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 × 10⁶ and that they have a very low affinity for its preferred substrate ($K_m = 592 \mu M$). 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 Gyp6p(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 eukaryotic species (5). These sequences are localized within the C-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 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 >10⁵-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 pEGKKT-GYP6, the plasmid pGEX-GYP6 (bearing a BamHI restriction site 5’ adjacent to the ATG start codon and an EcoRI site directly following the TAA translational stop codon of GYP6) was digested with EcoRI, treated with the Klenow fragment, and cleaved with BamHI. The isolated GYP6-containing fragment was ligated into the BamHI- and XbaI-cleaved vector pEGKKT (14) after filling in the overhanging ends of the XbaI cleavage site with the Klenow enzyme. The C-terminal deletions of Gyp6p were generated by inserting the following GYP6 sequence-containing fragments into pEGKKT that are derived from pGEX-GYP6: BamHI-AccI (for Gyp6(1–277) p), BamHI-SphI (for Gyp6(1–323)p), and BamHI-EcoNI (for Gyp6(1–382)p). For N-terminal deletions, a HindIII-EcoRI fragment (for Gyp6(454–458)p), a BspMI-EcoRI fragment (for Gyp6(182–458)p), or a BglII-EcoRI fragment (for Gyp6(209–458)p) was isolated from pGEX-GYP6, and a BanI-XbaI fragment (for Gyp6(72–458)p) was isolated from pRS326-GYP6 (3). The fragments were blunt-end inserted into pEGKKT (K) 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-DB, Gal4 DNA binding domain; CHAPS, 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonic acid.

² S. Albert, A. DeAntoni, and D. Gallwitz, unpublished observations.
Production and Purification of Proteins—Ypt6p was produced in *Escherichia coli* using the pET vector system (Novagen) and purified as described previously (15). GST-Ypt6p fusion proteins were produced in the yeast strain BY5445 (MATa ura3–52 trp1 lys2–801 leu2–3,121 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-Ypt6p (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, 1 M KCl. GST-Ypt6p was eluted from glutathione-Sepharose at 4 °C in 50 mM Tris-HCl, pH 7.5, 20 mM reduced glutathione, pH 7.5. Concentration and purity of the GST-Gyp6p fusion in a proteinase-deficient yeast culture used in the assay.

### GAP Activity Assays—GAP activity in crude yeast cell extracts was determined using the filter assay and [γ-32P]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 Michalis-Menten equation (23) 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-βD) with Ypt6p interaction 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-Ypt6p and an initial substrate concentration of 200 μM Ypt6p-GTP, we determined K₉ = 592 μM and k₉ = 18.8 s⁻¹ for GST-Ypt6p. Given the slow intrinsic GTP hydrolysis rate of Ypt6p (0.002 min⁻¹), this means a maximal acceleration of 5.6 × 10⁶-fold. Thus, compared with the catalytic properties of other GAPs for Ypt/GTPases, Ypt6p 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 motif B (Fig. 1), which is required for the catalytic activity of other Ypt/GTPases (Table I). We have shown by mutational analysis that the conserved arginine in sequence segment B of the Ypt/Rab-GAP (Table I).

### RESULTS

Catalytic Properties of the Gyp6p/Ypt6p Interaction—We had previously shown that bacterial expression of full-length Gyp6p 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-Ypt6p fusion protein in *E. coli* in reasonable quantity. Therefore, we expressed the GST-Ypt6p 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 fusion 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 GAP hydrolysis determined by high pressure liquid chromatography-based quantification of GAP 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 K₉ and k₉ 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-Ypt6p and an initial substrate concentration of 200 μM Ypt6p-GTP, we determined K₉ = 592 μM and k₉ = 18.8 s⁻¹ for GST-Ypt6p. Given the slow intrinsic GTP hydrolysis rate of Ypt6p (0.002 min⁻¹), this means a maximal acceleration of 5.6 × 10⁶-fold. Thus, compared with the catalytic properties of other GAPs for Ypt/GTPases, Ypt6p 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).

| GAP                  | GTPase        | K₉ (μM) | k₉ (s⁻¹) | Activation | Reference |
|----------------------|---------------|---------|----------|------------|-----------|
| Gyp1–46              | Ypt51p        | 143     | 2.9      | 4.5 × 10⁴  | (6)       |
| Msh3/Gyp3p*         | Sec4p         | 154     | 13.3     | 5.0 × 10⁴  | (9)       |
| Gyp7p*              | Ypt7p         | 400     | 7.5      | 2.0 × 10⁰  | (6)       |
| Gyp7–47p*           | Ypt7p         | 42      | 30.0     | 7.8 × 10⁶  | (6)       |
| GST-Gyp6p           | Ypt6p         | 592     | 18.8     | 5.6 × 10⁰  | (6)       |

* Proteins were C-terminal His₆-tagged.

### TABLE I

Catalytic properties of Ypt-specific GTPase activating proteins

The K₉ and k₉ values of the different GAPs were measured at 30 °C.
Residues are numbered 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. 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 GAPase binding are shown at the top. Relevant amino acid residues are numbered.

Table II

| GST-Gyp6p | GDP hydrolysis ratea | Acceleration |
|-----------|----------------------|--------------|
| ___ | min⁻¹ | fold |
| Wild type | 0.0002 | — |
| R38A | 0.039 | 16,650 |
| R155A | <0.0002 | 0 |
| R155K | <0.0002 | 0 |
| R290A | 0.0035 | 18 |
| R298A | 0.044 | 220 |

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

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 III

| Interaction of Gyp6p and Ypt6p in the two-hybrid system | “Bait” plasmid | “Prey” plasmid | β-Galactosidase activitya |
|--------------------------------------------------------|----------------|----------------|--------------------------|
| pAS2 pACTII-GYP6 | 0.08 (± 0.05) | — | 0.09 (± 0.09) |
| pAS2 pACTII-GYP6(R155A) | 0.10 (± 0.09) | — | 0.01 (± 0.01) |
| pAS2-YPT6 | 1.60 (± 0.31) | — | 2.90 (± 0.90) |
| pAS2-YPT6 | 5.13 (± 1.73) | — | 2.30 (± 0.70) |
| pAS2-YPT6(G20S) | 0.46 (± 0.31) | — | 0.15 (± 0.05) |
| pAS2-YPT6(G20S) | 10.91 (± 3.75) | — | 3.10 (± 1.10) |
| pAS2-YPT6(Q69L) | 11.40 (± 0.15) | — | 3.20 (± 0.80) |
| pAS1-YPT6(Q69L) | 12.25 (± 4.69) | — | 3.30 (± 1.20) |

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. 4A). 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 GYP 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-32P]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-Gyp6p 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 Gyp6p proteins fused to Gal4

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transcription activation domain were coexpressed with Gal4-BD-Ypt6p or Gal4-BD-Ypt6p(Q69L)p, expression was confirmed by Western blotting analysis, and the interaction was analyzed using the X-gal filter test (Fig. 4B). The expression level of the truncated GAPs was comparable to that of full-length Gyp6p (data not shown). Very weak interaction of Ypt6p(Q69L)p but not wild-type Ypt6p was detected with Gyp6p(45–458)p. No interaction was observed with two larger N-terminal deletions or with the C-terminal truncation mutant Gyp6p(1–325)p, which lacked most of the sequences distal of the GYP domain (Fig. 1). These results suggest that the GAP 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 YFP family members, does not inhibit GAP activity nor does it affect substrate specificity (6). 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, Gyp6p(45–458)p and Gyp6p(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 Gyp6p(1–323)p) is not sufficient to allow binding to the substrate GTPase. Even Gyp6p(1–382)p with 72 amino acids following the GAP 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 ($K_m > 500 \mu M$) for its preferred substrate Ypt6p. In fact, with a $5 \times 10^6$-fold acceleration of the

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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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