAATGGTGATGGTGATGGTGGTCACCCTTGTCTTTTTT-3′; MDR1 primer 1, 5′-AATATGGATCCCATATGTCATTTTTTGATTCTTTAC-3′; and MDR1 primer 2, 5′-ACTCTCGAGGCTAGCTTAATGGTGATGGTGATGGTGAGCTTCAAATTCAATAAG-3′.

To minimize possible errors, polymerase chain reactions were performed in duplicate and using DNA polymerase with proofreading activity. Amplified fragments were cleaved with *Bam*HI and *Nhe*I and separately cloned into the *Bam*HI and *Xba*I-cleaved yeast expression

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[1] The abbreviations used are: GAP, GTPase-activating protein; ORF, open reading frame; PAGE, polyacrylamide gel electrophoresis.

[2] Serpe, M., and Kosman, D. J. (1996) *Saccharomyces* Genome Database entry YGR100W.

FIG. 1. **Alignment of GAP domains of Gyp1p and Gyp7p with related sequence segments of Mdr1/Gyp2p and Msb3/Gyp3p.** The sequences were aligned using the program Pileup from GCG package (29) and edited manually using the program Seqapp (30). Amino acid residues conserved among three of the four sequences are on *black background*, and conservative substitutions are on *gray background*. *Boxes* indicate the conserved regions as identified by Neuwald (15). The arginines conserved among all sequences are marked with an *asterisk*. The arginine in box B was previously shown to be essential for the catalytic activity of Gyp1 and Gyp7 proteins (10).

vector pYES2 (Invitrogen). This brings the ORFs under transcriptional control of the strong GAL10 promoter. The recombinant vectors pYES-MSB3 and pYES-MDR1 were used to transform the proteinase-deficient yeast strain BJ5459 (MAT**a** *ura3–52 trp1 lys2–801 leu2Δ1 his3Δ200 pep4::HIS3 prb1Δ1.6R can1* GAL) (Yeast Genetic Stock Center, University of California, Berkeley). The expression of full-length recombinant proteins after induction with galactose was verified by Western blot analysis with an anti-His_6 antibody.

Purification of Recombinant Proteins—One liter cultures of transformed yeast expressing His_6-tagged Msb3p or Mdr1p were grown in galactose-containing media to an A_{600} of 4–5. Cells were pelleted, resuspended in 40 ml of buffer A (10 mM imidazole, 50 mM NaH_2PO_4, pH 8.0, 0.3 M NaCl, 1 mM Pefabloc and a mix of other protease inhibitors), and disrupted in a French press. After centrifugation at 100,000 × *g* for 45 min, the supernatant (containing about 10 mg of protein/ml) was incubated for 2 h at 4 °C with 0.5 ml of nickel-nitrilotriacetic acid (Ni-NTA) agarose (Qiagen) and washed successively with 50 ml each of buffer B (buffer A lacking the protease inhibitor mix, but containing 10% glycerol) and buffer B containing 20 mM imidazole. Bound proteins were eluted with buffer B containing 250 mM imidazole, and 1-ml fractions were collected. Two fractions containing most of the protein were pooled, diluted with 4 volumes of buffer C (10 mM Tris-HCl, pH 8.0, 2 mM $MgCl_2$, 1 mM EGTA, 1 mM dithiothreitol, 10% glycerol) and applied to a MonoQ HR5/5 column (Amersham Pharmacia Biotech). After washing with buffer C, proteins adsorbed to the ion exchanger were eluted with a gradient of 0–0.6 M NaCl in buffer C at a flow rate of 0.25 ml/min. Fractions containing the recombinant proteins were pooled and the proteins concentrated to 2 ml by centrifugation through Centricon 30 columns (Amicon). Final protein preparations were aliquoted, shock-frozen in liquid nitrogen, and stored at −75 °C. Soluble proteins from untransformed yeast cells were treated according to the same protocol but omitting the ion exchange chromatography.

To assess the purity of the Msb3-His_6 and Mdr1-His_6 protein preparations, Coomassie Blue-stained SDS gels were scanned densitometrically and evaluated with the help of the NIH IMAGE program. Protein concentrations were determined according to the Bradford method using a Bio-Rad Protein Assay Kit. Ypt-GTPases were produced in *Escherichia coli* using the pET vector system (Novagen) and purified as described previously (18).

GTPase Assays—Filter assays with [γ-^{32}P]GTP-loaded GTPases were performed as described previously (7). For substrate specificities and the parameters of the Msb3p-catalyzed hydrolysis of Sec4p-bound GTP, a quantitative high performance liquid chromatography-based method was employed (19). For this assay, purified GTPases were pre-loaded with GTP and, after removing non-bound nucleotide, frozen in aliquots and stored at −75 °C (10).

RESULTS

Inspired by the sequence relatedness with previously identified Ypt/Rab-specific GAPs (6–9, 11) which is confined primarily to their catalytically active domains (10), we analyzed the protein products of two open reading frames whose functions are still obscure. In a computer search, the primary sequences deduced from YNL293w (*MSB3*) and from YGR100w (*MDR1*) had already been found to contain several segments related to the Ypt/Rab-GAPs Gyp6p and Gyp7p (15). These sequence fragments are in fact part of the catalytic domain of Gyp1p and Gyp7p (10). A sequence comparison of the catalytically active fragments of the two Ypt/Rab-GAPs and the related sequence segments of Msb3p and Mdr1p is presented in Fig. 1.

We therefore decided to investigate if Msb3p and/or Mdr1p have GAP activity for members of the Ypt/Rab family of transport GTPases. Because of our previously experienced difficulties in producing reasonable quantities of full-length yeast Ypt/Rab-GAPs in *E. coli*, we attempted to overproduce the 72.99-kDa Msb3p and the 109.23-kDa Mdr1p in yeast from the *GAL10* promoter-controlled genes. To ease the purification of the proteins, they were synthesized as C-terminal His_6-tagged versions. The modified ORFs were amplified by polymerase chain reaction and inserted into the 2-μm based vector pYES2. After yeast transformation, positive clones containing the recombinant vectors were grown either in glucose- (uninduced) or in galactose-containing media (induced). Using a filter assay, protein extracts (10,000 × *g* supernatants of broken cells) from galactose-induced cells were first screened for enhanced GAP activity with different [γ-^{32}P]GTP-loaded Ypt GTPases as substrate. It was found that several positive clones induced to synthesize either of the two His_6-tagged proteins exhibited a significantly increased apparent GAP activity (loss of GTPase-bound radioactivity) toward some of the GTPases tested.

For further analysis, soluble proteins from selected transformants were passed over Ni^{2+}-agarose and, after column washing with loading buffer, affinity matrix-bound proteins were released with 250 mM imidazole. These were then subjected to ion exchange chromatography on MonoQ, and fractions containing the recombinant proteins were concentrated by Centri-

con 30-filtration. As can be seen from SDS-PAGE (Fig. 2*A*), His_6-tagged Msb3p and Mdr1p could be purified to about 60–70% by this two-step procedure, the main contaminants being two proteins with an apparent molecular mass of ~25 kDa.

The partially purified Msb3p and Mdr1p were then used to characterize the proteins with respect to their preferred substrate GTPases. GAP activities were determined under standard conditions in which about 200 nM of either Msb3-His_6 or Mdr1-His_6 protein, calculated from densitometric scanning of SDS gels of partially purified preparations (Fig. 2*A*), was incubated at 30 °C with various 20 μM GTP-loaded GTPases. GTP hydrolysis was monitored by high performance liquid chromatography analysis of the remaining GTP and the GDP generated with time. The initial GTP hydrolysis rates were compared with the intrinsic rates of the GTPases analyzed (Fig. 2, *B* and *C*). As a control, Ni^{2+}-agarose-bound proteins from yeast cells not expressing the His_6-tagged proteins were subjected to GAP activity determinations using Sec4p as substrate. As shown in Fig. 2*B*, GAPs at least for this GTPase were not enriched fortuitously from wild-type yeast by Ni^{2+}-agarose chromatography. As summarized in Table I, the Msb3 protein accelerated the intrinsic GTPase activity of most Ypt proteins tested, the preferred substrates being Sec4p, Ypt6p, Ypt51p, and Ypt31p. The Mdr1 protein was found to be a more specific GAP which significantly accelerated the intrinsic GTPase activity of only Ypt6p and Sec4p. An activation of less than 10-fold determined under the conditions described is not regarded as significant.

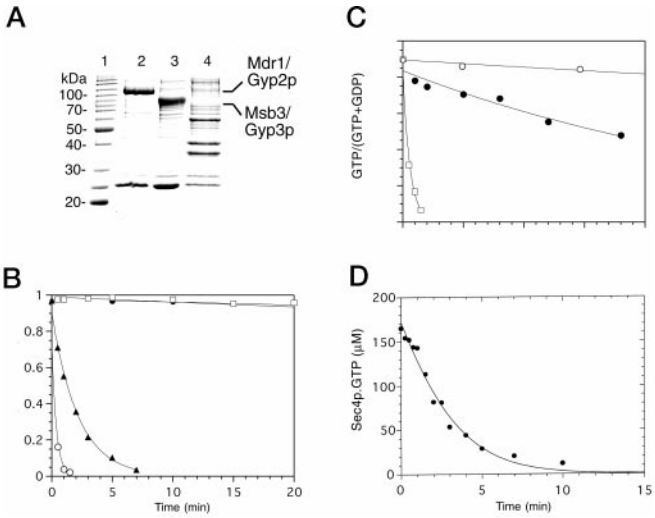

FIG. 2. **Acceleration of GTPase activities of Sec4p and Ypt51p by Mdr1/Gyp2p and Msb3/Gyp3p.** *A*, Mdr1/Gyp2-His_6 (*lane 2*) and Msb3/Gyp3-His_6 (*lane 3*) proteins were purified from overexpressing yeast strains by affinity chromatography on a Ni-NTA column, followed by MonoQ anion exchange chromatography. To check the purity, samples were separated by SDS-PAGE. Proteins from non-transformed yeast that bound to Ni-NTA (*lane 4*) served as control for GAP activity measurements. Molecular mass markers are shown in *lane 1*. *B*, Sec4p·GTP (20 μM) was incubated at 30 °C with 200 nM Mdr1/Gyp2p (▲) or Msb3/Gyp3p (○), with 2.8 μg of proteins from wild-type yeast bound to and eluted from a Ni-NTA column (●), or with buffer only (□). The amount of GTP hydrolyzed with time was determined by high performance liquid chromatography analysis. *C*, demonstration of significantly higher potency of Msb3/Gyp3p (□) *versus* Mdr1/Gyp2p (●) to accelerate the intrinsic GTPase activity (○) of Ypt51p. The analysis was done as described in *panel B*. *D*, determination of catalytic properties of Msb3/Gyp3p from time-dependent hydrolysis of Sec4p-bound GTP. Sec4p·GTP (165 μM) was incubated at 30 °C with 100 nM Msb3/Gyp3p and K_m, and k_{cat} values were calculated from the resulting GTP hydrolysis curve according to the integrated Michaelis-Menten equation using the program Scientist (10).

Remarkably, Sec4p and Ypt51p were recently shown to be the most efficient substrates for the Ypt/Rab-GAP Gyp1p as well (8, 10). In comparing the degree of activation of the Sec4p GTP hydrolysis rate catalyzed under identical conditions by either the Gyp1p catalytic domain (10) or the full-length Msb3 protein described here, we noticed that Msb3p appeared to be a more efficient GAP for Sec4p than Gyp1p. We therefore sought to determine the catalytic properties of Msb3p using the integrated Michaelis-Menten equation (20) which allows the K_m and k_{cat} values to be determined from single time curves and which has been previously applied to study Ras/Ras-GAP (19) and Ypt7p/Gyp7p interactions (10). For this study, 100 nM Msb3-His_6 protein were incubated with 165 μM GTP-loaded Sec4p and the GTP hydrolysis rates determined at short intervals. The time curve shown in Fig. 2*D* was used to calculate the K_m (154 μM) and the k_{cat} (13.3 s^{-1}) with the help of the program "Scientist." The data obtained demonstrate that like Gyp1p and Gyp7p (10), Msb3p has a relatively low affinity for its preferred substrate GTPase. On the other hand, Msb3p is catalytically very active. Given the intrinsic GTP hydrolysis rate of Sec4p (0.0016 min^{-1}) and the k_{cat} measured (13.3 s^{-1}), the activation by Msb3p is as high as 5×10^5-fold.

DISCUSSION

We have shown here that two yeast proteins of so far unknown function, Mdr1p and Msb3p, are very efficient GTPase-activating proteins for transport GTPases of the Ypt/Rab family. The search for GAP activity of these proteins was prompted by the fact that the originally discovered sequence motifs that Mdr1p and Msb3p share with the known Ypt/Rab-GAPs Gyp1p, Gyp6p, and Gyp7p (15) lie within the catalytic domain of the Gyp proteins (10). Like Gyp1p, Gyp6p, and Gyp7p (6, 8–10), Mdr1p and Msb3p are not entirely specific for only one of the eleven yeast Ypt/Rab-GTPases. Although Gyp6p (6) and Gyp7p (7, 10) display a clear substrate preference for Ypt6p and Ypt7p, respectively, Gyp1p (8, 10) and the novel GAP Msb3p, which we suggest to name Gyp3p, are remarkably

TABLE I
Substrate specificity of Mdr1/Gyp2p and Msb3/Gyp3p

GTPase	GTP hydrolysis rate[a]		Acceleration	GTP hydrolysis rate[a]	Acceleration
	Intrinsic	Mdr1/Gyp2p-accelerated		Msb3/Gyp3p-accelerated	
	min^{-1}		*-fold*	*min^{-1}*	*-fold*
Ypt1p	0.0025[b]	0.0073	2.9	0.1116	44.6
Sec4p	0.0016[b]	0.4594	287.1	2.5897	1618.6
Ypt31p	0.0064[b]	0.0357	5.6	0.7103	111.0
Ypt32p	0.0083	0.0424	5.1	0.3928	47.3
Ypt51p	0.0052[b]	0.0247	4.7	1.6919	325.4
Ypt52p	0.0862	0.0829	1.0	0.2489	2.9
Ypt53p	0.0102	0.0221	2.1	0.1503	14.7
Ypt6p	0.0002[b]	0.0689	344.5	0.1113	556.5
Ypt7p	0.0023[b]	0.0126	5.5	0.0453	19.7

[a] GTP hydrolysis rates were determined at 30 °C.
[b] Values taken from Albert *et al.* (1999).

promiscuous with respect to their substrate GTPases. Msb3/Gyp3p is a potent GAP for Sec4p, accelerating its intrinsic GTPase activity by a factor of 5×10^5. This compares well with the activation rates measured for Gyp7p (10), for Ras- and Ran-GAPs (21–23), and it significantly exceeds the activation rate reported for Gyp1p (10) and Rho-GAPs (24). Mdr1p, which we suggest to name Gyp2p, is, like Msb3/Gyp3p, also a potent GAP for Sec4p and Ypt6p. But in contrast to Msb3/Gyp3p, Mdr1/Gyp2p does not activate significantly the intrinsic GTPase activity of any other Ypt protein (Table I). Notably, Msb3/Gyp3p is the only of the known five yeast Ypt/Rab-GAPs which activates the intrinsic GTPase activity of all three essential Ypt-GTPases, Ypt1p, Sec4p and the redundant pair Ypt31p/Ypt32p. This is at least true *in vitro*. It is unclear at present which sequences of the different GTPases are important for binding to a given Ypt/Rab-GAP. Lack of GAP activity toward a given GTPase might not necessarily mean that a Ypt/Rab-GAP does not bind to that GTPase. It is known, for example, that Ras-GAP binds with even higher affinity to Rap1 than to Ras without accelerating its intrinsic GTPase activity (25). It might well be that within the cell, substrate specificity of Ypt/Rab-GAPs is attained by their recruitment to specific organelles on which specific GTPases reside. In this context it is important to note that although for the catalytic activity and substrate specificity of Gyp1p and of Gyp7p a fragment of about 45 kDa is sufficient, leaving nearly half of the proteins for other purposes, perhaps for the recruitment of the GAPs to specific membranes (10). The same might be true for the newly discovered Ypt/Rab-GAPs having molecular masses of 73 kDa (Msb3/Gyp3p) or 109 kDa (Mdr1/Gyp2p).

What is known about Mdr1p and Msb3p that we have now identified to be potent Ypt/Rab-GAPs? According to structure prediction programs, both proteins contain putative transmembrane domains. Mdr1/Gyp2p and Msb3/Gyp3p, however, are perfectly soluble proteins as we have observed in the present study. Mdr1p is thought to interact with Mac1p (16), a transcriptional regulator required for copper permease expression and copper uptake (26, 27). As no detailed studies have been reported on Mdr1p, we will not speculate on a possible functional relationship of the putative transcription factor-binding protein and its proven efficient Ypt/Rab-GAP activity. Msb3p has been isolated as a multicopy suppressor of bud emergence mutants with specific defects in the GTPase Cdc42p and its nucleotide exchange factor Cdc24p (16). Interestingly, Msb3p/Gyp3p localizes to the bud tip, the site of polarized secretion (16). At the plasma membrane of the bud tip, Sec3p has been recently shown to organize the multi-protein complex required for fusion of secretory vesicles (28). Sec4p colocalizes with Sec3p and other proteins at the bud tip, and it appears to be an attractive possibility that Msb3/Gyp3p acts as Sec4p-GAP at the site of polarized exocytosis.

Msb3p has a homologue, Msb4p, with which it shares more than 50% of identical sequence. Like Msb3/Gyp3p but less efficient, Msb4p at high intracellular concentration acts as suppressor of a specific *cdc24* mutant (16). In line with this, our preliminary studies show that Msb4p might also be a GTPase-activating protein for Ypt/Rab-GTPases. Details of the functional link between the Cdc42-regulated pathway(s) and the Ypt/Rab-GAPs have still to be resolved.

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