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Biochemical Characterization of Gyp6p, a Ypt/Rab-specific GTPase-activating Protein from Yeast*

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Gyp6p from yeast belongs to the GYP family of Ypt/Rab-specific GTPase-activating proteins, and Ypt6p is its preferred substrate (Strom, M., Vollmer, P., Tan, T. J., and Gallwitz, D. (1993) *Nature* 361, 736–739). We have investigated the kinetic parameters of Gyp6p/Ypt6p interactions and find that Gyp6p accelerates the intrinsic GTPase activity of Ypt6p (0.0002 min^{-1}) by a factor of 5×10^6 and that they have a very low affinity for its preferred substrate (K_m = 592 μM). Substitution with alanine of several arginines, which Gyp6p shares with other GYP family members, resulted in significant inhibition of GAP activity. Replacement of arginine-155 with either alanine or lysine abolished its GAP activity, indicating a direct involvement of this strictly conserved arginine in catalysis. Physical interaction of the catalytically inactive Gyp6(R155A) mutant GAP with Ypt6 wild-type and Ypt6 mutant proteins could be demonstrated with the two-hybrid system. Short N-terminal and C-terminal truncations of Gyp6p resulted in a complete loss of GAP activity and Ypt6p binding, showing that in contrast to two other Gyp proteins studied previously, most of the 458 amino acid-long Gyp6p sequence is required to form a three-dimensional structure that allows substrate binding and catalysis.

Monomeric GTPases of the Ras superfamily act as regulators in many vital cellular processes. They switch their conformation depending on the nucleotide being bound. Ras and Ras-like proteins bind GDP and GTP specifically and with high affinity, and they are able to hydrolyze the bound GTP but with low efficiency. In general, as the switch from the GTP-bound to the GDP-bound conformation results in the termination of the functional stimulus by a given GTPase, the acceleration of the slow intrinsic GTPase activity (often far below 1 min^{-1}) must be an important device to regulate the activity of the regulator. GTPase-activating proteins (GAPs),[1] specific for Ras, Rho, and Ypt/Rab family members that are able to activate the hydrolysis rate of GTPase-bound GTP by several orders of magnitude, have been isolated from many eukaryotes and found to be important for the functional cycle of GTPase (for review, see Refs. 1 and 2).

GTPase-activating proteins for Ypt/Rab transport GTPases were first discovered in yeast (3, 4) and shown to share several conserved sequence motifs with a variety of proteins from other eukaryotic species (5). These sequences are localized within the catalytically active region of the yeast GAPs, Gyp1p and Gyp7p (6), and we refer to this region as the GYP domain. However, additional sequences C-terminal of the GYP domain are required for GAP activity (6). Eight yeast proteins and one mammalian protein containing the GYP domain are known to be Ypt/Rab-specific GAPs (3, 4, 6–11).[2] The length of the eight GYP family members in yeast ranges from 458 to 950 amino acid residues. The GYP domain, which in Gyp1p and Gyp7p is localized within the C-terminal half of the proteins, covers a region of approximately 300 amino acids. The N-terminal halves of both GAPs can be deleted without affecting the catalytic activity or the substrate specificity *in vitro* (6). Therefore, it appears that these sequences serve other purposes within the cell, such as the interaction with other cellular components to direct the GAPs to their scene of action.

With 458 amino acids, the Ypt6p-specific Gyp6p (3) is the smallest of the eight known Ypt/Rab GAPs from yeast and apparently has little sequence space outside the GAP catalytic domain. This observation and the fact that its overall sequence deviates more significantly from the other GAPs prompted us to study the biochemical characteristics of this protein. We found that short N-terminal or C-terminal deletions inactivate Gyp6p and that Gyp6p accelerates the intrinsic GTPase activity of Ypt6p $>10^6$-fold and most probably uses a catalytic arginine, which is in the corresponding position of the critical arginine identified in Gyp1p and Gyp7p (6, 12).

EXPERIMENTAL PROCEDURES

Cloning Strategies—All cloning procedures were performed using standard protocols (13). For construction of the yeast expression vector pEG(KT)-GYP6, the plasmid pGEX-GYP6 (bearing a *Bam*HI restriction site 5′ adjacent to the ATG start codon and an *Eco*RI site directly following the TAA translational stop codon of *GYP6*) was digested with *Eco*RI, treated with the Klenow fragment, and cleaved with *Bam*HI. The isolated *GYP6*-containing fragment was ligated into the *Bam*HI- and *Xba*I-cleaved vector pEG(KT) (14) after filling in the overhanging ends of the *Xba*I cleavage site with the Klenow enzyme. The C-terminal deletions of Gyp6p were generated by inserting the following *GYP6* sequence-containing fragments into pEG(KT) that are derived from pGEX-GYP6: *Bam*HI-*Acc*I (for Gyp6(1–277)p), *Bam*HI-*Sph*I (for Gyp6(1–323)p), and *Bam*HI-*Eco*NI (for Gyp6(1–382)p). For N-terminal deletions, a *Hin*dIII-*Eco*RI fragment (for Gyp6(45–458)p), a *Bsp*MI-*Eco*RI fragment (for Gyp6(182–458)p), or a *Bgl*II-*Eco*RI fragment (for Gyp6(209–458)p) was isolated from pGEX-GYP6, and a *Ban*I-*Xba*I fragment (for Gyp6(72–458)p) was isolated from pRS326-GYP6 (3). The fragments were blunt-end inserted into pEG(KT) in frame with the

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[1] The abbreviations used are: GAP, GTPase-activating protein; GST, glutathione *S*-transferase; X-gal, 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside; Gal4-BD, Gal4 DNA binding domain; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid.

[2] S. Albert, A. DeAntoni, and D. Gallwitz, unpublished observations.

TABLE I
Catalytic properties of Ypt-specific GTPase activating proteins
The K_m and k_{cat} values of the different GAPs were measured at 30 °C.

GAP	GTPase	K_m	k_{cat}	Activation	Reference
		μM	s^{-1}	-fold	
Gyp1–46	Ypt51p	143	3.9	4.5×10^4	(6)
Msb3/Gyp3p[a]	Sec4p	154	13.3	5.0×10^5	(9)
Gyp7p[a]	Ypt7p	400	7.5	2.0×10^5	(6)
Gyp7–47p[a]	Ypt7p	42	30.0	7.8×10^5	(6)
GST-Gyp6p	Ypt6p	592	18.8	5.6×10^6	This study

[a] Proteins were C-terminal His_6-tagged.

GST-encoding sequence. Proper expression of the truncated GST-Gyp6 fusion proteins was verified by Western blot analysis using anti-GST antisera (Amersham Pharmacia Biotech).

Production and Purification of Proteins—Ypt6p was produced in *Escherichia coli* using the pET vector system (Novagen) and purified as described previously (15). GST-Gyp6 fusion proteins were produced in the 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 at Berkeley) and purified as described previously for the preparations of GST-Gyp7p (8). To get active protein, buffers used had to be free of CHAPS. Yeast cell lysis was done in buffer consisting of 50 mM Tris-HCl, pH 7.5, 5 mM EDTA, 100 mM KCl, 1 mM Pefabloc and 1× complete proteinase inhibitors (Roche Molecular Biochemicals), column washes in 50 mM Tris-HCl pH 7.5, 5 mM EDTA, 1 M KCl. GST-Gyp6p was eluted from glutathione-Sepharose at 4 °C in 50 mM Tris-HCl, 20 mM reduced glutathione, pH 7.5. Concentration and purity of the GST fusion proteins were determined as described previously (6).

GAP Activity Assays—GAP activity in crude yeast cell extracts was determined using the filter assay and [γ^{32}P]GTP-loaded Ypt6p (4). The high pressure liquid chromatography-based quantitative GAP assay used for analysis of purified proteins and the evaluation of the Gyp6p/Ypt6p interaction using the integrated Michaelis-Menten equation were performed as described recently in detail (6, 16).

Two-hybrid Analysis—The generation of pAS2-YPT6, pAS1-YPT6(Q69L), and pACTII-GYP6 has been described previously (17). The fusion of the Gal4 DNA binding domain (Gal4-BD) with Ypt6(G20S)p was performed by the insertion of an *Nco*I/*Bgl*II fragment from pACTII-YPT6(G20S) into *Nco*I/*Bam*HI-cleaved pAS2, and the codon exchange was verified by sequence analysis. Truncated Gyp6 proteins were fused to the Gal4 transcription activation domain in the two-hybrid vector pACTII. Proper expression of the "bait" and "prey" plasmids in the yeast reporter strain Y190 (18) was controlled by Western blot analysis using hemagglutinin monoclonal antibody (Roche Molecular Biochemicals). Double transformants expressing both bait and prey hybrid proteins were analyzed for β-galactosidase activity by the X-gal filter assay as described previously (19). Quantification of the β-galactosidase activity in liquid cultures using *o*-nitrophenyl-β-D-galactosidase was performed as described by Guarente (20) and evaluated according to Miller (21), units = $A_{420} / (t \times V \times A_{600})$ where t is the reaction time (in min) at 30 °C, and V is the volume (in ml) of the yeast culture used in the assay.

Site-directed Mutagenesis of GYP6—Substitution of arginine to alanine or lysine codons in *GYP6* was achieved by a PCR-based overlap extension method (22) using the following oligonucleotides as primers (mutated codons are underlined): R39A, 5′-CTTTAAAGAAAATAGTGCCGGCTGGCTCTGGAA-3′ and 5′-TTCCAGAGCCAGCCGGCACTATTTTCTTTAAAG-3′; R155A, 5′-AGATTTGGACCTCTCGGCCATAATGCTTGACGAT-3′ and 5′-ATCGTCAAGCATTATGGCCGAGAGGTCCAAATCT-3′; R155K, 5′-GGACCTCTCAAAGATAATGCTTGACG-3′ and 5′-CGTCAAGCATTATCTTTGAGAGGTCC-3′; R290A, 5′-AATCTGGCTCATCGCCTGGACAAGGTTGTT-3′ and 5′-AACAACCTTGTCCAGGCGATGAGCCAGATT-3′; R298A, 5′-GGACAAGGTTGTTATTTTTGGCCGAATTACCCTTAAAAT-3′ and 5′-ATTTTAAGGGTAATTCGGCCAAAAATAACAACCTTGTCC-3′.

RESULTS

Catalytic Properties of the Ypt6-GAP Gyp6p—We had previously shown that bacterial expression of full-length Gyp6 protein allowed us to identify its activity as a Ypt/Rab-specific GAP (3), but large scale production of the protein in *E. coli* failed. Likewise, we were unable to produce a GST-Gyp6 fusion protein in *E. coli* in reasonable quantity. Therefore, we expressed the GST-Gyp6p fusion in a proteinase-deficient yeast strain under control of the strong galactose-inducible GAL10 promoter. As thrombin cleavage of the affinity-purified fusion protein resulted in a significant loss of GAP activity, the biochemical analyses were performed with GST-Gyp6p fusions that were >80% pure. GAP activity was determined with varying amounts of the fusion protein and a 10–100-fold excess (20 μM) of GTP-bound Ypt6p. From the initial rates of GTP hydrolysis determined by high pressure liquid chromatography-based quantification of GTP and GDP, a specific activity of 72.2 (± 4.8) units/nmol GST-Gyp6p was calculated where one unit of GAP was defined as the hydrolysis of 1 nmol of Ypt6p-bound GTP in 1 min at 30 °C. This value compares well with specific activities that we recently determined for two other yeast Ypt/Rab GAPs (6).

For further characterization of the Gyp6p-Ypt6p interaction, the K_m and k_{cat} values were determined from single time curves using the integrated Michaelis-Menten equation (23) as described for the analysis of the catalytic properties of Gyp1p, Gyp7p, and Gyp3p (6, 9). In a representative experiment using 100 nM GST-Gyp6p and an initial substrate concentration of 200 μM Ypt6p-GTP, we determined K_m = 592 μM and k_{cat} = 18.8 s^{-1} for GST-Gyp6p. Given the slow intrinsic GTP hydrolysis rate of Ypt6p (0.0002 min^{-1}), this means a maximal acceleration of 5.6 × 10^6-fold. Thus, compared with the catalytic properties of other GAPs for Ypt-GTPases, Gyp6p seems to bind its substrate with very low affinity but causes the highest maximal acceleration of GTP hydrolysis measured so far for a Ypt/Rab-GAP (Table I).

Impairment of GAP Activity by Single Point and Truncation Mutations—Within the GYP family of yeast Ypt/Rab-GAPs, Gyp6p is an exception in that it lacks a larger N-terminal sequence segment preceding the GYP domain (Fig. 1). The GYP domain of the Ypt6-GAP with its six elements A–F, related in primary sequence among all GYP family members (5, 12), shares with that of Gyp1p and Gyp7p four arginine residues in equivalent positions. Two of these residues, Arg-39 and Arg-155, of Gyp6p are strictly conserved in all known Ypt/Rab-GAPs of the GYP family. We have shown by mutational analysis that the conserved arginine in sequence segment B of the GYP domain (Fig. 1) is required for the catalytic activity of Gyp1p and Gyp7p (6). We generated GST-Gyp6 fusion proteins with alanine substitutions for the four arginines in position 39 (*motif A*), 155 (*motif B*), and 290 and 298 (*motif F*) in addition to a mutant GAP with the conservative R155K exchange. From kinetic analyses under standard conditions (Fig. 2), we observed that all substitutions led to significant inhibition of Gyp6p GAP activity (Table II). Importantly, both the R155K and the R155A substitution resulted in an apparently complete inactivation of the Gyp6 mutant proteins.

Because we were unable to achieve GST-Gyp6p concentrations high enough to measure the K_m and k_{cat} values for the two mutant proteins, a distinction between an impairment of catalysis and that of substrate affinity was attempted using the two-hybrid system. To test for substrate protein binding, Gyp6p and Ypt6p were fused to the Gal4 transcription activa-

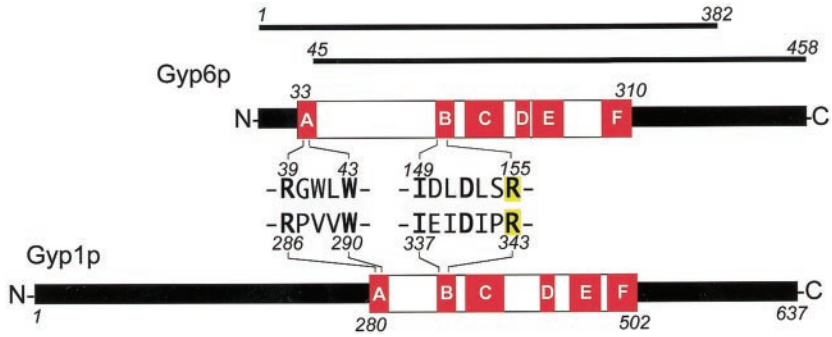

FIG. 1. **Schematic representation of the structural makeup of yeast Ypt/Rab-specific GAPs.** The location of the GYP domain with the related sequence segments *A–F highlighted* in *red* is compared between Gyp6p and Gyp1p. Amino acid residues strictly conserved in segments *A* and *B* of all GYP family members are shown; the arginine likely to be involved in catalysis is *highlighted* in *yellow*. Two mutant Gyp6 proteins (with the shortest C-terminal and N-terminal deletions) that are catalytically inactive in Ypt6 GTPase binding are shown at the *top*. Relevant amino acid residues are *numbered*.

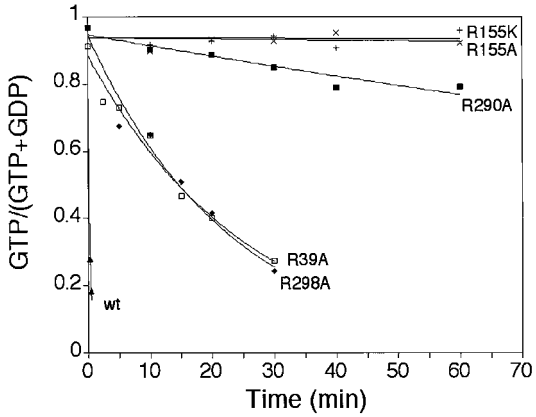

FIG. 2. **Effect of arginine substitutions on the catalytic activity of Gyp6p.** 1 μM of either wild-type (*wt*) or mutant Gyp6p fused to GST was incubated with 20 μM GTP-loaded Ypt6p at 30 °C. At the time points indicated, aliquots of the incubation mixtures were shock frozen, and GTP and GDP concentrations were determined by high pressure liquid chromatography analysis. Note that R155A and R155K substitutions only led to an apparent complete loss of Gyp6p catalytic activity.

TABLE II
Activity of GST-Gyp6 proteins with arginine substitutions

GST-Gyp6p	GTP hydrolysis rate[a]	Acceleration
	min^{-1}	*-fold*
—	0.0002	—
Wild type	3.33	16,650
R39A	0.039	195
R155A	<0.0002	0
R155K	<0.0002	0
R290A	0.0035	18
R298A	0.044	220

[a] GTP hydrolysis rates were determined at 30 °C with 20 μM GTP-loaded Ypt6p and 1 μM GST-Gyp6 wild type or mutant proteins.

tion domain and Gal4-BD, respectively, and protein-protein interactions were identified by monitoring β-galactosidase activity in a reporter yeast strain transformed with both expression plasmids. Expression of wild-type and mutant proteins was verified by Western blotting analysis using appropriate antibodies. Although GAP/GTPase interactions must be extremely short-lived because of the fast GTP hydrolysis reaction, Gyp6p-interaction with its substrate GTPase Ypt6p could be demonstrated (Table III). We also observed a weaker interaction of the GAP with Ypt6p mutated in the P-loop (G20S substitution), and as expected, a considerable enhancement of GAP interaction with Ypt6(Q69L)p, a mutant GTPase that like other Ras superfamily members with the same substitution in the equivalent position, has a significantly reduced intrinsic

TABLE III
Interaction of Gyp6p and Ypt6p in the two-hybrid system

"Bait" plasmid	"Prey" plasmid	β-Galactosidase activity[a]
		units $\times$ 10^{-3}
pAS2	pACTII-GYP6	0.08 (± 0.05)
pAS2	pACTII-GYP6(R155A)	0.10 (± 0.09)
pAS2-YPT6	pACTII-GYP6	1.60 (± 0.31)
pAS2-YPT6	pACTII-GYP6(R155A)	5.13 (± 1.73)
pAS2-YPT6(G20S)	pACTII-GYP6	0.46 (± 0.31)
pAS2-YPT6(G20S)	pACTII-GYP6(R155A)	10.91 (± 3.75)
pAS1-YPT6(Q69L)	pACTII-GYP6	11.40 (± 0.15)
pAS1-YPT6(Q69L)	pACTII-GYP6(R155A)	12.25 (± 4.69)

[a] The β-galactosidase activity produced in double transformants grown in liquid cultures was quantified using *o*-nitrophenyl-β-D-galactosidase as substrate. Three single colonies were tested for each interaction.

GTPase activity (Fig. 4*A*). When the catalytically inactive Gyp6(R155A) mutant GAP was analyzed with respect to its binding to wild-type and mutant Ypt6 GTPases, we found that the GAP/GTPase interactions were significantly stronger than they were with the wild-type Gyp6 protein. This was especially apparent with the P-loop mutant Ypt6(G20S)p (Table III). From this study, it follows that substitutions of Arg-155 in Gyp6p affect the catalytic activity of GAP rather than its substrate binding.

Because the large N-terminal segments preceding the GYP domain in Gyp1p and Gyp7p could be deleted without reduction of the catalytic activity (6), we addressed the question of whether Gyp6p contained N-terminal or C-terminal sequences dispensable for catalytic activity or substrate GTPase binding. Successive N-terminal and C-terminal deletions were created using available restriction enzyme cutting sites of the *GYP6* protein-coding region. GST fusions of the truncated Gyp6 proteins were expressed in yeast (Fig. 3), and their apparent GAP activity was assessed in cellular extracts with [γ-^{32}P]GTP-loaded Ypt6p using a filter assay. Whereas cell extracts containing full-length GST-Gyp6p generally resulted in the hydrolysis of >80% Ypt6p-bound GTP within 10 min at 30 °C, extracts containing truncated GST-Gyp6 fusion proteins exhibited only background activity in at least three independent experiments. This finding shows that GAP activity of Gyp6p is already lost or significantly reduced in mutant proteins lacking either the N-terminal 44 amino acid residues (including most of the segment A sequence of the GYP domain) or the C-terminal 76 residues (still leaving intact a block of 72 amino acids distal of the GYP domain) (see Fig. 1).

Substrate binding of Gyp6p truncation mutants was assessed by two-hybrid analyses to determine whether the lack of GAP activity was the result of an impaired physical GAP/GTPase interaction. Truncated Gyp6 proteins fused to Gal4

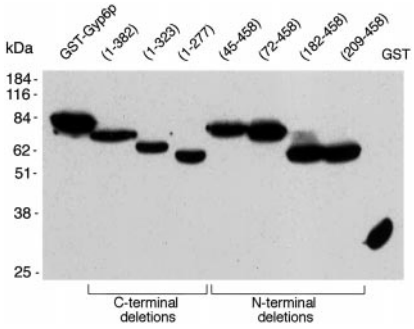

FIG. 3. **N-terminal and C-terminal truncated Gyp6 proteins produced in yeast.** Wild-type Gyp6p and its truncated versions were synthesized in a proteinase-deficient yeast strain from a multi-copy plasmid under the control of the galactose-inducible GAL10 promoter. Total cellular protein was separated by SDS-polyacrylamide gel electrophoresis, transferred to nitrocellulose, and GST and GST-Gyp6 fusion proteins were identified immunologically with anti-GST antibodies. *Numbers* in *parentheses* indicate the N-terminal and C-terminal Gyp6p residues of the truncation mutants.

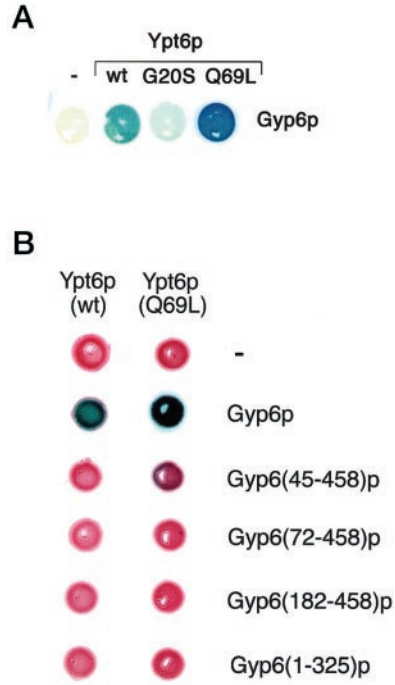

FIG. 4. **Two-hybrid interactions of Gyp6p and its substrate GTPase Ypt6p.** *A*, the Y190 reporter strain was transformed with pACTII-GYP6 as prey in combination with empty pAS2, pAS2-YPT6 (wild-type), pAS1-YPT6(G20S), or pAS1-YPT6(Q69L) as baits. *B*, prey constructs encoding truncated Gyp6 hybrid proteins were combined with either pAS2-YPT6 wild-type (*Ypt6p*(*wt*)) or pAS1-YPT6(Q69L). β-Galactosidase activity of double transformants grown on agar plates for 2 days was tested by the X-gal filter assay. Production of all fusion proteins was verified by Western blot analysis with total protein of the transformants.

transcription activation domain were coexpressed with Gal4-BD-Ypt6p or Gal4-BD-Ypt6(Q69L)p, expression was confirmed by Western blotting analysis, and the interaction was analyzed using the X-gal filter test (Fig. 4*B*). The expression level of the truncated GAPs was comparable to that of full-length Gyp6p (data not shown). Very weak interaction of Ypt6(Q69L)p but not wild-type Ypt6p was detected with Gyp6(45–458)p. No interaction was observed with two larger N-terminal deletions or with the C-terminal truncation mutant Gyp6(1–325)p, which lacked most of the sequences distal of the GYP domain (Fig. 1). These results suggest that the GYP domain as well as sequences C-terminal of the GYP domain contribute to efficient substrate binding.

DISCUSSION

Our recent studies have shown that the deletion of extended regions, which are located N-terminal of the catalytic domain of most GYP family members, does not inhibit GAP activity nor does it affect substrate specificity (6).[3] In Gyp1p and Gyp7p, this GAP-dispensable region amounts to 39 and 48%, respectively, of the total length of the protein. In fact, the catalytically active fragment of both proteins is more active than the full-length GAPs. As *gyp1* (7), *gyp6* (3), *gyp7* (8), and other Ypt-GAP null mutants are phenotypically inconspicuous in complete growth media, it is not an easy task to elucidate the function(s) of the N-terminal domains *in vivo*. We have argued (6) that because of the low affinity of Ypt/Rab-GAPs to their substrate GTPases, high concentrations of the GAPs would be required at those membranes where they are likely to act and that the N-terminal extensions of the GAPs might be required for their recruitment to specific cellular locations. If this were the case, Gyp6p without a fragment of appreciable length preceding the GYP domain would need to employ other part(s) of the molecule for localization purposes.

All of the N-terminal and C-terminal truncations of Gyp6p we have described here significantly inhibited or even abolished GAP activity. The shortest of the truncations, Gyp6(45–458)p and Gyp6(1–382)p, are of special interest. The deletion of the N-terminal 44 amino acids included most of the 15 amino acid-long sequence motif A with Arg-39 and Trp-43, two residues strictly conserved in all GYP family members (Fig. 1). This mutant not only lost GAP activity but also its ability to bind its substrate GTPase Ypt6p as shown by two-hybrid analysis. The latter finding strengthens the argument, derived from our recently solved x-ray structure of the Gyp1p catalytic domain, that the conserved arginine and tryptophan residues in motif A contribute to the stabilization of the tertiary structure of the GAP domain and presumably to the formation of the GTPase-binding epitope (12). The C-terminal truncation mutants of Gyp1p and Gyp7p, which we previously studied and found to be catalytically inactive, terminated only 31 and 17 amino acids, respectively, distal of the motif F of the GYP domain. But in this study, we had not addressed the question of whether the truncated GAPs were still able to bind their substrate proteins. The work with Gyp6p now shows that a segment of 13 amino acid residues C-terminal of the GYP domain (truncation mutant Gyp6(1–323)p) is not sufficient to allow binding to the substrate GTPase. Even Gyp6(1–382)p with 72 amino acids following the GYP domain was inactive most probably because of its deficiency for substrate binding. The crystal structure of Gyp1p and the proposed Gyp1p·Ypt51p complex (12) suggests that at least one α-helical region located approximately 100 amino acid residues C-terminal of the GYP domain could contribute to GTPase binding. The C-terminal region distal of the GYP domain of different GYP family members is at least 150 amino acid residues long, but the primary sequences are quite divergent. This work clearly indicates that a significant part of this region is required for the overall architecture of an active GAP and for the binding of the substrate GTPases.

Apart from its exceptional N terminus among the GYP family members, Gyp6p, which for technical reasons had to be analyzed as a GST fusion protein, shares similar biochemical properties with other Ypt/Rab-specific GAPs studied. It accelerates the low intrinsic GTP hydrolysis rate very potently but displays very low affinity ($K_m > 500$ μM) for its preferred substrate Ypt6p. In fact, with a 5×10^6-fold acceleration of the

[3] S. Albert, A. DeAntoni, and D. Gallwitz, unpublished observations.

basic GTPase activity of Ypt6p, Gyp6p is the most potent of the Ypt/Rab-GAPs that we have analyzed so far. Although the substitution with alanine of four arginine residues within the shared sequence motifs of the GYP domain led to a significant loss of Gyp6p GAP activity, only the strictly conserved arginine in position 155 proved to be essential for GAP activity. Importantly, as we could demonstrate for Gyp6(R155A) in a two-hybrid analysis, substitutions of Arg-155 do not interfere with GAP/GTPase interaction. This finding is further evidence for the suggestion, based on our mutational (6, 9, 11) and structural (12) investigations, that this particular arginine is directly involved in catalysis.

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