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Distinct Subclasses of Small GTPases Interact with Guanine Nucleotide Exchange Factors in a Similar Manner

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The Ras-related GTPases are small, 20- to 25-kDa proteins which cycle between an inactive GDP-bound form and an active GTP-bound state. The Ras superfamily includes the Ras, Rho, Ran, Arf, and Rab/YPT1 families, each of which controls distinct cellular functions. The crystal structures of Ras, Rac, Arf, and Ran reveal a nearly superimposible structure surrounding the GTP-binding pocket, and it is generally presumed that the Rab/YPT1 family shares this core structure. The Ras, Rac, Ran, Arf, and Rab/YPT1 families are activated by interaction with family-specific guanine nucleotide exchange factors (GEFs). The structural determinants of GTPases required for interaction with family-specific GEFs have begun to emerge. We sought to determine the sites on YPT1 which interact with GEFs. We found that mutations of YPT1 at position 42, 43, or 49 (effector loop; switch I), position 69, 71, 73, or 75 (switch II), and position 107, 109, or 115 (alpha-helix 3–loop 7 [α3-L7]) are intragenic suppressors of dominant interfering YPT1 mutant N22 (YPT1-N22), suggesting these mutations prevent YPT1-N22 from binding to and sequestering an endogenous GEF. Mutations at these positions prevent interaction with the DSS4 GEF in vitro. Mutations in the switch II and α3-L7 regions do not prevent downstream signaling in yeast when combined with a GTPase-defective (activating) mutation. Together, these results show that the YPT1 GTPase interacts with GEFs in a manner reminiscent of that for Ras and Arf in that these GTPases use divergent sequences corresponding to the switch I and II regions and α3-L7 of Ras to interact with family-specific GEFs. This finding suggests that GTPases of the Ras superfamily each may share common features of GEF-mediated guanine nucleotide exchange even though the GEFs for each of the Ras subfamilies appear evolutionarily unrelated.

The small GTPases of the Ras superfamily are involved in regulating many intracellular processes, including cell growth and division, cell morphology and movement, vesicular transport, and nuclear events (4, 40, 41). These proteins, which act as molecular switches to control various functions in the cell, are in the active, or "on," state when bound to GTP and the inactive, or "off," state when bound to GDP. The immediate control of these GTPase-mediated events resides in the proteins which regulate their GTP- or GDP-binding status. Two classes of regulatory proteins have been identified: the guanine nucleotide exchange factors (GEFs), whose physiological function is to convert GTPases from a GDP-bound state to a GTP-bound state, and the GTPase-activating proteins (GAPs), which turn off the GTPases by activating an intrinsic GTPase activity (3, 42, 44). The GEFs stimulate guanine nucleotide release to yield a GEF–apo-GTPase reaction intermediate and, in part because the GTP concentration in cells is higher than that of GDP, the formation of active GTP-bound GTPase is favored (61).

Most of our understanding of the physical interaction of these regulatory molecules with the small GTPases is based on studies of the Ras protein (3, 42–44). For example, it is known that Ras GAPs bind to the effector loop of Ras (3, 42–44). The Ras effector loop, comprising residues 30 to 45, also interacts with the known downstream targets of Ras (42–44, 79).

Numerous groups have contributed to the effort to identify Ras residues which are involved in interactions with GEFs. Residues 62 to 75 in the switch II region of H-ras were found to be involved, as were residues 103 and 105 in the alpha-helix 3–loop 7 (α3-L7) region (16, 38, 49, 57, 59, 60, 68, 69, 73). The effector loop (switch I region) of Ras was also implicated in direct interactions with GEFs (5, 38, 47, 79). The switch I, switch II, and α3-L7 regions of H-ras are found adjacent to each other on the surface of the molecule, as would be expected for a surface domain involved in GEF binding (see Fig. 7) (36). The recently described crystal structure of H-ras complexed with Sos demonstrates that each of these three regions is indeed at the interface of the Ras-Sos complex (5).

Ras GEFs exhibit a modest preference for binding GDP-bound forms of Ras, whereas Ras GAPs preferentially bind GTP-bound forms (28, 37, 45, 49, 74). Thus, the GEFs and GAPs which affect the nucleotide-binding status of Ras preferentially bind their respective substrates rather than their products. The high affinities for substrates likely reflect structural differences between the two nucleotide-bound forms of Ras. Significantly, the switch I and switch II regions of H-ras, known to have altered structures when bound to either GDP or GTP, fall within the regions implicated in interactions with GEFs and GAPs (66).

Recently, the crystal structure of the Sec7 domain of human Arno, a GEF for the Arf GTPase, and an analysis of the interaction sites of these two proteins have been reported (48). The analysis revealed that Arf interacts with its exchange factor in a manner reminiscent of the Ras interaction with its GEFs. Arf appears to use three noncontiguous segments of its polypeptide to interact with Sec7. Importantly, these three regions of the Arf protein are analogous to those used by Ras to interact with its GEFs. The switch I region (effector loop) and switch II region of Arf and Ras interact with their GEFs (5, 38, 47, 48, 79). Also, Ras residues 103 to 105 in the α3-L7 region and the corresponding residues of Arf (residues 113 to

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115) appear to bind GEFs (5, 24, 48, 68, 69). While the GEF-binding sequences of Arf and Ras are at analogous positions in the GTPases, GEF-binding sequences of Ras do not show homology with the Arf sequences. The finding that these two distantly related GTPases use analogous regions to interact with their GEFs raises several questions relating to other subclasses of GTPases. For example, do the Rho and Rab/YPT1 families of GTPases interact with their GEFs by using domains analogous to those used by Ras and Arf? Do the different families of GEF use a similar mechanism for catalyzing guanine nucleotide exchange on small GTPases?

We undertook the present study to ask whether other small GTPases use the regions corresponding to the GEF-binding domain of H-ras to interact with their cognate GEFs. For this study, we chose the yeast YPT1 protein, which is a member of the Rab family of small GTPases (22, 29, 70). This family of proteins is involved in regulating vesicular transport (54, 55). Previously we used a yeast genetic screen to identify Ras residues which were involved in binding to Ras GEFs (49). This screen uses both a dominant interfering mutant and a constitutively active mutant of Ras. Here we created analogous YPT1 mutants and demonstrated that they could be used in a similar genetic screen. We demonstrated that the mechanism of dominant interference of YPT1 mutant N22 (YPT1-N22) is sequestration of an endogenous essential GEF for YPT1 such that a lethal phenotype occurs because endogenous YPT1 cannot be activated. Using both site-directed and random mutagenesis procedures, we identified a series of intragenic suppressors of YPT1-N22, among which we predicted would be mutants which fail to sequester essential GEFs for YPT1 due to the loss of a complete GEF-binding domain.

Among the intragenic suppressor mutations, we identified 10 residues, at positions 42, 43, 49, 69, 71, 73, 75, 107, 109, and 115, which were involved in in vitro binding to DSS4, a GEF which can stimulate nucleotide exchange on YPT1 in vitro (10, 50). The positions of these residues correspond to the switch I, switch II, and α3-L7 regions of Ras, the same regions found to be important for Ras interaction with GEFs.

Our findings suggest that the interaction of Ras with its specific GEFs may prove to be a useful model for analyzing the structural basis underlying the interaction of other small GTPases with their cognate GEFs. Further, our findings, together with an analysis of the interactions of Ras and Arf GTPases with their GEFs, indicate that small GTPases of the Ras superfamily use similar regions for interactions with GEFs, suggesting a similar catalytic mechanism of guanine nucleotide exchange for all small GTPases.

MATERIALS AND METHODS

Molecular cloning of YPT1 and DSS4. Wild-type YPT1 and the mutants YPT1-V17 and YPT1-N22 were amplified by PCR and subcloned into the yeast expression vector pYES2 (Invitrogen) for yeast genetic experiments and into the *Escherichia coli* expression vector pRSETA (Invitrogen) or pGEX-2T (Pharmacia) for the generation of histidine (His)-tagged fusion proteins or glutathione *S*-transferase (GST) fusion proteins, respectively. Plasmids harboring the original wild-type YPT1 and YPT1-V17 alleles were obtained from Sara Jones and Nava Segev. YPT1-N22 was generated by site-directed mutagenesis of wild-type YPT1 DNA by PCR as described below. pYES2 contains the yeast 2μm origin of replication, the *GAL1* promoter, and *URA3* as a selectable marker. Yeast genomic DSS4 DNA was amplified by PCR, subcloned into pBluescript (Stratagene), and then further subcloned into the yeast expression vector pAD4 in the correct (DSS4) and incorrect (rev-DSS4) orientations and into the *E. coli* expression vector pMAL (New England Biolabs) for production of the maltose-binding protein (MBP) fusion MBP-DSS4. pAD4 contains the yeast 2μm origin of replication, the alcohol dehydrogenase (ADH) promoter, and *LEU2* as a selectable marker. All constructs made by PCR were verified by DNA sequence analysis.

Random hydroxylamine mutagenesis of YPT1-N22. Hydroxlamine mutagenesis was performed as described before (49). Briefly, 10 μg of pYES2 YPT1-N22 DNA in 400 μl of 0.25 M K_2PO_4 (pH 6.0)–5 mM EDTA was mixed with 800 μl of freshly made 1.0 M hydroxlamine-HCl in 0.4 N NaOH and heated for 1 h at 75°C. After dialysis overnight at 4°C against three changes of 2 liters of 10 mM Tris-HCl (pH 8.0)–1 mM EDTA, the DNA was precipitated with 2.5 volumes of 100% alcohol and washed in 70% alcohol. The precipitated DNA was recovered by centrifugation, dried under vacuum, dissolved in 20 μl of 10 mM Tris-HCl (pH 8.0)–0.1 mM EDTA, and then used to generate an amplified library of mutagenized plasmid DNA in *E. coli* DH5α.

Site-directed mutagenesis of YPT1. Second-site mutations in YPT1-N22 were generated by PCR with oligonucleotide primers containing base substitutions as described before (49, 56). Overlapping PCR fragments each containing the new substitution were generated and then combined in a second round of PCR with oligonucleotide primers flanking the intact coding sequence and encoding *Hin*dIII and *Bam*HI restriction sites at the 5′ and 3′ ends of YPT1, respectively. The resulting fragments containing both N22 and the new YPT1 mutations were subcloned into *Hin*dIII-*Bam*HI-digested pYES2. New mutations were confirmed by DNA sequence analysis (K68, C71, T73, L75, C79, K105, A107, D109, I111, A113, and R115) and then transferred from the pYES2 YPT1-N22 plasmids into pYES2 YPT1 (wild type) or pYES2 YPT1-V17 via a *Mun*I-*Spe*I restriction fragment (codons 40 to 151) and into pRSETA YPT1 (wild type) via a *Mun*I-*Xho*I restriction fragment (codons 40 to 206). pYES2 constructs were used for yeast genetic experiments, and pRSETA constructs were used to produce His-tagged fusion proteins in *E. coli* BL21(DE3).

Suppression of the dominant interfering lethal phenotype of YPT1-N22 by overexpression of DSS4. Lithium acetate-competent cells of yeast strain W303-1B (*MAT*α *ade2 can1 his3 leu2 trp1 ura3*) (from Doug Johnson) (81) were transformed simultaneously with pYES2 plasmid DNA carrying YPT1-N22 and pAD4 plasmid DNA carrying DSS4 in either the correct (DSS4) or the incorrect (rev-DSS4) orientation or the empty pAD4 plasmid. The transformed cells were spread on synthetic complete (SC) medium without uracil and leucine ($SC^{-Ura-Leu}$ medium) but containing 2% glucose and incubated for 3 to 4 days at 37°C. Two to four independent colonies from each transformation were patched on $SC^{-Ura-Leu}$ medium containing either 2% glucose or 2% galactose and incubated for 3 to 4 days at 37°C. Growth of transformants on galactose medium at 37°C was an indication that overexpression of DSS4 had suppressed the dominant interfering lethal phenotype of YPT1-N22.

Selection of intragenic suppressors of YPT1-N22. Lithium acetate-competent cells of yeast strain W303-1B were transformed with hydroxylamine-mutagenized pYES2 YPT1-N22 plasmid DNA, spread on SC^{-Ura} medium containing 2% glucose, and incubated for 3 days at 28°C. About 10,000 transformants were replica plated onto SC^{-Ura} medium containing either 2% glucose or 2% galactose and incubated for 3 to 4 days at 37°C. Individual transformants picked from the galactose medium were streaked on the same medium and incubated for 3 days at 37°C. Plasmid DNA recovered from these transformants was amplified in *E. coli* DH5α and then used to transform yeast strain W303-1B. In each case, the plasmid DNA conferred the ability to suppress the lethal phenotype of the dominant interfering YPT1-N22 mutation. New YPT1 mutations were identified by DNA sequence analysis (K42, E42, M43, I49, C69, R83, N89, I91, L95, and I101) and then transferred into pYES2 YPT1 (wild type), pYES2 YPT1-V17, or pGEX-2T YPT1 (wild type) via a *Mun*I-*Spe*I restriction fragment (codons 40 to 151) and into pRSETA YPT1 (wild type) via a *Mun*I-*Xho*I restriction fragment (codons 40 to 206).

Suppression of the dominant interfering lethal phenotype of YPT1-N22 by site-directed mutations. Lithium acetate-competent cells of yeast strain W303-1B were transformed with pYES2 YPT1 plasmids containing the N22 mutation and one of the site-directed mutations described above (K68, C71, T73, L75, C79, K105, A107, D109, I111, A113, and R115). The cells were then spread on SC^{-Ura} medium containing 2% glucose and incubated for 3 to 4 days at 28°C. Three independent colonies from each transformation were patched on SC^{-Ura} medium containing 2% glucose or 2% galactose and incubated for 3 to 4 days at 37°C. Growth of transformants on galactose medium at 37°C was an indication that the site-directed mutation had suppressed the dominant interfering lethal phenotype of the YPT1-N22 mutation.

Suppression of the loss of YPT1 function in temperature-sensitive yeast strains. Lithium acetate-competent cells of the temperature-sensitive yeast strain NSY161 (*MAT*α *his4-539 ura3-52 ypt1-A136D*) (from Sara Jones and Nava Segev) (32) were transformed with the pYES2 empty vector or pYES2 plasmid DNA carrying YPT1 (wild type) and a YPT1 mutation (C69, C71, T73, L75, A107, D109, or R115) or pYES2 plasmid DNA carrying YPT1-V17 and a YPT1 mutation (K68, C69, C71, T73, L75, C79, R83, N89, I91, L95, I101, K105, A107, D109, I111, A113, or R115). The cells were then spread on SC^{-Ura} medium containing 2% glucose and incubated for 3 to 4 days at 28°C. Two independent colonies from each transformation were patched on SC^{-Ura} medium containing 2% glucose (YPT1-V17 or wild type YPT1 with one of the mutations), 2% galactose (YPT1-V17 with one of the mutations), or 1.99% glycerol plus 0.01% galactose (wild-type YPT1 with one of the mutations) and incubated for 3 to 4 days at 37°C. Growth of transformants on galactose medium at 37°C was an indication that the pYES2 YPT1 mutant plasmid had suppressed the loss of YPT1 function in yeast.

Preparation of His-tagged YPT1, His-tagged H-ras, GST-YPT1, and MBP-DSS4 proteins. Wild-type H-ras, wild-type YPT1, and mutant YPT1 cDNAs were cloned into the bacterial expression vector pRSETA and expressed in *E.*

coli BL21(DE3) or cloned into pGEX-2T and expressed in *E. coli* DH5α. Induction and purification of the His-tagged fusion and GST fusion proteins were performed as described previously (56). Wild-type DSS4 cDNA cloned into the bacterial expression vector pMAL was expressed in *E. coli* DH5α. Induction and purification of the MBP-DSS4 fusion protein were performed as suggested by the manufacturer. The final concentration and purity of expressed proteins were determined by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) with Coomassie blue staining.

In vitro binding assay for YPT1 (His tagged or GST fusion) and MBP-DSS4 proteins. MBP-DSS4 fusion protein (20 pmol) bound to amylose resin in 25 μl of binding buffer (buffer G, which consisted of 50 mM Tris-HCl [pH 7.5], 5 mM $MgCl_2$, 20 mM KCl, and 1 mM dithiothreitol [DTT] containing 500 μg of bovine serum albumin [BSA] per ml and 1 mM $ZnCl_2$) was added to 300 μl of binding buffer containing 100 pmol of His-tagged YPT1, His-tagged Ras, GST-YPT1, or GST proteins in the nucleotide-free state or bound to GDP or GTP. The mixture was rotated for 90 min at room temperature. The amylose resin was pelleted by brief centrifugation and washed five times in 1 ml of buffer G containing 1% Triton X-100 and once in 1 ml of buffer G. Resin-bound proteins were dissolved in 15 μl of sample loading buffer, heated for 3 min at 95°C, separated by SDS-PAGE and analyzed by Western blotting by use of anti-His tag antibodies (Qiagen) with goat anti-mouse immunoglobulin G or anti-GST antibodies (Santa Cruz) with goat anti-rabbit immunoglobulin G and an Immun-Star kit as described by the manufacturer (Bio-Rad).

Intrinsic GTPase activity. The rate of intrinsic GTP hydrolysis of YPT1 (wild type) or YPT1-V17 protein was determined by a modification of a method described previously (71). His-tagged YPT1 (wild type) or YPT1-V17 protein (50 pmol) was incubated in a 50-μl reaction mixture containing 50 nM [γ-^{32}P]GTP (6,000 Ci/mmol), 20 mM Tris (pH 8.0), 2 mM DTT, and 1 mM EDTA for 5 min at 25°C to bind GTP. Four volumes of buffer A (20 mM Tris [pH 8.0], 100 mM NaCl, 5 mM $MgCl_2$, 2 mM DTT, 500 μg of BSA per ml) preheated to 35°C were added to the reaction mixture, which was then incubated at 35°C. At various times after mixing (0, 30, 60, 90, and 120 min), 25-μl samples were removed, diluted with 1 ml of ice-cold wash buffer (20 mM Tris [pH 7.5], 5 mM $MgCl_2$, 1 mM DTT), and then filtered through prewetted nitrocellulose membranes. The membranes were washed with 5 ml of ice-cold wash buffer and then dried under a heat lamp. The amount of unhydrolyzed, radioactive GTP remaining bound to the protein was determined by liquid scintillation counting as previously described (49).

GDP release assay for YPT1 proteins. Wild-type and mutant His-tagged YPT1 proteins (100 pmol) were incubated in 200 μl of buffer B (50 mM Tris-HCl [pH 7.5], 2.5 mM EDTA, 1 mM DTT, 20 mM KCl, 500 mg of BSA per ml, 1 mM $ZnCl_2$) containing 5 nM [^{3}H]GDP (10 mCi/mmol) for 15 min at 30°C. After incubation, $MgCl_2$ was added to a final concentration of 5 mM, and the mixture was placed on ice for 10 min to allow nucleotide binding. Each [^{3}H]GDP-labeled YPT1 protein was incubated at room temperature with 100 pmol of immobilized MBP-DSS4 or MBP as a control in reaction buffer (50 mM Tris-HCl [pH 7.5], 1 mM DTT, 20 mM KCl, 500 mg of BSA per ml, 1 mM $ZnCl_2$, 100 mM GTP). At 0 and 60 min, 50 μl of the reaction mixture was removed and [^{3}H]GDP binding was measured by a filter-binding assay and liquid scintillation counting.

Other materials and methods. Yeast transformations were performed by the lithium acetate method (31), and *E. coli* transformations were performed by electroporation as described by the Gene Pulser manufacturer (Bio-Rad) or by the $CaCl_2$ method (65). *E. coli* strains were grown in Luria-Bertani medium (65) containing 100 μg of ampicillin per ml. Yeast strains were grown in yeast extract-peptone-dextrose medium or SC medium (72).

RESULTS

Yeast genetic analysis of GTPases binding to endogenous GEFs. Previously we exploited a yeast genetic system to identify amino acid residues in the switch II region of Ras which interact with GEFs (49). The rationale for this genetic system is based on the mechanism by which dominant interfering mutants of Ras cause growth arrest in yeast, that is, by binding to and sequestering essential GEFs (14, 20, 34, 58, 67). Thus, mutations in dominant interfering Ras which disrupt its interaction with GEFs are expected to suppress the dominant interfering lethal phenotype. However, mutations which cause global structural change are expected to suppress the dominant interfering phenotype without yielding information relevant to the protein-protein interaction sites. For this reason, the yeast genetic system that we used revealed intragenic suppressor mutants which were defective in GEF interactions without affecting other essential functions of Ras.

Segal et al. (68, 69) demonstrated that in addition to the switch II region of Ras, residues 103 and 105 in the α3-L7 region are similarly involved in binding GEFs, in agreement with the crystal structure of the H-ras–Sos complex (5). If the yeast genetic system that we developed does indeed accurately identify GEF-binding residues, we predicted that mutations in the α3-L7 region would suppress the lethal phenotype of the dominant interfering H-ras–N17 mutant. Further, we predicted that the α3-L7 mutations introduced into H-ras–V12 would not affect the ability of activated Ras to suppress the loss of Ras function in yeast. We found that the α3-L7 mutations (at residues 103 and 105) were intragenic suppressors of the dominant interfering H-ras–N17 mutant (data not shown). Further, when the α3-L7 mutations were introduced into H-ras–V12, these mutations did not prevent suppression of the loss of Ras function in yeast (data not shown).

Can a yeast genetic system be developed to identify amino acid residues in YPT1 which interact with GEFs? A yeast genetic system for identifying amino acid residues in YPT1 which interact with GEFs would have three general requirements. First, the system would require a dominant interfering mutant of YPT1 which induces a lethal phenotype. Second, the mechanism underlying the dominant interfering lethal phenotype would need to involve sequestering of GEFs. Third, a constitutively active YPT1 mutation, analogous to the H-ras–V12 mutation, would be needed in order to test the suppression of the loss of YPT1 function independent of endogenous GEF activity.

The dominant interfering H-ras–N17 mutation has been well characterized and is known to act through sequestering of essential GEFs (14, 20, 34, 67). We created the analogous YPT1-N22 mutant and determined whether it could induce a YPT1-null (lethal) phenotype in yeast. As shown in Fig. 1A, YPT1-N22, but not wild-type YPT1, induced a lethal phenotype when overexpressed under the control of the *GAL1* promoter.

If the mode of action of the dominant interfering YPT1-N22 mutation is through sequestering of essential GEFs, then overexpression of a GEF, which can lead to activation of endogenous wild-type YPT1, would be expected to suppress the dominant interfering lethal phenotype. Although the GEF that regulates YPT1 in *Saccharomyces cerevisiae* has not been identified, DSS4 (dominant suppressor of Sec4-8), a known SEC4 GEF, can activate YPT1 in vitro (10, 33, 50). Thus, we determined whether overexpression of DSS4 in yeast can suppress the lethal phenotype induced by YPT1-N22. Overexpression of DSS4 suppressed the YPT1-N22-induced lethal phenotype (Fig. 1A). This finding suggests that the mechanism by which YPT1-N22 induces a lethal phenotype is through sequestering of essential GEFs such that endogenous YPT1 can be activated only by overexpression of an exogenous GEF.

The interaction of GEFs with Ras-GDP induces nucleotide release, yielding a nucleotide-free apo-Ras GEF reaction intermediate. This stable reaction intermediate was demonstrated by the observation that a complex of Ras and GEF is most stable in the absence of guanine nucleotides (13, 28, 34, 37, 49). Dominant interfering mutants of Ras which sequester GEFs in vivo have been shown to bind strongly to GEFs in vitro under conditions where the interaction of wild-type Ras with GEFs is disrupted by binding to GDP or GTP (14, 34). We determined whether YPT1-N22 displayed similar altered binding characteristics with DSS4.

We examined the ability of soluble His-tagged YPT1 (His-YPT1) proteins to bind immobilized MBP-DSS4 fusion protein under different conditions. In these experiments, the presence of GDP or GTP efficiently disrupted the complex formed between nucleotide-free His-YPT1 and MBP-DSS4 (Fig. 1B). MBP-DSS4 bound strongly to nucleotide-free His-YPT1. In contrast, MBP-DSS4 was found to bind His-YPT1-GDP and

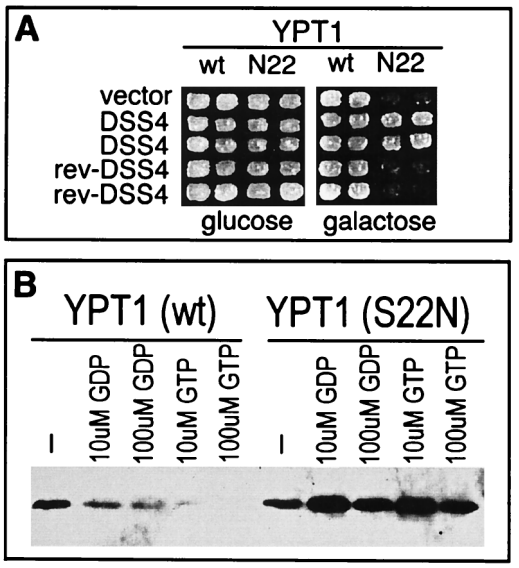

FIG. 1. Analysis of dominant interfering mutants of YPT1. (A) YPT1-N22 induces a dominant interfering lethal phenotype, and overexpression of the DSS4 GEF suppresses the dominant interfering lethality induced by YPT1-N22. Wild-type (wt) yeast strain W303-1B was cotransformed with vectors for galactose-induced expression of the indicated YPT1 protein together with ADH promotor-based vectors containing the DSS4 coding sequences in the correct (DSS4) or incorrect (rev-DSS4) orientation. Either two or four independent transformants were analyzed for growth on glucose- or galactose-containing media. Similar results were obtained in two independent experiments. (B) The dominant interfering YPT1-N22 protein binds DSS4 under conditions where the interaction of wild-type YPT1 and DSS4 is disrupted. His-tagged wild-type YPT1 or YPT1-N22 protein in the nucleotide-free state (−), GDP-bound state (10 or 100 μM GDP), or GTP-bound state (10 or 100 μM GTP) (100 pmol) was incubated (90 min, room temperature) with an MBP-DSS4 fusion protein (20 pmol) immobilized on amylose resin. Following extensive washing, resin-bound His-tagged YPT1 proteins were detected by SDS-PAGE and Western blotting with monoclonal anti-His tag antibody. Similar results were obtained in two independent experiments.

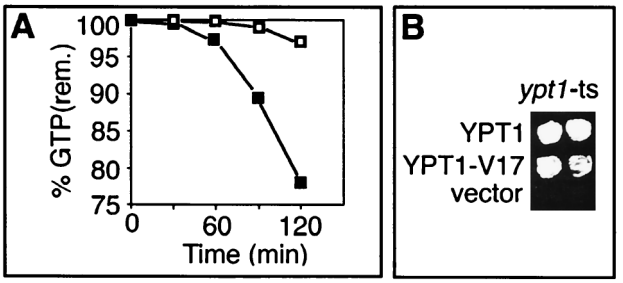

FIG. 2. Analysis of an activated allele of YPT1. (A) The intrinsic GTPase activities of purified His-tagged wild-type YPT1 (filled squares) and YPT1-V17 (open squares) proteins were determined as described in Materials and Methods. The percentages of unhydrolyzed, radioactive GTP remaining (rem.) bound to the YPT1 or YPT1-V17 protein are shown. Initial values corresponded to approximately 22,000 cpm. The values shown are the averages of duplicate measurements in a single experiment. Similar results were obtained in three independent experiments. (B) The YPT1-V17 mutant suppresses the loss of YPT1 function in yeast. Yeast strain NSY161 harboring a temperature-sensitive (ts) allele of YPT1 (*ypt1-A136D*) was transformed with plasmids for galactose-induced expression of the indicated YPT1 protein or with an empty vector. Two independent transformants were tested for growth on galactose at the nonpermissive temperature (37°C). Similar results were obtainedin two independent experiments.

His-YPT1-GTP in significantly smaller amounts (Fig. 1B). In contrast to wild-type His-YPT1, His-YPT1-N22 bound tightly to MBP-DSS4 even in the presence of high concentrations of GDP or GTP. This finding is similar to the defect reported for Ras dominant interfering mutants Ras-N17 and Ras-Y57 (14, 34). Together, these results indicate that the dominant interfering lethal phenotype of YPT1-N22 is due to sequestering of endogenous GEFs essential for YPT1 functions.

We next determined whether a constitutively active YPT1 mutant could be generated. The Ras-V12 mutant has defective GTPase activity such that it can achieve an active GTP-bound state in the cell independent of GEF activation (1, 49, 58). Glycine 12 of H-ras, as well as the corresponding position in other small GTPases, is involved in GTP hydrolysis (23, 39, 77, 78). We created the analogous YPT1-V17 mutant and determined whether it has characteristics similar to those of H-ras–V12. In contrast to wild-type His-YPT1, His-YPT1-V17 displayed undetectable GTPase activity (Fig. 2A). We also determined whether YPT1-V17 could suppress the temperature-sensitive *ypt1-A136D* mutation in yeast (32). Recently, a YPT1-67L mutant was shown to be defective in GTP hydrolysis and shown to suppress the loss of YPT1 function in yeast (62). Similarly, YPT1-V17 enabled the growth of a *ypt1-A136D*-harboring strain at the nonpermissive temperature (Fig. 2B).

Intragenic suppressors of YPT1-N22. Using the genetic system described above, we sought mutations which disrupt the interaction of YPT1 with endogenous yeast GEFs but do not interfere with the ability of YPT1 to activate downstream targets. As a first step, we identified intragenic suppressors of the dominant interfering YPT1-N22 mutation. We predicted that Ras and YPT1 might interact with their cognate GEFs by using analogous domains (see Discussion). The three regions of YPT1 that we thought might interact with GEFs are those corresponding to Ras residues 30 to 42, 62 to 73, and 99 to 109. Therefore, we created in the dominant interfering YPT1-N22 mutant a series of site-directed mutations in the regions encompassing these residues. In addition, we generated a pool of random mutations by hydroxylamine treatment of a yeast expression vector containing YPT1-N22 under the control of the *GAL1* promoter. Among the site-directed and random mutations obtained, we identified 17 intragenic suppressors of the lethal phenotype of the dominant interfering YPT1-N22 mutant that did not contain premature stop codons (Table 1). Eleven of these mutations involved residues of YPT1 in regions corresponding to the switch I (positions 42, 43, and 49), switch II (positions 69, 71, 73, and 75), and α3-L7 (positions 107, 109, and 115) regions of Ras.

Intragenic suppressor mutations which do not interfere with downstream signaling. We next examined whether the intragenic suppressor mutations when combined with the YPT1-V17 mutation would retain the ability to interact with downstream targets and suppress the loss of YPT1 function in yeast. Four of the intragenic suppressor mutations (E42, K42, M43, and I49) that we identified are located in the effector loop region of YPT1; thus, because these mutations were unlikely to be able to interact with downstream targets, they were not tested in these experiments. However, in the discussion below, we argue that residues in the YPT1 effector loop region are involved in binding GEFs, consistent with the reports of Burstein and Macara (8) and Burton et al. (11).

Nine of the 13 intragenic suppressor mutations that we tested did not affect the ability of YPT1-V17 to interact with downstream targets, as judged by suppression of the *ypt1-A136D* mutation (Table 1 and Fig. 3A). Among these nine mutations, seven affected residues at positions 69, 71, 73, 75, 107, 109, and 115, which correspond to surface residues in the crystal structure of Ras (see Fig. 7). Four of these mutations (C69, C71, T73, and L75) are located in a region corresponding

TABLE 1. YPT1 mutations tested for their ability to suppress YPT1-N22 or *ypt1*-A136D

YPT1 mutation	Suppression of:		
	YPT1-N22[a]	*ypt1*-A136D[b]	
		V17	None
None	−	+++	+++
E42	+++	ND	ND
K42	+++	ND	ND
M43	+++	ND	ND
I49	+++	ND	ND
K68	−	+++	ND
C69	+++	+++	+
C71	+++	+++	−
T73	+++	+++	−
L75	+++	+++	−
C79	+++	−	ND
R83	+++	−	ND
N89	+++	+++	ND
I91	+++	−	ND
L95	+++	+++	ND
I101	+++	−	ND
K105	−	+++	ND
A107	+++	+++	−
D109	+++	+++	−
I111	−	+++	ND
A113	−	+++	ND
R115	+++	+++	++

[a] The indicated YPT1 mutations were tested for intragenic suppression of the dominant interfering phenotype of the YPT1-N22 allele. Symbols: +++, the indicated mutation is an intragenic suppressor of YPT1-N22; −, the indicated mutation fails to suppress YPT1-N22.

[b] The indicated YPT1 mutations in the wild-type background (none) or combined with the YPT1-V17 activated allele (as double mutants) were tested for suppression of yeast strain NSY161 harboring the temperature-sensitive *ypt1-A136D* allele. Symbols: +++, the indicated mutant is a strong suppressor; ++, the indicated mutant restores growth to near wild-type levels; +, the indicated mutant is a very weak suppressor; −, the indicated mutant fails to suppress the loss of YPT1; ND, not determined.

to the switch II region of Ras (residues 62 to 76), and three of them (A107, D109, and R115) are located in a region corresponding to the α3-L7 region of Ras (residues 101 to 109). These seven altered residues are located in regions of YPT1 analogous to the regions of Ras which are believed to be involved in binding GEFs. The other two intragenic suppressor mutations which did not affect the ability of YPT1-V17 to interact with downstream targets (N89 and L95) correspond to H-ras–GTP residues partially exposed and lying beneath residues 11, 12, and 13 of H-ras, which Boriack-Sjodin et al. recently reported to interact with Sos1 (5). Thus, residues at these positions of YPT1 are probably not directly involved in binding GEFs. However, we cannot rule out the possibility that residues 89 and 95 of YPT1 become exposed by interaction with GEFs.

We next determined whether the expression of YPT1 (wild type at residues 17 and 22) with point mutations at positions 69, 71, 73, 75, 107, 109, and 115 could suppress the loss of YPT1 function in yeast (Table 1 and Fig. 3B). It has been shown that a GEF is required for YPT1 functions in vitro and in vivo (32, 61). Thus, we predicted that mutants defective in binding yeast GEFs would be unable to provide YPT1 function in the cell. Under conditions where wild-type YPT1 or YPT1-V17 complemented the loss of YPT1 function, expression of YPT1 mutants with mutations at positions 71, 73, 75, 107, and 109 failed to complement the *ypt1-A136D* allele. The YPT1-C69 mutant conferred very weak suppression, while the YPT1-R115 mutant restored growth to nearly wild-type levels (Table 1 and Fig. 3B). Together, these results suggest that residues 42, 43, 49, 69, 71, 73, 75, 107, 109, and 115 of YPT1 are involved in binding endogenous GEFs for YPT1 (see Discussion).

In vitro interaction of DSS4 with YPT1 proteins with mutations in the switch I, switch II, or α3-L7 region. We examined the ability of GST-YPT1 proteins with mutations in the region corresponding to switch I (positions 42 and 49) and His-YPT1 proteins with mutations in the switch II region (positions 68, 69, 71, 73, and 75) or the α3-L7 region (positions 107, 109, and 115) to bind MPB-DSS4 protein in vitro in the absence of guanine nucleotides. Under conditions where the wild-type GST-YPT1 protein, wild-type His-YPT1 protein, and mutant His-YPT1-K68 protein (YPT1-K68 is not an intragenic suppressor of dominant interfering YPT1-N22) were capable of binding MBP-DSS4, GST-YPT1 or His-YPT1 mutant proteins with mutations at residues 42, 49, 69, 71, 73, 75, 107, 109, and 115 bound MBP-DSS4 significantly less well (Fig. 4).

We further examined the ability of MBP-DSS4 to stimulate guanine nucleotide release from wild-type His-YPT1 protein and from His-YPT1 proteins with mutations in the switch II or α3-L7 region (Fig. 5). We first characterized the ability of MBP-DSS4 to stimulate the release of [^{3}H]GDP from wild-type His-YPT1 in the presence or absence of excess unlabeled GTP. Consistent with previous reports, DSS4 could stimulate the release of [^{3}H]GDP from YPT1 in the presence of excess guanine nucleotide (9, 50) but not in its absence (data not shown). Because an excess of guanine nucleotide is necessary, this finding suggests that after DSS4 stimulates the release of [^{3}H]GDP from YPT1, a guanine nucleotide (provided here by excess GTP) must bind to a DSS4–apo-YPT1 reaction intermediate before DSS4 can dissociate from YPT1 and thereby act (in a catalytic fashion) on additional YPT1-[^{3}H]GDP molecules.

We next examined the ability of MBP-DSS4 to stimulate guanine nucleotide ([^{3}H]GDP) release from wild-type His-YPT1 protein and from His-YPT1 proteins with mutations in the switch II or α3-L7 region in the presence of excess GTP. In these experiments, 35 to 40% of [^{3}H]GDP dissociated from the His-YPT1 proteins in the presence of MBP, reflecting the

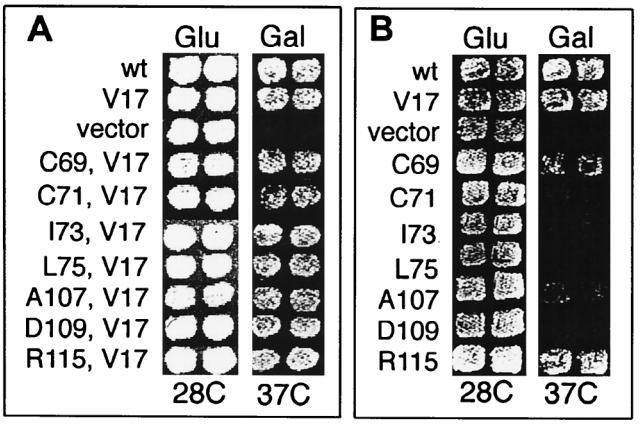

FIG. 3. Suppression of the loss of YPT1 function in yeast. (A) Suppression of the loss of YPT1 function in a yeast strain (NSY161) which contains the temperature-sensitive mutation *ypt1-A136D* was tested as described in Materials and Methods. YPT1-V17 with a mutation at residue 69, 71, 73, 75, 107, 109, or 115 suppresses the loss of YPT1 function and thus does not affect downstream signaling in yeast. (B) In parallel experiments, YPT1 with a mutation at residue 69, 71, 73, 75, 107, or 109 does not suppress the loss of YPT1 function in yeast. Similar results were obtained in two independent experiments for panels A and B. wt, wild type.

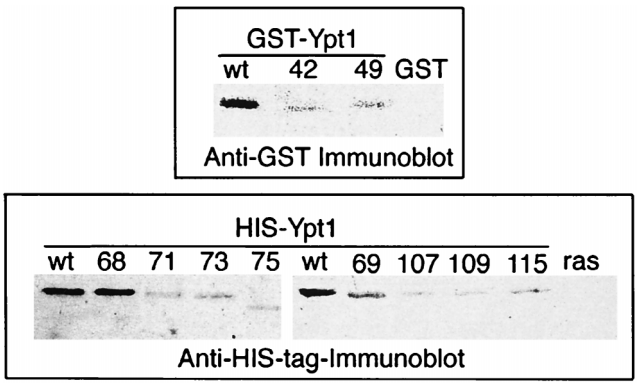

FIG. 4. Mutation of residues 42, 49, 69, 71, 73, 75, 107, 109, and 115 of YPT1 disrupts the interaction of YPT1 with DSS4. GST–wild-type YPT1 (wt), GST–YPT1-E42, or GST–YPT1-I49 in the nucleotide-free state or GST protein as a negative control (100 pmol) was incubated for 90 min at room temperature with 20 pmol of MBP-DSS4 fusion protein immobilized on amylose resin. Following extensive washing, resin-bound GST-YPT1 proteins were analyzed by SDS-PAGE and Western blotting with anti-GST antibody (top panel). Analysis of the binding of His-YPT1 proteins to MBP-DSS4 was similar to that for GST-YPT1 proteins, except for the source of the YPT1 proteins (His-tagged wild-type YPT1 [wt] or YPT1 mutants K68, C69, C71, T73, L75, A107, D109, and R115 or His-tagged H-ras protein as a negative control) and the use of monoclonal anti-His tag antibody in Western blotting (bottom panel). Similar results were obtained in two independent experiments.

intrinsic loss of bound guanine nucleotide that was not significantly affected by the mutations studied here. In the presence of MBP-DSS4, 80% of the bound [^{3}H]GDP dissociated from wild-type YPT1. MBP-DSS4 failed to dissociate [^{3}H]GDP from mutants of His-YPT1 to the same extent as it did for wild-type His-YPT1. The I73, L75, A107, D109, and R115 mutants of YPT1 were not significantly recognized as substrates for MBP-DSS4. The YPT1-C69 and YPT1-C71 mutants were recognized as substrates by MBP-DSS4, although the extent of stimulated release of [^{3}H]GDP was significantly less (about one-half) than that observed with wild-type YPT1.

DISCUSSION

YPT1, like Ras, interacts with cellular GEFs by use of switch I, switch II, and α3-L7 sequences. Our genetic analysis of small GTPases suggests that Ras and YPT1 interact with GEFs through analogous domains (but not homologous in terms of primary sequences). Several mutations altering residues corresponding to the Ras switch I, switch II, and α3-L7 regions abolish the dominant interfering lethal phenotype of the YPT1-N22 mutant (Table 1). This result suggests that these mutations prevent YPT1-N22 from binding the endogenous yeast GEFs required for normal YPT1 function. When the switch II and α3-L7 mutations were introduced into the GTPase-defective YPT1-V17 mutant, the double mutants retained the ability to suppress the loss of YPT1 function in yeast (Table 1 and Fig. 3A). This result indicates that these mutations do not prevent YPT1 from interacting with downstream effector molecules. Mutations in the switch II and α3-L7 regions in the context of nucleotide-free YPT1 prevented interactions with DSS4 in vitro (Fig. 4). Also, mutations in these regions in the context of YPT1-GDP significantly reduced the ability of DSS4 to promote nucleotide exchange (Fig. 5). Two mutants, YPT1-C69 and YPT1-C71, did not exhibit profound defects in recognition by DSS4 in the GDP release assay but did exhibit profound defects in the formation of a stable complex with DSS4 in the absence of guanine nucleotide in an in vitro binding assay. Differences in the structural requirements for DSS4 recognition of YPT1-GDP versus DSS4 binding to apo-YPT1 may explain the differences between these assays. The corresponding region of H-ras (residues 63 and 65) has been shown to be significantly altered in nucleotide-free Ras-Sos structures versus Ras-GDP or Ras-GTP structures (5).

Several reports have indicated that Ras and Rab/YPT1 effector loop residues are defective in interaction with GEFs or guanine nucleotide release factors (GRFs) in vitro (5, 9, 11, 38, 47, 79). Further, we found that effector loop mutations are intragenic suppressors of the dominant interfering YPT1-N22 mutant shown here to sequester GEFs. However, because the effector loop is also involved in stimulating downstream targets, it was not possible to determine the protein stability of the effector loop mutants in a yeast-based assay which monitors the suppression of a YPT1 temperature-sensitive mutant. Nonetheless, we argue that many effector loop mutations of GTPase in general do not disrupt global protein structure or stability. First, Ras effector loop mutants with mutations at positions 35, 37 (the position analogous to the mutation in YPT1-E42 studied here), and 40 are each defective in binding some of the known Ras effectors while retaining the ability to bind other Ras effectors in vitro and in cells (35, 63, 79). Second, an H-ras mutation at position 37 disrupts interactions with the yeast Ras GEF CDC25 while not affecting the ability to bind the yeast adenylyl cyclase or vertebrate Raf kinase (79). Third, Rac effector mutants which are also selectively defective in binding some, but not all, Rac targets have been identified (78). Fourth, Rab3A effector loop mutants which are defective in interactions with the Rab3A GRF or Rab3A GEF while not affecting the ability of GAP to stimulate GTP hydrolysis have been identified (9).

While each of these points suggests that effector loop mutations of small GTPases do not generally affect global protein structure, the last point is perhaps most relevant to a discussion of YPT1. Residue glycine 56 (G56) of Rab3A is conserved in

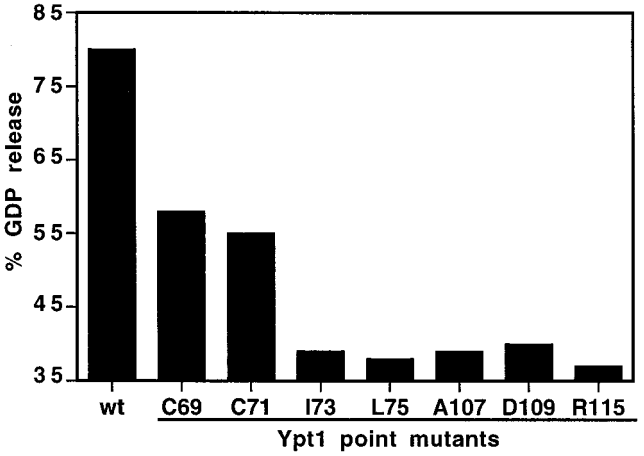

FIG. 5. His-YPT1 proteins with mutations at position 69, 71, 73, 75, 107, 109, and 115 are defective in MBP-DSS4-mediated stimulation of GDP release. His-YPT1 proteins were loaded with [^{3}H]GDP and incubated with MBP or MBP-DSS4 proteins. After 60 min, samples were removed and the amount of [^{3}H]GDP released was determined as described in Materials and Methods. Values are the averages of two independent determinations. In these experiments, about 35 to 40% of GDP was released from YPT1 proteins when incubated with MBP, reflecting the intrinsic release of GDP from YPT1 proteins. Approximately 80% of GDP was released from wild-type YPT1 protein by MBP-DSS4. In contrast, the amount of GDP released from each mutant YPT1 protein by MBP-DSS4 was significantly smaller than that released from wild-type YPT1 protein.

most Rab family GTPases, including YPT1 (9). Burstein et al. (9) reported that a Rab3A-G56D mutant is insensitive to Rab3A GRF while exhibiting only a modest reduction in responsiveness to GAP-stimulated GTP hydrolysis. This finding argues convincingly that this mutation does not produce global structural defects but results in a protein defective in GEF binding but capable of GAP interactions. We found that a mutation at the corresponding residue of YPT1, namely, a G42E substitution, was an intragenic suppressor of YPT1-N22 and that it abolished interactions with DSS4 in vitro. We argue that the results of Burstein et al. (9) together with the results presented here strongly suggest that the effector loop of Rab/YPT1 interacts not only with effector molecules but with GEFs or GRFs as well.

In the presence of a V17 (GTPase-defective) mutation, YPT1 mutations in the switch II or α3-L7 region suppressed the loss of YPT1 function in yeast. In the absence of the V17 mutation, YPT1 mutations at positions 71, 73, 75, 107, and 109 failed to suppress the loss of YPT1 function, suggesting a requirement for YPT1 to bind a GEF in order to be activated (Table 1 and Fig. 3B). The GTPase-defective YPT1 mutants thus appear to bypass this requirement. The YPT1-R115 mutant appears to bypass this requirement for a cellular GEF. This result does not appear to be due to an increased intrinsic exchange rate or a defect in GTPase activity (Fig. 5 and data not shown). All of the in vitro assays were done at room temperature due to the instability of YPT1-GDP and the lack of DSS4 activity at higher temperatures. Thus, we cannot rule out the possibility that at 37°C the YPT1-R115 mutant exhibits a temperature-sensitive defect that permits it to achieve a GTP-bound state in yeast. Together, the yeast genetic and biochemical analyses described here are consistent with the conclusion that YPT1 proteins with normal GTPase activity must interact with an essential GEF through residues in the switch II and α3-L7 regions in order to become active (GTP bound) (62).

Ras as a structural model for the interaction of small GTPases with GEFs. The Ras superfamily of small GTPases can be subdivided into five families: the Ras, Rho, Ran, Arf, and Rab/YPT1 families (4, 25). GTPases in each of these families share a conserved core structure which forms a GTP-binding pocket (6). The common structural motifs are reflected in the homologous sequences shared among the different families (6). For example, 44 of the first 178 residues of H-ras are conserved in all five families.

While the GTPases in the five different families have some common properties, each is capable of family-specific functions. The Ras effector loop comprising residues 30 to 45 is highly conserved within the Ras family, but the corresponding sequences in the Rho, Ran, and Rab families are different from those in the Ras family (Fig. 6). However, within each family the sequences corresponding to these effector loop residues are highly conserved, suggesting a common function for this region among members of the same family. These sequences within the Ras, Rho, Arf, Ran, and Rab families are known to interact with downstream targets (2, 9, 39, 78). Thus, each family is characterized by interactions with family-specific targets via family-specific sequences corresponding to the Ras effector loop. Thus, Ras has proven to be a good model for the interactions of other small GTPases with their targets.

In addition to interactions with family-specific targets, GTPases within each family are activated by family-specific GEFs. The Rho family is activated by Dbl-related GEFs, the Ran family is activated by RCC1-related GEFs, and the Ras family is activated by the CDC25- or Sos-related GEFs (3, 4, 19, 61, 80). The large Rab family of GTPases may be subdivided into those activated by DSS4-related GEFs, Sec2 GEFs, or other Rab family GEFs (10, 30, 33, 50, 64, 75, 76).

As discussed above, Ras and YPT1 interact with GEFs through three distinct regions of the Ras protein. The switch I (effector loop), switch II, and α3-L7 regions together form a GEF-binding domain (Fig. 7). The sequences in these three regions are highly conserved within the Ras family but are clearly distinct from corresponding sequences in other GTPase families (Fig. 6). As shown in Fig. 6, the Rho, Ran, and Rab families have unique consensus sequences corresponding to the Ras switch I region, switch II region, and α3-L7 region. These family-specific sequences may provide GTPases in each family the ability to interact with family-specific GEFs.

Are there other Rab family-specific sequences that might be involved in binding family-specific proteins? To address this question, we identified sequences predicted to be located on the surface of Rab proteins; these sequences distinguish Rab proteins from other GTPase families and thus may also be involved in YPT1-specific functions, such as GEF binding. From a sequence alignment of seven Rab proteins, five Ras proteins, five Rho proteins, and four Ran proteins, we identified 35 amino acid residues which, by analogy to the crystal structure of Ras, are predicted to be surface residues and which are conserved in the Rab family but not in the other GTPases (see the legend to Fig. 5 for a list of GTPases and the rules of comparison). Twelve of the 35 residues correspond to Ras switch I (effector loop) (residues 30 to 45) (Fig. 7). In addition, 5 of the 35 residues correspond to Ras residues located on the surface adjacent to the effector domain and thus, in concert with the effector loop, may be involved in interactions with both target and GEF proteins. Twelve of the 35 residues correspond to the switch II and α3-L7 regions of Ras (Fig. 7). Thus, among the 35 residues that distinguish Rab molecules from other GTPases, 29 correspond to a cluster of surface residues. The six remaining residues are scattered over the surface of Ras and are not clustered in a single domain, which might be expected for a GEF-binding domain. Further, relative to the orientation of Ras shown in Fig. 7C and D, these six residues are all found on the "back side" of the molecule. Therefore, from this sequence alignment analysis, we are able to identify only one large YPT1 surface region that possesses YPT1-specific sequences. Importantly, this analysis identifies each of the sequences found to be involved in binding GEFs.

Arf and Rac GTPases may interact with GEFs by use of switch I, switch II, and α3-L7 regions. Recently, Mossessova et al. (48) reported that the Arf GTPase may interact with its GEF, Arno, by use of regions of the polypeptide which correspond to those used by Ras to bind GEFs. This suggestion was based on the ability of Arno to protect these three regions of Arf from hydroxyl radical cleavage. However, the possibility that the protection from cleavage by Arno induced structural changes in Arf cannot be ruled out. The observation that Ras, YPT1, and possibly Arf use analogous domains to interact with their family-specific GEFs raises the interesting possibility that all small GTPases use a similar approach to bind GEFs. In further support of this suggestion, we recently found preliminary evidence that the Rac GTPases use switch I, switch II, and α3-L7 sequences to bind endogenous GEFs in vivo as well as the Vav GEF in vitro (17). Point mutations introduced into each of these three domains in the dominant interfering Rac-N17 mutant abolished its inhibitory phenotype in cells. Rac proteins with mutations in these three regions failed to interact with the Vav GEF under conditions where wild-type Rac binds to Vav (17).

The GEFs or GRFs for the Ras, Rab, Rho, and Arf families of GTPases have common properties. The release of guanine

A

H-ras	(30)DEYDPTIEDSYR.KQVV(45)	(62)EEYSAMRDQYMRTGE(76)	(101)KRVKDSDDVP(109)
N-ras	(30)DEYDPTIEDSYR.KQVV(45)	(62)EEYSAMRDQYMRTGE(76)	(101)KRVKDSDDVP(109)
RAS1	(37)DEYDPTIEDSYR.KQVV(52)	(68)EEYSAMREQYMRTGE(82)	(107)QRVKDSDYIP(115)
RAS2	(36)DEYDPTIEDSYR.KQVV(51)	(67)EEYSAMREQYMRNGE(81)	(106)QRVKDTDYVP(114)
YPT1	(35)NDYISTIGVDFKIKTVE(51)	(68)ERFRTITSSYYRGSH(82)	(107)DRYA.TSTVL(115)
rab1a	(38)ESYISTIGVDFKIRTIE(54)	(71)ERFRTITSSYYRGAH(85)	(110)DRYA.SENVN(118)
rab1b	(35)ESYISTIGVDFKIRTVE(51)	(68)ERFRTVTSSYYRGAH(82)	(107)DRYA.SENVN(115)
rab8	(35)STFISTIGIDFKIRTIE(51)	(68)ERFRTITTAYYRGAM(82)	(107)EEHA.SADVE(115)
cdc42	(30)SEYVPTVFDNY.AVTVM(45)	(62)EDYDRLRPLSYPQTD(76)	(102)THHCP..KTP(109)
CDC42	(30)ADYVPTVFDNY.AVTVM(45)	(62)EDYDRLRPLSYPSTD(76)	(102)HHHCP..GVP(109)
rac1	(30)GEYIPTVFDNY.SANVM(45)	(62)EDYDRLRPLSYPQTD(76)	(102)RHHCP..NTP(109)
rhoA	(32)EVYVPTVFENY.VADIE(47)	(64)EDYDRLRPLSYPDTD(78)	(104)KHFCP..NVP(111)
ran	(37)KKYVATLGVEVHPLVFH(53)	(70)EKFGGLRDGYYIQAQ(84)	(109)VRVC..ENIP(116)
gsp1	(39)KKYIATIGVEVHPLSFY(55)	(72)EKFGGLRDGYYINAQ(86)	(111)VRVC..ENIP(118)
ranB	(40)KKYEPTIGVEVHPLDFF(56)	(73)EKFGGLRDGYYIHGQ(87)	(112)CRVC..ENIP(119)
ranTp	(37)KKYIATQGVNVSNMILH(53)	(70)EKLGGLREGYYIGAD(84)	(109)TRIC..ENVP(116)

B

ras consensus	DE**Y**DP**TI**EDSYR.KQVV	**EE**Y**F**AMREQYMRxGE	xRVKDSDx**V**P
rab consensus	xx**Y**IS**TI**GVDFKIRTVE	**E**R**F**RTITSSYYRGAx	DxYA.Sxx**V**x
rac consensus	xx**Y**VP**TV**FDNY.xxxVx	**E**D**Y**DRLRPLSYPxTD	xHHCP..xxP
ran consensus	KK**Y**xx**Ti**GVEVxxxxFx	**E**K**F**GGLRDGYYIxAQ	xRVC..EN**I**P

FIG. 6. Alignment of amino acid sequences of different GTPases in the regions corresponding to the switch I (effector loop), switch II, and α3-L7 regions of Ras. (A) Amino acid sequences distinguishing the Ras; Rab/YPT1; Rac, Rho, and Cdc42; and Ran families of GTPases. Amino acid sequences were aligned for 16 Ras-related GTPase proteins (summarized from 7 Rab-related proteins, 5 Ras-related proteins, 5 Rho-related proteins, and 4 Ran-related proteins; see below) for the regions corresponding to the effector loop region (residues 30 to 45), switch II region (residues 62 to 76), and α3-L7 region (residues 101 to 109) of human H-ras. These sequences are identical or highly conserved within each of the small GTPase families but clearly divergent between the different small GTPase families. The positions of known mutations (squares) affecting the interaction of Ras with its GEF and the positions of mutations (circles) identified in this study as being critical for the YPT1 interaction with the DSS4 GEF are indicated. Residue numbering is indicated at the beginning and the end of each block of sequence. (B) Consensus sequences for the Ras; rab/YPT1; Rac, Rho, and Cdc42; and Ran families for the regions shown in panel A. Boldface indicates residues highly conserved in all of the GTPases shown in panel A, which may reflect functions common to all GTPases rather than family-specific functions. A lowercase letter indicates that one exception to the consensus is found when considering all of the GTPases shown. A lowercase x denotes the lack of a consensus. The consensus sequences were derived with the following rules. (i) An uppercase letter indicates that the four aligned sequences each have the identical or conserved amino acid at that position. (ii) The following groups of amino acids were considered conservative residues: L, I, V, and M; K and R; D, E, N, and Q; S, T, A, and G; and F, Y, and H. The names and the sources of the protein sequences used are as follows: Ras family—H-ras (human Ha-ras), N-ras (human N-ras), RAS1 (*S. cerevisiae* RAS1), RAS2 (*S. cerevisiae* RAS2), K-ras (human K-ras); Rab family—YPT1 (*S. cerevisiae* YPT1), rab1a (rat Rab1a), rab1b (rat Rab1b), rab8 (human Rab8), rab3A (rat Rab3A), Cfrab10 (canine Rab10), and Sec4 (*S. cerevisiae* Sec4); Rho family—cdc42 (human CDC42), CDC42 (*S. cerevisiae* CDC42), rac1 (human Rac1), rhoA (human RhoA), and rho1 (*S. cerevisiae* Rho1); Ran family—ran (human Ran), gsp1 (*S. cerevisiae* Gsp1), ranB (tobacco RanB), and RanTp (*Tetrahymena pyriformis* Ran). The protein sequences of the GTPases were derived from Chardin (12), except for RanB (46) and Ran (52).

nucleotides bound to the Ras, YPT1/Rab, Arf, and Rac families of GTPases can be stimulated by GEFs or GRFs specific for each of these distinct families of GTPases. Based on their amino acid sequences, the CDC25-type GEFs for Ras, the DSS4-type and Sec2-type GEFs for Rab, the Sec7-type GEFs for Arf, and the Dbl-type GEFs for Rho appear structurally unrelated. However, these distinct GEFs or GRFs share a number of similar functional properties. First, the physiological role of these GEFs or GRFs is to convert a GDP-bound GTPase to a GTP-bound GTPase. Second, each of these GEFs or GRFs stimulates the release of bound guanine nucleotides from their respective GTPase substrates. The major contribution to the overall exchange reaction mediated by each of these GEFs or GRFs is stimulation of the release of a bound nucleotide rather than stimulation of the uptake of a new nucleotide. Third, each of these GEFs or GRFs binds preferentially to nucleotide-free GTPases. This binary protein complex is generally thought to reflect an enzymatic reaction intermediate. Some GEFs or GRFs may bind equally well to GDP-bound, GTP-bound, and epo-GTPases, but this fact may reflect the use of GEF molecules that have not been properly modified for full activity, as we have noted for the Vav GEF (26). Fourth, the ability of each of these GEFs or GRFs to catalytically stimulate the release of [^{3}H]GDP from their respective substrates requires the presence of excess guanine nucleotides (GDP or GTP). We previously referred to this activity as guanine nucleotide exchange activity because of the requirement of a guanine nucleotide to replace (or "exchange with") the released [^{3}H]GDP (7). Fifth, mutations in the Ras-related GTPases at positions corresponding to Ras residue 17 result in GTPases with dominant interfering properties (i.e., null phenotype of the GTPase), and the mode of dominant interference is thought to be sequesteration of cellular GEFs or GRFs. Sixth, as discussed below, each of these GEFs or GRFs recognizes similar structural elements on the surface of the GTPases.

The Ras, YPT1/Rab, Arf, and Rac families of GTPases each appear to interact with GEFs by use of similar structural domains, namely, the switch I, switch II, and α3-L7 regions. This proposal could have important implications for the mechanism of GEF-mediated nucleotide exchange on all small GTPases as well as implications for the use of GTPases with mutations in the GEF interaction domains. The interaction of distinct families of GEFs with a common set of structural elements on the GTPases suggests that some aspects of GEF-mediated GDP or GTP exchange are common to the various families of GTPases. For example, what is learned concerning the mech-

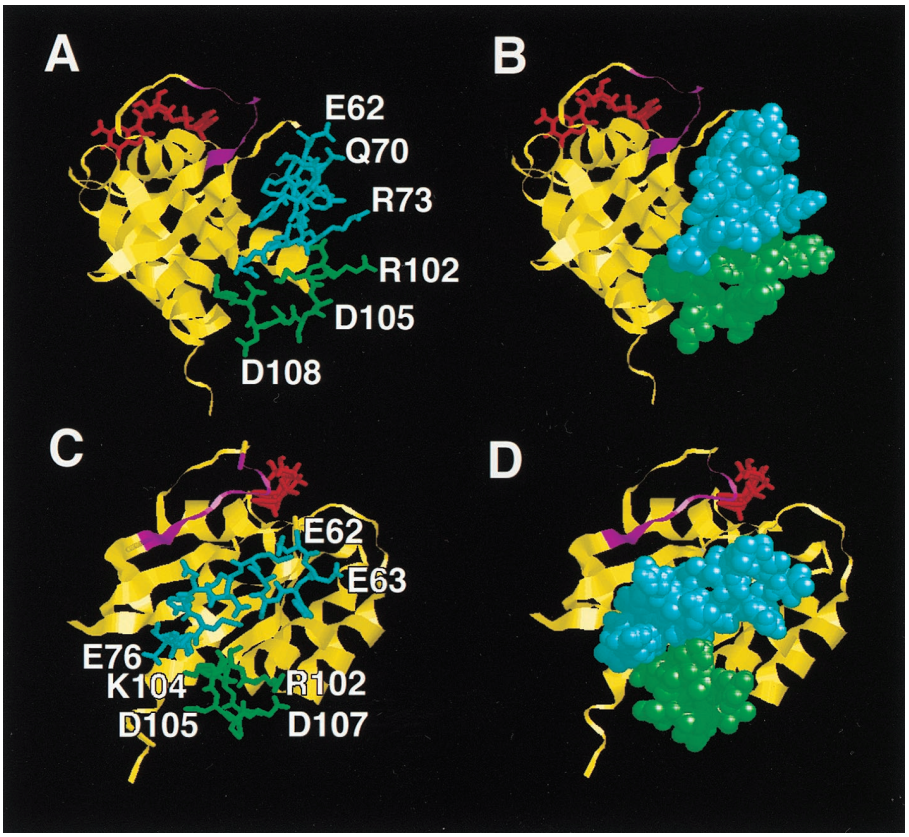

FIG. 7. Diagram showing the structure of H-ras bound to GDP. The effector loop (residues 35 to 42) (magenta), switch II region (residues 62 to 76) (cyan blue), and α3-L7 region (residues 101 to 109) (green) of Ras are on the surface of the molecule and are located next to each other. The remaining H-ras structure is shown in yellow. GDP is shown in red. Two orientations of the molecules are presented: A and B show one orientation, and C and D show the other orientation. The locations of several amino acid residues of H-ras in regions involved in binding GEFs are indicated. The switch II region and the α3-L7 region are presented as either stick models (A and C) or space-filling models (B and D).

anism of the Sos GEF-stimulated exchange on Ras (5) is likely to be relevant to the mechanism of GEF-mediated exchange on Rac, Rab, and Arf GTPases.

The intrinsic properties of most (if not all) of the GEFs for Ras-related GTPases do not exhibit a strong directionality; i.e., they convert GTPase-GDP to GTPase-GTP only modestly better than the reverse reaction. As the physiological role of the GEFs is generally thought to produce GTPase-GTP molecules (rather than GTPase-GDP molecules), additional factors have been thought to affect the direction of the GEF-mediated reaction in the cell. Each of the Ras-related GTPases is proposed to interact with GEFs, GAPs, and target molecules through the switch I (effector loop) region. Therefore, consistent with in vitro biochemical analysis (27), the interaction of a downstream target with a GTP-bound GTPase in vivo is expected to block the interaction with GEFs. The ability of target molecules to block GEF interactions with GTPase-GTP molecules would then prevent GEFs from acting on GTP-bound, but not GDP-bound, GTPases. This activity would contribute to the overall direction of the GEF-mediated reaction to favor a GTP-bound state. Also, because the GTP concentration in the cell almost always exceeds the GDP concentration by 10-fold or more, the GEF- or GRF-mediated reaction favors the formation of GTP-bound GTPase. There are two intrinsic properties of GEFs and GTPases which could also affect the direction of the exchange reaction. First, GDP-bound GTPases appear to be recognized by GEFs more readily than GTP-bound GTPases (Fig. 1B) (26, 37, 51). Second, reaction intermediates (apo-Ras2–CDC25) are more readily disrupted by GTP than by GDP (37, 49). Most detailed analyses of GEF-mediated reactions have used fragments of the GEF molecule; consequently, possible regions of GEF molecules that might have contributed to the directionality of the reaction may have been overlooked (47, 49, 69, 73).

The use of point mutants of GTPases is a widely used approach in the signaling field. For example, Ras switch I mutations have been widely used to assess the contribution of various Ras effectors to the phenotypes induced by Ras (35, 63, 79). A caveat to the use of switch I mutations of Ras in the study of Ras effectors is that these mutations also affect interactions with GEFs. Thus, in theory, the partial loss of Ras signaling by these mutations could be due in part to a loss of interactions with Ras GEFs. GTPase effector mutations are often used in the context of a second mutation that impairs the GTP hydrolysis activity of the GTPase; this effect is often suggested to render the GTPase active independent of GEF activity. This suggestion may not be completely accurate. Although RAS2-V19 (GTPase defective) can bypass the requirement of CDC25 for cell viability, it is still responsive to the yeast CDC25 GEF (7). Also, many GEFs have the potential to interact with signaling molecules other than GTPases. Vav, a GEF for Rac GTPase, interacts with phosphoinositide 3-kinase, Grb2, Crk, Shc, Xyzin, ZAP-70, and SLP-76, as well as other molecules (15). Also, Ras GEFs possess both a Ras GEF domain and a Rac GEF domain and thus activate both Ras signaling and Rac signaling (53, 61, 80). If any of the signaling

properties of these GEF-interacting proteins requires an interaction with GTPases, then these signals would likely be affected by GTPase switch I mutants, even though these signals are not generally considered to be mediated by effectors. For example, in the case of Ras GRF1, a Rac effector mutant might not bind to the DH domain of the Ras GRF1, and if a DH-Rac interaction affected the activity of the CDC25 domain for Ras, the phenotype of the Rac effector mutant could in theory differ from wild-type Rac phenotypes (18, 21). Interestingly, the activity of the Ras GEF domain of Ras GRF was shown to be dependent on the activity of the DH domain (18, 21).

Not all mutations of residues involved in protein-protein interactions will significantly reduce the affinities of the proteins involved. This idea is well illustrated by the use of effector mutations of GTPases which, while preventing interactions with some target molecules, permit interactions with others. In this regard, not all mutations in the GEF-binding domain on GTPases are expected to prevent interactions with all the GEFs for a GTPase. For example, a mutation in Ras that prevents interactions with the yeast SCD25 GEF may not affect interactions with GEFs for yeast CDC25 or vertebrate Sos1, Sos2, or cdc25 or Ras GRF. Thus, it should be possible to isolate mutations of GTPases which selectively prevent interactions with a subset of the GEFs for a GTPase. Just as the use of effector mutations of GTPases has proven useful for defining the signals downstream of GTPases, the use of GEF interaction site mutations in the switch II or α3-L7 regions of GTPases should prove useful in defining the contributions of various GEFs to the activation of GTPases.

The Ras, Rab, and Rho families of GTPases have been shown to be regulated by GEFs which do not activate all members of the families. The specificity of GEFs for some but not all GTPases in a family could reflect structural differences in the GEF-binding domains of the GTPases. As the emerging view of GEF-binding domains on GTPases has defined the switch I, switch II, and α3-L7 regions, sequence differences in these regions between different GTPases in the same family could underlie the specificity of GEFs. As indicated in Fig. 6, consensus sequences are present in the families of GTPases. However, there are sequence differences among the members of the families of GTPases. These sequence differences are likely to be involved in the observed specificity of GEFs. For example, we have begun a mutational analysis of the switch I, switch II, and α3-L7 regions which distinguish the Rho, CDC42, and Rac GTPases (17). This analysis could identify differences in Rho, CDC42, and Rac which determine specific recognition by lbc, FGD1, and the DH domain of Sos1, respectively (80).

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