Identification of the catalytic domains and their functionally critical arginine residues of two yeast GTPase-activating proteins specific for Ypt/Rab transport GTPases

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Ypt/Rab proteins constitute the largest subfamily of the Ras superfamily of monomeric GTPases and are regulators of vesicular protein transport. Their slow intrinsic GTPase activity (10⁻¹-10⁻³ min⁻¹ at 30°C) has to be accelerated to switch the active to the inactive conformation. We have identified the catalytic domain within the C-terminal halves of two yeast GTPase-activating proteins (GAPs), Gyp1p and Gyp7p, with specificity for Ypt/Rab GTPases. The catalytically active fragments of Gyp1p and Gyp7p were more active than the full-length proteins and accelerated the intrinsic GTP hydrolysis rates of their preferred substrates by factors of 4.5 × 10⁴ and 7.8 × 10⁵, respectively. The Kₘ values for the Gyp1p and Gyp7p active fragments (143 and 42 µM, respectively) indicate that the affinities of those GAPs for their substrates are very low. The catalytic domains of Gyp1p and Gyp7p contain five invariant arginine residues; substitutions of only one of them (R343 in Gyp1p and R458 in the analogously positioned Gyp7p) rendered the GAPs almost completely inactive. We suggest that Ypt/Rab–GAPs, like Ras– and Rho–GAPs, follow the same mode of action and provide a catalytic arginine (‘arginine finger’) in trans to accelerate the GTP hydrolysis rate of the transport GTPases.

Keywords: GTP hydrolysis/GTPase-activating protein/Rab GTPase/vesicular protein transport/Ypt GTPase

Introduction

Rab proteins, called Ypt in yeast, constitute the largest family of the Ras superfamily of monomeric GTPases. They are regulators of vesicular protein transport in all eukaryotic cells with different members of this family acting at defined steps of exo- and endocytic trafficking (Lazar et al., 1997; Novick and Zerial, 1997; Schimmöller et al., 1998, for a review).

Like the signal-transducing Ras and Rho proteins, Ypt/Rab GTPases cycle between a membrane-associated and a cytosolic state and they are active in a GTP-loaded form and rendered inactive by the hydrolysis of the bound GTP. The functional cycle involves several proteins, among them specific guanine nucleotide exchange factors (GEFs) which accelerate the dissociation of Ypt/Rab-bound GDP and its replacement by GTP (Horiuchi et al., 1997; Wada et al., 1997; Walch-Solimena et al., 1997; Hama et al., 1999), and GTPase-activating proteins (GAPs) which increase the slow intrinsic GTPase activity of the proteins (Strom et al., 1993; Vollmer and Gallwitz, 1995; Fukui et al., 1997; Du et al., 1998; Cuif et al., 1999; Vollmer et al., 1999). As the membrane-associated, GTP-bound forms of Ypt/Rab GTPases are supposed to interact with specific effector proteins (Shirakata et al., 1993; Ren et al., 1996; Diaz et al., 1997; Simonsen et al., 1997; Wang et al., 1997; Echard et al., 1998), and the hydrolysis of the GTPase-bound GTP makes the proteins extractable from membranes by the guanine nucleotide dissociation inhibitor (GDI) (Araki et al., 1990; Soldati et al., 1993; Ullrich et al., 1993), studies on the specificity and the mechanism of action of the Ypt/Rab protein regulators GEF and GAP are of prime importance.

Ras GTPase (p21 ras), the best-characterized member of the superfamily of small GTPases, is regarded as the prototype for all proteins of the family. Several GAPs for Ras have been isolated and characterized in great detail. They share several structural motifs termed Ras–GAP fingerprints (Trahey and McCormick, 1987; Xu et al., 1990; Maewaka et al., 1994; Cullen et al., 1995). The activity of p120-GAP can lead to an enhancement of the Ras intrinsic GTP hydrolysis rate by up to 10⁵-fold (Gideon et al., 1992). The combination of biochemical and structural data led to the so-called ‘arginine finger’ hypothesis (Ahmadian et al., 1997; Scheffzek et al., 1998) according to which an invariant arginine residue, situated in a loop structure of Ras–GAP, points into the active site of the GTPase and stabilizes the transition state of the GTPase reaction. This hypothesis has gained strong support from the atomic structure of a Ras–GDP–AlF₃–GAP complex (Scheffzek et al., 1997). Although Ras- and Rho-specific GAPs exhibit no primary sequence and only limited tertiary structure similarities, Rho–GAP uses a catalytic arginine residue in a way similar to that of Ras–GAP (Rittinger et al., 1997).

The first GAPs with specificity for Ypt/Rab GTPases, termed Gyp1p, Gyp6p and Gyp7p, were isolated from yeast using the strategy of high-expression cloning (Strom et al., 1993; Vollmer and Gallwitz, 1995; Vollmer et al., 1999). On the basis of the sequence relatedness of the Gyp proteins, Gyp1p was also identified in a database search by others and shown to enhance the GTP hydrolysis rate of several Ypt GTPases (Du et al., 1998). In contrast to Gyp1p, partially purified Gyp6p and Gyp7p exhibited clear substrate preference for Ypt6p and Ypt7p, respectively. Recently a human GAP acting on the Rab6 GTPase was identified and found to contain a central 200-amino-acid-long segment with sequence similarity to the yeast Gyp proteins (Cuif et al., 1999). The only other known mammalian Rab–GAP, apparently specific for Rab3 subfamily members, has a primary structure which is entirely
the catalytic domain of Ypt/Rab–GAPs (Fukui et al., 1997). All known Ypt/Rab–GAPs are not related in primary structure to either Ras– or Rho–GAPs.

In an attempt to characterize the transport GTPase-specific GAPs with respect to their catalytic properties, substrate specificities and binding affinities, we have delineated the catalytic domains of the yeast Gyp1p and Gyp7p and studied some of the kinetic properties of wildtype and mutant forms. We demonstrate that the Ypt/Rab–GAPs are similar to Ras–GAPs in their potency to accelerate the GTP hydrolysis rate of their substrate GTPases and, most likely, in their mechanism of action.

Results

The catalytic domains of Gyp1p and Gyp7 proteins

A sequence comparison of the Saccharomyces cerevisiae Ypt/Rab–GAPs Gyp1p and Gyp7p revealed that segments of highest homology are clustered in the C-terminal halves of the 637- and 746-amino-acid-long proteins. This is also true for the Gyp7 proteins from budding yeast and the evolutionarily very distant dimorphic yeast Yarrowia lipolytica (Vollmer et al., 1999). It therefore appeared that the catalytic domain was confined to a C-terminal region in all three GAPs.

This assumption was tested by deleting segments of varying length from the N- and C-terminal ends of Gyp1p and Gyp7p and determining the GAP activities of the truncated proteins after expression in yeast (Figure 1). GST fusions of Gyp1p and some of its truncated versions were catalytically active. As shown in Figure 1A, the N-terminal 248 amino acids of Gyp1p were dispensable for GAP activity, whereas the deletion of the N-terminal 300 or the C-terminal 104 amino acids rendered this GAP inactive. GST–Gyp1p and GST–Gyp1(249–637)p (referred to further as GST–Gyp1-46p, because of its molecular mass of 46 kDa) were affinity-purified from overexpressing S.cerevisiae. The yield and the purity of full-length GST–Gyp1p were significantly lower than those of GST–Gyp1-46p and varied from preparation to preparation. When the GAP activities were determined in crude preparations with comparable amounts of full-length Gyp1p and its active fragment (tested by Western blot analysis with anti-GST antibodies), it was noted that full-length GST–Gyp1p was always less active than the N-terminally truncated, active fragment GST–Gyp1-46p (data not shown). The same observation was made with Gyp7p (see below).

To test a possible effect of the GST protein fused to the N-terminus of Gyp1p-46p, we compared the specific activities of GST–Gyp1-46p and of Gyp1-46p from which the GST portion had been removed by thrombin cleavage. Using Ypt51p as substrate, the partially purified proteins like those shown in Figure 2A had similar specific activities (108 and 120 U/nmol GAP, respectively). The purity of these GAP preparations was evaluated by scanning Coomassie Blue-stained gels (see Materials and methods).

N- and C-terminal deletions of Gyp7p were constructed using naturally occurring restriction sites of the GYP7 gene. Surprisingly, Gyp7p lost its activity when fused with GST. However, active full-length and truncated Gyp7 proteins could be expressed in yeast as C-terminally His6-tagged proteins. They were analysed for GAP activity
using the Ypt7 GTPase as substrate. As can be seen in Figure 1B, all the N-terminally truncated versions tested that eliminated up to 358 amino acid residues (48% of Gyp7p) retained full GAP activity. As was the case for Gyp1p, the deletion of ~100 amino acids from the C-terminus of Gyp7p resulted in a complete loss of GAP activity. The active domain, Gyp7(359–745)–His6 (termed Gyp7-47p), was enriched by affinity purification to ~70% purity; full-length Gyp7–His6 prepared in the same way was ~80% pure (Figure 2B).

Notably, the truncated version had >10 times higher specific activity than the full-length Gyp7p (520 versus 42 U/nmol). This observation was reproduced in three independent experiments. Quantitative analysis of purified Gyp7(209–745)–His6 and Gyp7(263–745)–His6 revealed that the activity of these proteins was comparable to that of full-length protein (not shown). This suggests that the removal of amino acids 263–358 is responsible for the increased GAP activity of Gyp7-47p.

**Substrate specificity of Gyp1p and Gyp7p and their catalytically active fragments**

The budding yeast *S.cerevisiae* has 11 Ypt/Rab GTPases (Lazar et al., 1997), of which some (Ypt31p/Ypt32p and Ypt51p/Ypt52p/Ypt53p) are functionally redundant (Singer-Krüger, 1994; Benli et al., 1996). Using [γ32P]GTP-loaded Ypt1p, Ypt31p, Ypt32p, Sec4p, Ypt51p, Ypt53p, Ypt6p or Ypt7p as substrates and a Gyp1p-enriched yeast protein extract as enzyme source in a GAP filter assay, the intrinsic GTPase activity of Ypt51p, Ypt53p and Ypt1p (in this order) was accelerated significantly, whereas that of the others was activated only slightly or not at all. Comparable results were obtained with GST–Gyp1p and GST–Gyp1-46p. This was at odds with a recent study of Du et al. (1998), who found Sec4p to be the best substrate for partially purified Gyp1p. We therefore turned to a more quantitative assay in which GTP and GDP were quantified by HPLC analysis following incubation of GTP-loaded GTPases with purified Ypt/Rab–GAPs.

As the full-length GST–Gyp1p was difficult to express in reasonable amounts and purity, the catalytically active fragment was used to determine the substrate specificity. For the semiquantitative assay, 250 nM GST–Gyp1-46p was incubated with 20 µM GTPase–GTP complex. GTP hydrolysis was recorded with time. Initial rates of these reactions were compared with intrinsic hydrolysis rates of the tested GTPases that were determined under identical conditions. As shown in Table I, Sec4p and Ypt51p were the best substrates for the isolated catalytic domain, followed by Ypt7p and Ypt1p. Mammalian Rab1A protein also served as a very efficient substrate. The intrinsic GTP hydrolysis rates of Ypt51p and Ypt6p were not significantly accelerated. The same experimental protocol was used to determine the substrate specificity of purified C-terminally His6-tagged Gyp7p and its catalytic domain Gyp7-47p. As the catalytic fragment was more active, 20 nM Gyp7-47–His6 or 250 nM Gyp7–His6 were incubated with 20 µM substrate to obtain comparable activation rates. No difference in substrate specificity of full-length Gyp7p and its catalytic domain was observed. Both proteins showed clear preference for Ypt7p as substrate (Table II). Weak activation of Ypt6p and Ypt31p was also detected.

**Table I. Intrinsic and Gyp1p-induced rates of GTP hydrolysis of tested Ypt/Rab GTPases**

<table>
<thead>
<tr>
<th>GTPase</th>
<th>Intrinsic GTP hydrolysis rate (min−1)</th>
<th>GST–Gyp1-46p accelerated GTP hydrolysis rate (min−1)</th>
<th>Acceleration (+fold)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sec4p</td>
<td>0.0016 (± 0.00021)</td>
<td>0.2912</td>
<td>182</td>
</tr>
<tr>
<td>Ypt51p</td>
<td>0.0052 (± 0.00137)</td>
<td>0.8015</td>
<td>154</td>
</tr>
<tr>
<td>Rab1Ap</td>
<td>0.0029 (± 0.00044)</td>
<td>0.4427</td>
<td>153</td>
</tr>
<tr>
<td>Ypt7p</td>
<td>0.0023 (± 0.00067)</td>
<td>0.1839</td>
<td>80</td>
</tr>
<tr>
<td>Ypt1p</td>
<td>0.0025 (± 0.00088)</td>
<td>0.1742</td>
<td>70</td>
</tr>
<tr>
<td>Ypt6p</td>
<td>0.0002 (± 0.00013)</td>
<td>0.0010</td>
<td>5</td>
</tr>
<tr>
<td>Ypt31p</td>
<td>0.0064 (± 0.00129)</td>
<td>0.0104</td>
<td>2</td>
</tr>
</tbody>
</table>

*20 µM GTP-loaded GTPases were incubated at 30°C with or without 250 nM GST–Gyp1-46p. GTP hydrolysis rates were measured using the HPLC method as described in Materials and methods.

The GTP hydrolysis rates of Sec4p, Ypt1p and Ypt51p were not significantly activated.

**Catalytic properties of Gyp1p and Gyp7p**

The catalytic properties of the Ypt/Rab–GAPs were studied either by following classical Michaelis–Menten kinetics...
The catalytic domain of Ypt/Rab–GAPs

Fig. 4. Sequence alignment of the catalytically active domains of Gyp1p and Gyp7p. Shared motifs (A–F) according to Neuwald (1997) were aligned manually, intermediate regions using the CLUSTAL V program (Higgins et al., 1992). Identical residues are highlighted on a black, invariant arginines on a blue and the essential arginine on a red background. Conservative substitutions are shaded. Arrows indicate the start of the shortest active fragments identified.

Table II. Substrate specificity of Gyp7p

<table>
<thead>
<tr>
<th>GTPase</th>
<th>Intrinsic GTP hydrolysis rate (min⁻¹)</th>
<th>+250 nM Gyp7p–His6</th>
<th>+20 nM Gyp7-47p–His6</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ypt7p</td>
<td>0.0023</td>
<td>0.5332</td>
<td>232</td>
</tr>
<tr>
<td>Ypt6p</td>
<td>0.0002</td>
<td>0.0070</td>
<td>35</td>
</tr>
<tr>
<td>Yp31p</td>
<td>0.0064</td>
<td>0.0789</td>
<td>12</td>
</tr>
<tr>
<td>Sec4p</td>
<td>0.0016</td>
<td>0.0075</td>
<td>5</td>
</tr>
<tr>
<td>Ypt1p</td>
<td>0.0025</td>
<td>0.0069</td>
<td>3</td>
</tr>
<tr>
<td>Ypt51p</td>
<td>0.0052</td>
<td>0.0116</td>
<td>2</td>
</tr>
</tbody>
</table>

*20 µM GTP-loaded GTPase was incubated at 30°C with 250 nM full-length Gyp7 or 20 nM active fragment. GTP hydrolysis rates were measured using the quantitative GAP assay as described in Materials and methods. The values are representative of two independent measurements.

or by using the integrated Michaelis–Menten equation (Duggleby and Clarke, 1991), which allows determination of $K_m$ and $k_{cat}$ values from single time curves (described in Materials and methods). This equation was originally designed to analyse stable enzymes catalysing an irreversible reaction with a single substrate where none of the reaction products is inhibitory. It was also applied successfully in studies of the interaction of Ras with p120Ras–GAP (Schweins et al., 1996). As His₆-tagged full-length Gyp7 and its catalytic domain could be prepared in sufficient quantities and purity, $K_m$ and $k_{cat}$ values were determined for both.

For classical Michaelis–Menten analysis, 10 nM Gyp7-47–His₆ ($E_o$) was incubated at 30°C with substrate concentrations (Ypt7p–GTP) of 2.5–200 µM. The initial GTP hydrolysis rates determined from separate time curves were plotted as $V/E_o$ against the substrate concentrations (Figure 3A), allowing calculation of the $K_m$ (44 µM) and

the $k_{cat}$ (33.2 s⁻¹) of the Gyp7 active domain. This means that the intrinsic GTP hydrolysis rate of Ypt7p (0.0023 min⁻¹) is accelerated by a factor of $8.7 \times 10^5$. For the determination of $K_m$ and $k_{cat}$ values using the integrated Michaelis–Menten equation, 20 nM Gyp7-47p was incubated with 100 µM GTP-loaded Ypt7p. From the time curve shown in Figure 3B, the $K_m$ and $k_{cat}$ values were calculated to be 40 µM and 25.8 s⁻¹, respectively, which is in good agreement with the values obtained by the classical method. Kinetic constants of the full-length Gyp7 were determined from single time curves obtained by incubating 200, 100 and 20 µM Ypt7p–GTP with either 125 nM or 250 nM Gyp7–His₆. Using the integrated Michaelis–Menten equation, $K_m$ values between 354 and 462 µM, and $k_{cat}$ values between 6.6 and 8.6 s⁻¹ were obtained, suggesting that the higher GAP activity of the isolated Gyp7 catalytic domain appears to be due primarily to its higher affinity for the substrate GTPase.
Fig. 5. Mutational analysis of conserved arginines in Gyp1p. (A) Crude extracts from yeast cells overexpressing GST fusions with the Gyp1p catalytic domain carrying the substitutions indicated were subjected to SDS–PAGE. GST, a tagged version of the non-mutated Gyp1p fragment, and the mutant forms were identified using an anti-GST antibody. (B) GAP activities of the proteins shown in (A) were determined with the filter assay using Ypt51p as substrate.

The catalytic properties of Gyp1p could be determined only for its active domain. This was purified from over-expressing yeast either as C-terminally His6-tagged protein or as GST fusion and then released by thrombin cleavage. $K_m$ (143 ± 10 µM) and $k_{cat}$ values (3.9 ± 2.5 s$^{-1}$) were calculated from single time curves obtained by incubating 100–200 µM Ypt51p–GTP, one of the preferred substrates of Gyp1p, with 250 nM Gyp1-46p. Given the intrinsic GTPase hydrolysis rate of Ypt51p (0.0052 min$^{-1}$; Table I), GTPase hydrolysis was accelerated by the Gyp1 catalytic domain by a factor of 4.5 $\times$ 10$^4$.

The dissociation of GTP from the GTPases used to determine the catalytic properties of the two GAPs was negligible and almost certainly could not have affected the kinetic parameters. The nucleotide dissociation was evaluated in a filter-binding test by measuring the time-dependent loss of radioactivity from $[\alpha^{32}\text{-}P]$GTP-loaded Ypt proteins (data not shown).

Mutational analysis of conserved arginines in the catalytic domain of Gyp1p and Gyp7p

Alignment of Gyp1p and Gyp7p revealed five conserved arginines within their catalytic domain (Figure 4). As specific arginine residues are essential for the catalytic activity of Ras– and Rho–GAPs (Rittinger et al., 1997; Scheffzek et al., 1997), it appeared possible that the GTPase activation mediated by Ypt/Rab–GAPs follows the same basic mechanism. The catalytic fragments of Gyp1p and Gyp7p were therefore subjected to a mutational analysis and all conserved arginine residues were substituted for either lysine or alanine.

The mutant forms of Gyp1p and Gyp7p were produced in yeast as GST fusion proteins. For simplicity, the filter assay was used first to assess the GAP activity of different mutants with protein extracts from positive transformants (Figure 5A) and either $[\gamma^{32}\text{-}P]$GTP-loaded Ypt51p or Ypt1p as substrate. As can be seen in Figure 5B, mutations to either lysine or alanine of two (R343 and R490) out of the five arginines led to a significant loss of GAP activity. In contrast, the corresponding substitutions of R286, R482 and R567 did not appear to alter Gyp1p catalytic activity significantly. The GST–Gyp1-46p mutant proteins whose GAP activities were most severely affected (substitutions R343A, R343K, R490A and R490K) were purified by affinity chromatography (Figure 6A) and their catalytic activities measured with Ypt51p–GTP as substrate. Single time curves of GTP hydrolysis (Figure 6B) were evaluated as described for the specificity test. It was found that...
GST–Gyp1-46(R490A)p activated the GTP hydrolysis rate about 33-fold, which is eight times less than the activation rate of the non-mutated protein. The decrease in GAP activity of GST–Gyp1-46(R490K)p was less pronounced: it had only four times lower activity than the wild-type protein. Importantly, substitution of R343 for either alanine or lysine led to Gyp1 proteins unable to accelerate the rate of hydrolysis of Ypt51p-bound GTP (Figure 6B). Measured GTP hydrolysis rates were identical with the intrinsic GTPase activity of Ypt51p even when measured at 10 times higher concentrations (2.5 µM) of mutant Gyp1-46 protein (not shown).

Substitutions of the conserved arginines to either alanine or lysine were also introduced into Gyp7-47p and evaluated with respect to their effect on GAP activity. His6-tagged mutant proteins having either R391, R458, R607, R615 or R683 substituted with alanine, or R458 or R615 replaced by lysine, were affinity-isolated from yeast and obtained with similar yield and purity (~50–70%) (Figure 7A). We noted, however, that the yield of Gyp7-47(R391A)p and of the corresponding Gyp1 mutant protein, Gyp1-46(R286A)p, was always low. The specific activities of all Gyp7-47 mutant proteins were clearly reduced (Figure 7B). Most importantly, the substitutions of R615 (which corresponds to R490 in Gyp1p) led to a severe loss of Gyp7 GAP activity, and substitutions of R458 (which corresponds to the essential R343 in Gyp1p) resulted in a protein completely inactive under the conditions tested. This is best documented by the time curves shown in Figure 7C, from which it was also calculated that substitutions of R615 resulted in a reduction of GAP activity by a factor of ~30.

The apparent inactivation by a specific arginine substitution of Gyp1p and of Gyp7p posed the question as to whether the interactions of the mutant proteins with their substrate GTPases were affected. This was investigated with the His6-tagged Gyp7-47(R458K) mutant protein, which could be easily produced in a soluble, non-aggregated form from yeast and purified to >90% by affinity chromatography on Ni²⁺-agarose, followed by gel filtration on Sephacryl S-200 and MonoQ ion exchange chromatography (Figure 8A). A classical Michaelis–Menten analysis was performed at a mutant protein concentration as high as 15 µM (Figure 8B). It was found that Gyp7-47(R458K)p was not completely inactive: the $k_{cat}$ was determined to be 0.019 s⁻¹, which means a 1.5 × 10³-fold reduction of GAP activity of the mutant compared with the wild-type protein (Table III). From the $K_m$ determined (125 µM), which is about three times higher than that of the wild-type protein, it follows that the interaction of the mutant Gyp7 catalytic domain with its substrate is only moderately affected.

**Discussion**

The replacement of Ras-protein-bound GDP by GTP is accompanied by a conformational switch which allows the GTPase to bind to its effector Raf kinase. The hydrolysis of GTP to GDP results in the dissociation of the GTPase–effector complex and terminates the act of signal transduction (for a review, see Wittinghofer and Nassar, 1996). GTP hydrolysis, therefore, is most critical for the regulatory role(s) that Ras proteins fulfil in eukaryotic cells. Ypt/Rab GTPases likewise adopt their active conformation in the GTP-bound state, which then allows them to associate with various effector proteins (Diaz et al., 1997; Simonsen et al., 1997; Echard et al., 1998; Ostermeier and Brunger, 1999). The highly conserved nucleotide-binding motif and the remarkable overall sequence similarities of Ras and Ras-related proteins...
suggest that the conformational switch mechanism of Ras and Ypt/Rab regulators is also conserved. The switch from the GTP- to the GDP-bound conformations of Ras and Ypt/Rab proteins involves specific GTPase-activating proteins required to significantly accelerate the slow intrinsic hydrolysis rates of the GTPases. The recently solved crystal structure of the GTP-bound form of Rab3A (Dumas et al., 1999; Ostermeier and Brunger, 1999) shows that the binding characteristics of the guanosine nucleotide and the magnesium ion are almost identical in Ras and Rab proteins. It was noticed, however, that the side chains of serine residues conserved in the P-loop and the switch I region of Ypt/Rab GTPases (S31 and S53 in Rab3A, which are analogous to G12 and P34, respectively, in p21 Ras) would be sterically unfavourable for the insertion of a potential catalytic arginine residue provided by a Rab–GAP. It was discussed (Dumas et al., 1999; in contrast to Ras– and Rho–GAPs, which provide a catalytic arginine (Rittinger et al., 1997; Scheffzek et al., 1997). Ypt/Rab–GAPs might accelerate GTP hydrolysis by stabilizing the transition state conformation of the switch regions in the way in which RGS4 activates G\textsubscript{at} (Tesmer et al., 1997; Srinivasa et al., 1998).

As we show in this report, the catalytically active domains of two yeast GTPase-activating proteins with specificity for Ypt/Rab GTPases contain five arginines of which one (R343 in Gyp1p and R458 in Gyp7p) proved absolutely essential for GAP activity. The almost complete loss of GAP activity following replacement of these arginine residues with either alanine or lysine was not due to either instability or aggregation of the mutant proteins. This was seen in the case of the Gyp7-47(R458K) mutant protein, which was perfectly soluble, behaved normally on gel filtration and had only a moderately affected affinity (3-fold higher \( K_m \)) for its substrate GTPase Ypt7p. For the acceleration of the slow intrinsic GTP hydrolysis rates of their substrate GTPases, R343 of Gyp1p and R458 in the corresponding position of Gyp7p (Figure 4) are likely to function in a way analogous to the finger-arginine residue in Ras– and Rho–GAPs. One might even speculate that the conserved R490 in Gyp1p and R615 in Gyp7p, whose conservative and non-conservative substitutions alike diminished GAP activity significantly, could play a role in stabilizing the ‘arginine finger’ loop similar to the invariant R903 and K122 in Ras–GAP and p50Rho–GAP, respectively (Rittinger et al., 1997; Scheffzek et al., 1997, 1998). Although this has to await the elucidation of the structure of a Ypt/Rab GTPase–Gyp protein complex, it now appears a strong possibility that Ras–, Rho– and Ypt/Rab–GAPs share a common mode of action.

Our study shows that the two yeast Ypt/Rab–GAPs are very potent activators and accelerate the intrinsic GTPase activity of their substrates by several orders of magnitude. The degree of activation compares well with that deter-
mined for other monomeric GTPases and their activating proteins (Table III). The finding that the catalytic domains of Gyp1p and Gyp7p, constituting ~60 and 50% of the two GAPs, respectively, were significantly more active than the full-length proteins has the interesting implication that the N-terminal regions of both GAPs might serve a role in regulating the activity through the interaction with other proteins. As implicated from the $K_{m}$ values (Table III), the affinities of the Gly proteins for their preferred substrates are very low. Therefore, the recruitment of Ypt/Rab–GAPs to specific membranes, where the local concentration of substrate GTPases would be high, appears to be necessary for the GAPs to function and might involve their N-terminal regions.

As we have shown here, the N-terminal halves of Gyp1p and Gyp7p, which are dispensable for GAP activity, do not appear to influence the substrate specificity, at least in vitro. Neither Gyp1p nor Gyp7p is specific for a given Ypt GTPase. Most surprisingly, Gyp1p as full-length protein (Du et al., 1998) and as isolated catalytic domain shown here accelerated the intrinsic GTP hydrolysis rates of exocytic (Sec4p) and endocytic GTPases (Ypt51p) with comparable efficiency. In a previous report using crude yeast extracts and a GAP filter assay, Gyp7p was found to activate significantly Ypt7p and Ypt31p/Ypt32p (Voellmer et al., 1999). However, the quantification of GAP activity of the purified Gyp7 full-length protein and its catalytic domain performed in the present study demonstrate that Ypt7p is by far the best substrate, and Ypt6p and Ypt31p are only marginally activated. Nevertheless, in vitro both GAPs are promiscuous with respect to their substrate GTPases. If this were also true within the cell, the lack of protein transport defects in yeast cells carrying GAP gene deletions (Strom et al., 1993; Du et al., 1998; Voellmer et al., 1999) could be explained by the compensation of a given Ypt/Rab–GAP by another member of a larger family. In fact, sequence alignment revealed that yeast contains at least five other proteins related to Gyp1p, Gyp6p and Gyp7p (Neuwald, 1997). Importantly, the essential arginine we have identified in Gyp1p and Gyp7p, and suggested to play a role comparable to the catalytic finger arginine in Ras– and Rho–GAPs, is present in all of them.

### Materials and methods

#### Cloning of the GYP1 gene

All cloning procedures were performed using standard protocols (Sambrook et al., 1989). A 2µ-based multicopy yeast genomic library was prepared and screened for Ypt/Rab–GAP containing genes as previously described (Voellmer and Gallwitz, 1995). The GYP1 gene was originally identified on an 8 kb DNA fragment whose high expression led to an acceleration of the Ypt1p GTPase activity. The candidate reading frame (YOR076c) was amplified and cloned under stringent PCR conditions using two primers flanking the coding region and containing the recognition sites for restriction endonucleases BamHI and HindIII (underlined), respectively. Primer #1 contained an additional NcoI restriction site (bold characters) that overlaps the ATG initiation codon of GYP1 gene: primer #1: 5′-CAATTGACTG-GAGATCATAATGGGTGAGATCCCCTGC-3′; primer #2: 5′-TAC-ACGGATACAGGCTTGTTATACGACGTCGAC-3′. Amplification was performed in 100 µl volume with 1 µg of plasmid DNA template, 100 pmol of both primers and 20 mmol of dNTPs. DNA polymerase with proofreading activity, Deep Vent (New England Biolabs), was added after initial denaturation (hot start). Thirty cycles (94°C, 30 s; 53°C, 30 s; 72°C, 3.5 min) were performed. Amplification products were purified on a Quiagick column (QIAGEN), digested with BamHI and HindIII, and ligated into BamHI- and HindIII-cleaved pYES2 T–His6 (modified Invitrogen pYES2 vector) template. The PCR product was cleaved with restriction endonucleases HpaI and BamHI and inserted into pET3a-GYP7 (Vollmer and Gallwitz, 1995) as template. The PCR product was cleaved with restriction endonucleases HpaI and BamHI and inserted into pET3a-GYP7 linearized with the same enzymes. The Ndel–BanHI fragment from modified pET3a-GYP7 was cloned into pYES2–His6 (modified InVitrogen pYES2 vector bearing six histidine codons and single Ndel and BamHI sites) to allow C-terminally His$_6$-tagged Gyp7p to be produced in yeast. The GYP1 coding sequence was elongated with six histidine codons by PCR using GYP1 primer #3 (5′-CTATAGATCTGCAGTATGGTCTGTTG-3′). PCR reactions were run for each deletion, and products of those were cloned into pET3a. GYP1-139: 5′-ACGGTGTTTACAGCCAGTGCGACG-3′; GYP1-248: 5′-GGATCCCACTTTTCGCTAGCGGA TA TGTGC-3′; GYP1-318: 5′-ACGGTGTTTACAGCCAGTGCGACG-3′.

#### Site-directed mutagenesis of GYP1 and GYP7

Change of arginine to alanine or lysine codons in GYP1 and GYP7 was achieved using a PCR-based overlap extension method (Ho et al., 1989). A 2µ-based multicopy yeast genomic library was prepared and screened for Ypt/Rab–GAP containing genes as previously described (Voellmer and Gallwitz, 1995). The GYP1 gene was originally identified on an 8 kb DNA fragment whose high expression led to an acceleration of the Ypt1p GTPase activity. The candidate reading frame (YOR076c) was amplified and cloned under stringent PCR conditions using two primers flanking the coding region and containing the recognition sites for restriction endonucleases BamHI and HindIII (underlined), respectively. Primer #1 contained an additional NcoI restriction site (bold characters) that overlaps the ATG initiation codon of GYP1 gene: primer #1: 5′-CAATTGACTG-GAGATCATAATGGGTGAGATCCCCTGC-3′; primer #2: 5′-TAC-ACGGATACAGGCTTGTTATACGACGTCGAC-3′. Amplification was performed in 100 µl volume with 1 µg of plasmid DNA template, 100 pmol of both primers and 20 mmol of dNTPs. DNA polymerase with proofreading activity, Deep Vent (New England Biolabs), was added after initial denaturation (hot start). Thirty cycles (94°C, 30 s; 53°C, 30 s; 72°C, 3.5 min) were performed. Amplification products were purified on a Quiagick column (QIAGEN), digested with BamHI and HindIII, and ligated into BamHI- and HindIII-cleaved pYES2 T–His6 (modified Invitrogen pYES2 vector) template. The PCR product was cleaved with restriction endonucleases HpaI and BamHI and inserted into pET3a-GYP7 linearized with the same enzymes. The Ndel–BanHI fragment from modified pET3a-GYP7 was cloned into pYES2–His6 (modified InVitrogen pYES2 vector bearing six histidine codons and single Ndel and BamHI sites) to allow C-terminally His$_6$-tagged Gyp7p to be produced in yeast. The GYP1 coding sequence was elongated with six histidine codons by PCR using GYP1 primer #3 (5′-CTATAGATCTGCAGTATGGTCTGTTG-3′). PCR reactions were run for each deletion, and products of those were cloned into pET3a. GYP1-139: 5′-ACGGTGTTTACAGCCAGTGCGACG-3′; GYP1-248: 5′-GGATCCCACTTTTCGCTAGCGGA TA TGTGC-3′; GYP1-318: 5′-ACGGTGTTTACAGCCAGTGCGACG-3′.
R482K: 5
9
on glutathione–Sepharose (Pharmacia) as described (Grabowski and
5224
galactose-containing media. GST fusion proteins were affinity purified
E.coli
by sequence analysis.
R615K: 5
R615A: 5
R607A: 5
GYP7:
R490K: 5
R567K: 5
R567A: 5
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GAP activity in crude cell extracts was determined with \([\gamma^{32P}]GTP-
S.A.Gal,
200 µl) of reaction


gene. We thank Mohammad Reza Ahmadian (Dortmund) for introducing to us the
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References
Ahmadian, M.R., Wiesmüller, L., Lautwein, A., Bischoff, F.R., and
Wittinghofer, A. (1996) Structural differences in the minimal catalytic
domains of the GTPase-activating proteins p120GAP and
Confirmation of the arginine-finger hypothesis for the GAP-stimulated
of the ras protein, to synaptic plasma membranes and vesicles by its specific
interaction with GAP at the concentrations used. The approach and the manner in which it is
implicated are similar to those when using the integrated Michaelis–Menten equation (Duggleby and Clarke, 1991), but with the advantage that it is generally applicable, i.e. not only when the substrate (Ypt/Rab–
GTP) is in large excess over the catalyst (GAP).

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Analysis of GAP–GTPase interaction
In order to obtain \(K_m\) and \(k_{cat}\) values from single time curves, the
program ‘Scientist’ (Micromath, Salt Lake City, UT) was used. The
model equation file used defines the concentration of the GAP–Ypt/Rab–
GTP complex as a function of the \(K_m\) value and the concentrations of
GAP and Ypt/Rab–GTP; and the rate at a given time by the product of
the concentration of the ternary complex and \(k_{cat}\). The rate is entered as
differential equation into the model file, which also contains equations
defining the distribution of concentrations among the various species.
The fitting procedure involves numerical integration and simulation, and
leads to a representation of the concentration of Ypt/Rab–GTP as a function of
time. For this procedure, the reasonable assumption is made that
the reaction product (Ypt/Rab–GDP) does not interact with GAP at
the concentrations used. The approach and the manner in which it is
implicated are similar to those when using the integrated Michaelis–
Menten equation (Duggleby and Clarke, 1991), but with the advantage that it is generally applicable, i.e. not only when the substrate (Ypt/Rab–
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