Sst2 Is a GTPase-Activating Protein for Gpa1: Purification and Characterization of a Cognate RGS−Gα Protein Pair in Yeast†

Donald M. Apanovitch,‡ Kevin C. Slep,§ Paul B. Sigler,§ and Henrik G. Dohlman*,‡

Department of Pharmacology and Molecular Cardiobiology Program, Boyer Center for Molecular Medicine, Yale University School of Medicine, New Haven, Connecticut 06536, and Department of Molecular Biophysics and Biochemistry, The Howard Hughes Medical Institute, Yale University, New Haven, Connecticut 06511

Received December 5, 1997; Revised Manuscript Received January 27, 1998

ABSTRACT: Genetic studies in the yeast *Saccharomyces cerevisiae* have shown that *SST2* promotes pheromone desensitization in vivo. Sst2 is the founding member of the RGS (regulators of G protein signaling) family of proteins, which in mammals act as GAPs (GTPase activating proteins) for several subfamilies of Gα proteins in vitro. A similar activity for Sst2 has not been demonstrated, and it is not self-evident from sequence homology arguments alone. Here we describe the purification of Sst2 and its cognate Gα protein (Gpa1) in yeast, and demonstrate Sst2-stimulated Gpa1 GTPase activity. His-tagged versions of Sst2 and Gpa1 were expressed in *E. coli*, and purified using Ni^{2+}−agarose and ion exchange chromatography. Time-course binding experiments reveal that Sst2 does not affect the binding or release of guanine nucleotides. Similarly, steady-state GTPase assays reveal that Sst2 does not alter the overall rate of hydrolysis, including the rate-limiting nucleotide exchange step. Single-turnover GTPase assays reveal, however, that Sst2 is a potent stimulator of GTP hydrolysis. Sst2 also exhibits GAP activity for mammalian $G_o\alpha$, and the mammalian RGS protein GAIP exhibits GAP activity for Gpa1. Finally, we show that Sst2 binds with highest affinity to the transition state of Gpa1 (GDP−AlF_4^--bound), and with much lower affinity to the inactive (GDP-bound) and active (GTPγS-bound) conformations. These experiments represent the first biochemical characterization of Gpa1 and Sst2, and provide a molecular basis for their well-established biological roles in signaling and desensitization.

Many signaling molecules act via cell surface receptors coupled to guanine nucleotide binding regulatory proteins (G proteins) (*1*). These receptors catalyze the exchange of GDP[1] for GTP on the G protein α subunit, which in turn leads to dissociation of Gα from the G protein βγ subunits, and the concomitant activation of downstream effector proteins (*2*). Signaling persists until GTP is hydrolyzed to GDP and the subunits reassociate, completing the cycle of activation. Thus, the strength of the G protein signal depends on (i) the rate of nucleotide exchange, (ii) the rate of GTP hydrolysis, and (iii) the rate of subunit reassociation.

A ubiquitous property of G protein signaling systems is that they are subject to desensitization (*1*). This process allows cells to adapt appropriately to changes in their environment, but can also have deleterious consequences leading to drug tolerance or addiction. Considerable work in the past has focused on regulation at the level of the receptor, most notably the role of phosphorylation in promoting receptor inactivation and sequestration from the plasma membrane (*3*). More recently, attention has turned to the regulation of G proteins, in particular regulation by the newly identified family of RGS proteins (*4*, *5*).

The first RGS protein was identified in yeast, through a genetic screen for mutants that block pheromone desensitization. The affected gene, called *SST2* (for supersensitivity to pheromone), was later cloned and found to encode a 698 residue polypeptide (Sst2) with no obvious sequence similarity to other known signaling proteins (*6*−*8*). It was later realized that pheromone signaling in yeast involves a receptor and G protein very similar to those in higher eukaryotes. Further genetic analysis, using activated alleles of *SST2* (*9*), revealed the G protein as the most likely target of RGS action, a supposition that was confirmed through colocalization and coprecipitation of the two proteins from yeast (*10*). However, neither Sst2 nor Gpa1 has previously been purified, and no information about their enzymatic properties has been available. Thus, it has not been possible to determine how Sst2 modulates G protein activity.

Homologues of Sst2 have since been identified through genetic screens in the filamentous fungus *Aspergillus nidulans* (FlbA) (*11*) and the nematode *Chaenorhabditis elegans* (EGL-10) (*12*). In mammals, Sst2 homologues were identified in two-hybrid screens using Gα as "bait" (GAIP and RGS10) (*13*, *14*), as well as through expression cloning, low

† This work was carried out with research grants from the American Heart Association (CT-96-FW-32 to D.M.A.), the National Institutes of Health (GM53316 to H.G.D., GM22324 to P.B.S.), and American Cyanamid Co. (to H.G.D.). K.C.S. is an NIH predoctoral trainee (GM 5T32-GM07223).

‡ Boyer Center for Molecular Medicine, Yale University School of Medicine.

§ The Howard Hughes Medical Institute, Yale University.

[1] Abbreviations: DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; GDP, guanosine 5′-diphosphate; GTP, guanosine 5′-triphosphate; GTPγS, guanosine 5′-*O*-(3-thiotriphosphate); GAP, GTPase activating protein; GAIP, Gα interacting protein; RGS, regulator of G protein signaling; PCR, polymerase chain reaction; SDS−PAGE, sodium dodecyl sulfate−polyacylamide gel electrophoresis.

S0006-2960(97)02996-6 CCC: $15.00
Published on Web 03/17/1998

stringency hybridization, PCR cloning, and systematic genomic sequencing efforts (*12*, *15*−*20*). Transfection of RGS proteins into mammalian cultured cells was shown to attenuate hormone signaling through G_i and G_q (*15*, *21*−*24*). Expression of mammalian RGS proteins in yeast was shown to block pheromone signaling through Gpa1 (*15*, *18*, *25*). These studies suggest that RGS proteins have similar biological roles in mammals and in yeast.

A number of mammalian RGS proteins have now been purified and tested for their ability to regulate G protein catalytic function. These studies revealed that RGS proteins are potent GAPs for a wide variety of heterotrimeric G proteins, including members of the G_i and G_q subfamilies (*14*, *19*, *20*, *26*−*32*). However, it is not clear which of these RGS−Gα pairs actually interact in vivo. Nevertheless, these experiments provide a likely mechanism by which RGS proteins attenuate signaling in cells; specifically, by accelerating Gα GTPase activity, RGS proteins would decrease the lifetime of the active GTP-bound species and attenuate the cellular response. The biochemical properties of mammalian RGS proteins are concordant with the known physiological role of Sst2 in yeast. However, the hypothesis that Sst2 is a GAP has never been tested, and such an activity is not obvious from sequence homology arguments alone. Most notably, Sst2 is considerably larger (698 residues) than other RGS proteins that have been tested for GAP function (∼200 residues). In addition, the core region of homology is larger in yeast (∼400 residues) than in mammals (∼125 residues), and sequence identity within this region is only 20−27% (*5*, *12*).

The principal aim of this study was to determine if Sst2 is a GAP for Gpa1. These experiments are important because Sst2 is the only RGS for which the cognate Gα is known. Yeast is also the only system in which "knock out" mutations of the Gα and RGS genes are available. The ability to purify these proteins will now permit us to determine the biochemical basis for the large number of pheromone-supersensitive and unresponsive alleles of *GPA1* and *SST2* that have been identified genetically in yeast. The ability to conduct in vitro biochemical experiments (such as those described here), in conjunction with sophisticated in vivo genetic experiments that are available in yeast, makes this a unique and powerful system to study signal regulation.

EXPERIMENTAL PROCEDURES

Strains and Plasmids. *Escherichia coli* strain DH10B was used for the propagation and maintenance of plasmids. Strain BL21(DE3) was used for expression of recombinant Gpa1, GAIP, and Sst2. Established methods were used for the growth of bacteria and the manipulation of plasmids (*33*). All molecular biology reagents were purchased from New England Biolabs, and used according to the manufacturer's instructions. All PCR amplification products were verified by DNA sequencing (Keck Biotechnology Facility, Yale University).

N-Terminal hexahistadine-tagged versions of Gpa1 were constructed by PCR amplification using primers that encode an *Nde*I site at the initiator ATG (5′-GCG AAG AAC CAT ATG GCG TGT ACA GTG ACG CAA AC-3′) and a *Bam*HI site just after the terminating TCA (5′- GGC ATT CGC GGA TCC TCA TAT AAT-3′). The PCR-amplified products were digested with *Nde*I and *Bam*HI and ligated into the corresponding sites of pET15b (Novagen) to yield pET15b-GPA1. A His-tagged version of $Gpa1^{Q323L}$ was constructed by ligating the *Nco*I−*Hin*dIII *GPA1* fragment from pET15b-GPA1, the *Hin*dIII−*Hin*dIII fragment of $gpa1^{Q323L}$ (*10*), and the *Nco*I−*Hin*dIII product of pET21d (Novagen) to yield pET21d-$gpa1^{Q323L}$.

A C-terminal hexahistidine-tagged version of Sst2 was constructed by PCR amplification using a primer that encodes an *Nco*I site at the initiator ATG (5′-GGC GTG CGC GCC ATG GTG GAT AAA AAT AGG ACG TTG CAT-3′) and a second primer at the terminating TAA (5′- GGC ATT CGC GAA TTC TTA GCA CTT TTC TTG GAT TTC TAT TAA-3′). The resulting *Nco*I−*Bst*BI-digested PCR product was ligated, together with a *Bst*BI−*Bam*HI digestion product of *SST2* [from pBS−SST2-GST (*34*) containing a mutant *Bam*HI site that replaces the *SST2* termination codon] into the corresponding *Nco*I and *Bam*HI sites of pQE60 (Qiagen). The resulting *SST2*−hexahistidine fusion was excised by *Nco*I and *Hin*dIII digestion, and ligated to the corresponding sites in pET21d. A non-His-tagged version of *SST2* was constructed as described above, except that the PCR product was digested with *Nco*I and *Eco*RI and ligated to the corresponding sites in pET21d.

An N-terminally truncated and heptahistidine-tagged version of GAIP (American Tissue Type Collection, clone 33393) was constructed by PCR amplification using one primer that encodes an artificial *Bam*HI site and initiator ATG at codon 78 (5′-GCG AAG AAC CAT ATG CCA AGT CCT GAG GAG CTG CAG AGC-3′), and a second primer that encodes a terminating TGA and an artificial *Eco*RI site (5′ GGC ATT CGC GGA TCC TCA CAG CAG GGC ACG GTA GGT GGG AG-3′). The PCR-amplified product was digested with *Bam*HI and *Eco*RI and ligated into the corresponding sites of a modified pET21d. The resulting construct contains the N-terminal artificial sequence MGHHHHHHHGSLVPRGS preceding residues 79−206 of GAIP.

Purification of Recombinant Gpa1 and RGS Proteins. Vectors encoding His-tagged Gpa1, GAIP, or Sst2 were transformed into strain BL21(DE3) and grown at 30 °C (Gpa1 and GAIP) or 24 °C (Sst2) in 12 L of enriched medium as described (*35*). At $A_{600\ nm} = 0.5-0.6$, IPTG was added (10 μM final) to the cultures, and allowed to grow for an additional 6 h. Cells were centrifuged and flash-frozen in liquid nitrogen. All subsequent steps were carried out at 4 °C. Cells were thawed in TBP buffer (50 mM Tris-HCl, pH 8.0, 10 mM 2-mercaptoethanol, 100 μM PMSF, 20 μM GDP, 1 mM $MgCl_2$), and lysozyme (Boehringer Mannheim) was added (0.2 mg/mL final) for 30 min to complete the lysis. Samples were clarified by centrifugation at 20000*g* (11 000 rpm) for 60 min (Beckman JA 14 rotor, J2 series centrifuge) at 4 °C, and the supernatant was applied (2.0 mL/min) to a 5 mL Ni−NTA (Qiagen) column equilibrated with TBP. The column was then washed (2.0 mL/min) with 100 mL of TBP, 250 mM NaCl, followed by a second wash with TBP, 10 mM imidazole, and eluted with a 30 mL linear imidazole gradient (10−250 mM) in TBP. Eluted fractions were diluted with 3 volumes of HD buffer (20 mM Hepes, pH 8.0, 2 mM DTT, 20 μM GDP, 1 mM $MgCl_2$), and loaded (1 mL/min) onto a 5 mL MonoQ column (Pharmacia). The column was then washed with 25 mL of HD, and eluted

with a 100 mL linear NaCl gradient (10−200 mM for Gpa1; 0−700 mM for Sst2; 0−400 mM for GAIP) in HD buffer. Peak column fractions were analyzed by SDS−PAGE, and protein staining (Bio-Rad Silver Stain Plus) or immunoblotting with specific polyclonal antibodies to Gpa1 (*36*) and Sst2 (*10*). Gpa1 was exchanged into Gα storage buffer (50 mM Hepes, pH 7.6, 1 mM EDTA, 2 mM DTT, 10 μM GDP) and concentrated using a Millipore ultrafree 15 membrane (10K NMWL). Sst2 and GAIP were exchanged into RGS storage buffer (50 mM Hepes, pH 8.0, 1 mM EDTA, 2 mM DTT) and concentrated using a Centriprep 30 or Centriprep 3 (Amicon) concentrator, respectively. Approximate protein concentrations were determined by the method of Lowry (DC protein assay, Bio-Rad) using bovine serum albumin as a standard.

Guanine Nucleotide Binding and Hydrolysis Assays. Recombinant purified Gpa1 (250 nM) in 30 μL of Gα storage buffer was mixed with an equal volume of 2× GTP buffer (50 mM Hepes, pH 8.0, 1 mM EDTA, 2 mM DTT, 2 μM GTP, 10 mM $MgCl_2$) containing [^{35}S]GTPγS (2500 cpm/pmol) (DuPont−NEN) at 30 °C for 0−90 min. Bound and free nucleotides were separated by filtration using a Multiscreen HA (0.45 μM) filtration system (Millipore) and rapidly rinsed 6 times with 0.30 mL of ice-cold wash buffer (100 mM NaCl, 20 mM Tris-HCl, pH 8.0, 25 mM $MgCl_2$). After the filters were vacuum-dried, 50 μL of scintillation cocktail (Safescint, American Bioanalytical) was added to each well, and radioactivity was measured by scintillation counting (Wallac 1450 Micro Beta counter).

For measurement of steady-state GTP hydrolysis, recombinant purified Gpa1 or Gpa1^{Q323L} (250 nM) in 25 μL of Gα storage buffer was mixed with an equal volume of 2× GTP buffer, supplemented with additional $MgCl_2$ (20 mM) and [γ-^{32}P]GTP (∼7500 cpm/pmol) (DuPont−NEN) at 30 °C for 0−60 min. The reaction was stopped by mixing with 750 μL of a 5% (w/v) charcoal solution (Norit A, Aldrich) in 50 mM NaH_2PO_4, and chilled on ice. Samples were centrifuged 4 min at 10000*g* (14 000 rpm), and 200 μL of the supernatant was mixed with scintillation cocktail and counted (Packard 1500 scintillation counter).

For measurement of single-turnover GTP hydrolysis, the reaction was carried out as above except that the 2× GTP buffer was prepared with C12E10 detergent (0.05%) and without $MgCl_2$. Gpa1 (250 nM) was incubated at 30 °C for 15 min to allow nucleotide binding, and transferred to ice for 5 min. The GTP hydrolysis reaction was initiated with the addition of $MgCl_2$ (15 mM final concentration, to initiate catalysis) in the presence or absence of Sst2 or GAIP in 2× GTP buffer supplemented with unlabeled GTP (150 μM final concentration, to prevent further hydrolysis of radiolabeled nucleotide). As a negative control, Sst2 and GAIP were heat-denatured by boiling for 10 min. The time-course of [^{32}P]-P_i release was determined by scintillation counting as described above.

Sst2 Binding to Gpa1. Sst2 binding assays were performed as described (*28*). Briefly, His-tagged Gα subunits (5 μg) were preincubated in buffer A (50 mM Tris-HCl, pH 8.0, 100 mM NaCl, 1 mM $MgSO_4$, 5 mM imidazole, 0.025% C12E10, 10 mM 2-mercaptoethanol, 10% glycerol), containing either GDP (10 μM), GTPγS (10 μM), or GDP and $AlCl_3$ (30 μM) and NaF (10 mM) for 30 min at 20 °C. Clarified lysates (500 μL in buffer A) from 10 mL of *E. coli* expressing native (non-His-tagged) Sst2 were added to Gα subunits and Ni−NTA resin [10 μL of a 50% slurry (v/v), in buffer A] for 60 min at 4 °C, washed 3 times with buffer A containing NaCl (200 mM final concentration), and then eluted by boiling with 50 μL of SDS−PAGE sample buffer (*37*). Protein samples were resolved on 8% SDS−PAGE and detected by immunoblotting using antibodies to Sst2 or Gpa1 as described (*10*, *36*).

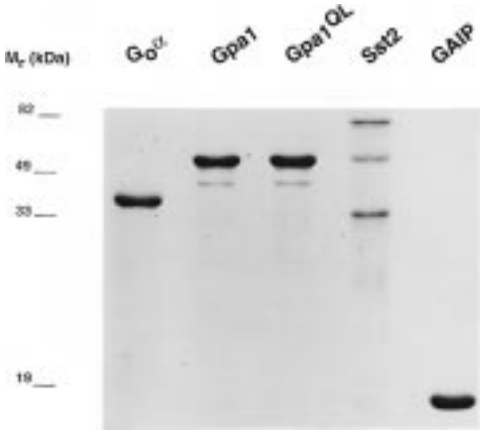

FIGURE 1: Purification of recombinant RGS and Gα proteins. His-tagged Gpa1, Gpa1^{Q323L}, GAIP, and Sst2 were expressed in *E. coli* and purified by Ni−NTA and Q Sepharose chromatography, as detailed under Experimental Procedures. Pooled and concentrated protein fractions (5 μg) were subjected to 15% SDS−PAGE and stained with Coomassie blue. Lane 1, $G_o\alpha$ (38 kDa); lane 2, Gpa1 (54 kDa); lane 3, Gpa1^{Q323L} (54 kDa); lane 4, Sst2 (81 kDa); lane 5, GAIP (18 kDa). M_r, relative mobility of prestained protein standards.

RESULTS

Expression of Sst2 and Gpa1. To determine if Sst2 acts as a GAP for Gpa1, we constructed plasmids for the expression and purification of both proteins from *E. coli*. A full-length version of each protein was expressed with a His-tag at either the N-terminus (Gpa1) or the C-terminus (Sst2). Both proteins were purified using sequential Ni−NTA−agarose affinity and Q Sepharose ion exchange chromatography. The yield of Sst2-$[His]_6$ and $[His]_6$-Gpa1 was 0.1 and 1.5 mg/L of bacterial culture, respectively. As a negative control, a mutant predicted to lack GTPase activity, Gpa1^{Q323L}, was also expressed and purified. The Q323L mutation alters a Gln residue that is conserved in all heterotrimeric G proteins, and is absolutely required for catalysis (*38*−*40*). As positive controls, recombinant forms of GAIP-$[His]_7$ and $[His]_6$-$G_o\alpha$ were purified in the same manner. All of the Gα proteins were >95% pure, as judged by protein staining after SDS−PAGE (Figure 1). Sst2 and GAIP were also nearly devoid of contaminant *E. coli* proteins, but the Sst2 preparation contained two prominent bands (molecular mass ∼51 and 32 kDa) that appear to result from degradation of the full-length protein, as judged from immunoblot analysis (see Experimental Procedures).

Sst2 Does Not Affect Dissociation of GDP from Gpa1. In principle, Sst2 could attenuate G protein signaling in either of two ways, by accelerating GTP hydrolysis or by inhibiting guanine nucleotide exchange. Of the mammalian RGS proteins tested so far (including GAIP), all appear to affect only the hydrolysis step. To rule out any effect of Sst2 on guanine nucleotide exchange, two types of experiments were

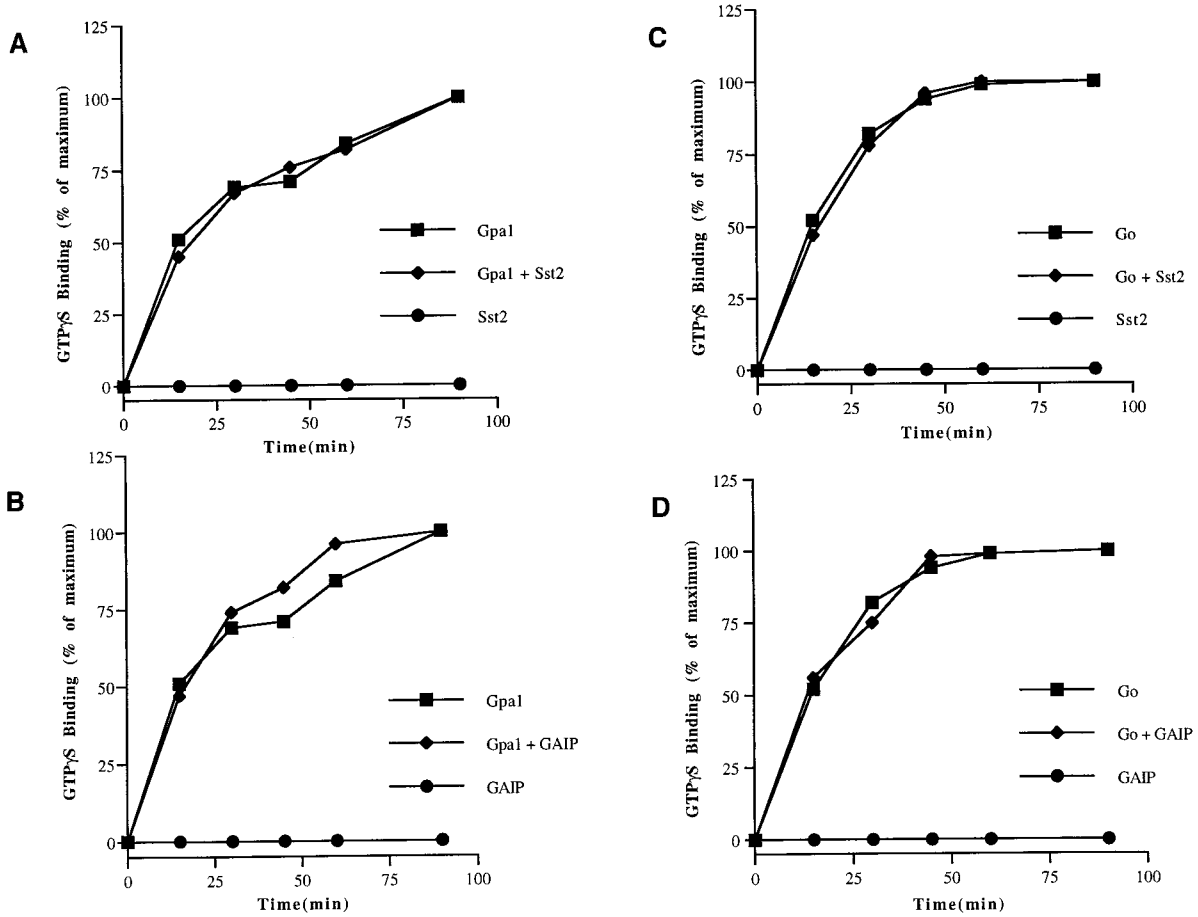

FIGURE 2: Sst2 does not affect the time-course of GTPγS binding to Gpa1. Gpa1 or $G_o\alpha$ (250 nM), in the absence or presence of Sst2 or GAIP (2 μM), was incubated with [^{35}S]GTPγS (5 μM) at 30 °C. At the indicated times, duplicate aliquots were withdrawn, filtered, washed, and counted as described under Experimental Procedures. Data are plotted as a percentage of the maximum GTPγS bound after subtraction of nonspecific binding. Data shown are the average of duplicate measurements, and are representative of at least two independent experiments.

performed. First, we measured the rate of pseudo-irreversible binding of [^{35}S]GTPγS, which is typically limited by the rate of GDP dissociation (*41*). As shown in Figure 2, [^{35}S]-GTPγS binding to Gpa1 was unaffected by the presence of a >8-fold molar excess of Sst2 (Figure 2A) or GAIP (Figure 2B). The rate of binding to Gpa1 was comparable to that observed for $G_o\alpha$, measured under the same conditions (Figure 2C,D). Sst2 and GAIP were devoid of any measurable GTP binding activity (Figure 2A−D).

As a second measure of the guanine nucleotide exchange step, we determined the steady-state rate of GTP hydrolysis by Gpa1. GTP binding is limited by the rate of GDP release, so the overall rate of hydrolysis reflects the rate of guanine nucleotide exchange (*41*). Thus, we measured the rate of [^{32}P]P_i release from [γ-^{32}P]GTP. As shown in Figure 3, the rate of Gpa1-catalyzed GTP hydrolysis was ~0.037 min^{-1}, and was unaltered by a molar excess of Sst2 (panel A) or GAIP (panel B). The rate of catalysis is comparable to that observed for $G_o\alpha$ measured under the same conditions (Figure 3C,D), and is within the range reported previously for mammalian $G_i\alpha$ and $G_o\alpha$ (*41*). The Gpa1^{Q323L} mutant was devoid of GTPase activity (Figure 3A), as were our preparations of Sst2 and GAIP (Figure 3A−D). Thus, there is no evidence that Sst2 affects the guanine nucleotide exchange step for Gpa1.

Sst2 Accelerates GTP Hydrolysis by Gpa1. It is evident from the experiments described above that Sst2 does not alter the rate of guanine nucleotide exchange, consistent with what has been reported for mammalian RGS proteins. However, mammalian RGS proteins have been found to accelerate the hydrolytic step of the Gα GTPase cycle; we sought to make a similar determination for Sst2. Such experiments require that exchange no longer be rate-limiting. In some cases, this has been accomplished by accelerating the exchange step, by reconstitution with agonist-occupied receptors. An alternative approach, used here, is to allow Gα to bind GTP and to initiate the reaction with the addition of Mg^{2+} (an essential cofactor), either in the presence or in the absence of RGS protein.

Accordingly, we measured the release of [^{32}P]P_i from Gpa1-bound [γ-^{32}P]GTP, at various time points after the addition of Mg^{2+}. As shown in Figure 4, the initial k_{cat} of hydrolysis was estimated to be 0.006 min^{-1} for Gpa1 and 0.042 min^{-1} for $G_o\alpha$. In either case, the rate of hydrolysis was greatly accelerated by the simultaneous addition of a molar excess of Sst2 (Figure 4A,C) or GAIP (Figure 4B,D). Reactions in the presence of RGS proteins were essentially complete at the earliest time points measured, precluding an accurate determination of the change in k_{cat} using this method. Since all of the reactions were performed on ice (vs 30 °C, in Figures 2 and 3), the rate of reaction could not be reduced any further. Nevertheless, acceleration of Gpa1 GTPase activity by either Sst2 (Figure 4A) or GAIP (Figure 4B) is at least 20-fold. A similar degree of acceleration was

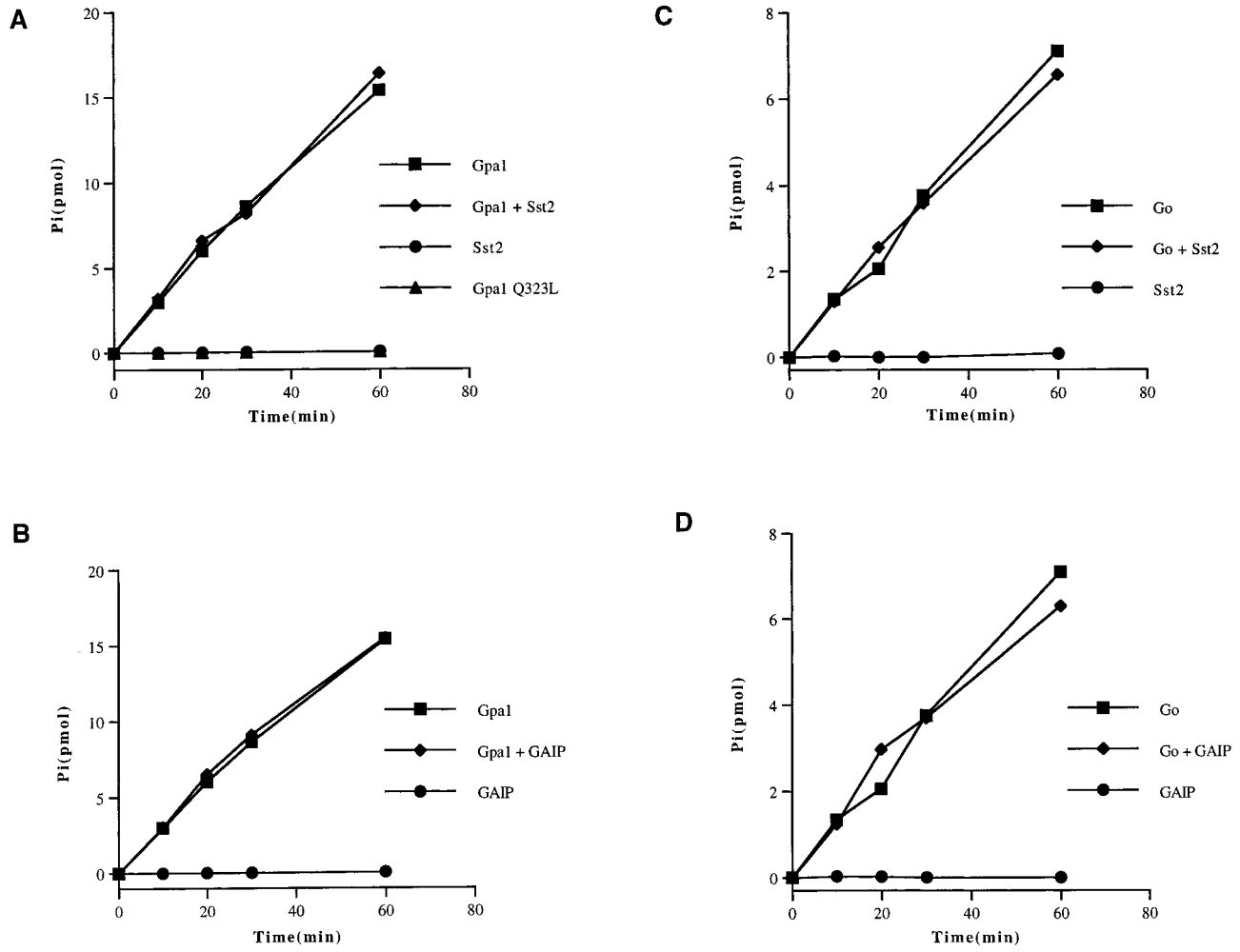

FIGURE 3: Sst2 does not affect steady-state GTP hydrolysis by Gpa1. Gpa1 or $G_o\alpha$ (250 nM), in the absence or presence of Sst2 or GAIP (2 μM), was incubated with [γ-^{32}P]GTP (5 μM) at 30 °C. At the indicated times, duplicate aliquots were withdrawn, charcoal-extracted, and counted as described under Experimental Procedures. Data shown are the average of duplicate measurements, and are representative of at least three independent experiments.

observed using mammalian $G_o\alpha$ (Figure 4C,D), and as reported previously using different RGS and Gα combinations (*14*, *19*, *20*, *26*−*32*). No acceleration was observed using boiled RGS protein. Thus, Sst2 is a GTPase-activating protein for Gpa1.

Sst2 Binds Preferentially to the Transition State of Gpa1. Several mammalian RGS proteins have been shown to bind preferentially to Gα complexed with GDP−AlF_4^- (*18*, *19*, *27*, *28*, *31*, *32*), which is thought to mimic the trigonal bipyramidal structure of the γ-phosphate at the transition state of hydrolysis (*39*, *40*). Indeed, the crystal structure of RGS4 complexed with $G_i\alpha$·GDP−AlF_4^- (*42*) revealed extensive contacts with two regions (switches I and II) that undergo conformational change after GTP hydrolysis (*39*, *40*, *43*−*47*). To determine if Sst2 recognizes a similar conformation in Gpa1, we examined the interaction of these proteins in the presence of GDP−AlF_4^-, GDP, or GTPγS.

The $[His]_6$-tagged form of Gpa1 in the presence of either GDP, GTPγS, or GDP−AlF_4^- (10 μM) was mixed with lysates from *E. coli* expressing full-length (nontagged) Sst2, and immobilized on Ni−NTA−agarose. After extensive washing, the remaining protein was eluted with SDS−PAGE sample buffer, and resolved by immunoblotting with antibodies to Sst2 and Gpa1. As shown in Figure 5, Sst2 bound specifically to the Gpa1·GDP−AlF_4^- complex but bound poorly to Gpa1·GDP and Gpa1·GTPγS. These results are in good agreement with similar experiments using mammalian Gα and RGS proteins (see below) (*18*, *19*, *27*, *28*, *31*, *32*). Thus, Sst2 binds preferentially to the transition state of Gpa1.

DISCUSSION

Many cellular processes use GTPases to regulate their activity. Further regulation is afforded by guanine nucleotide exchange stimulators, inhibitors, and GAPs. GAPs have long been known to exist for monomeric GTPase signaling proteins such as Ras (*48*). For heterotrimeric G proteins, GAP activity has been ascribed to downstream effectors such as phospholipase C-β1 (for $G_q\alpha$) (*49*, *50*) and a cellular complex that includes the retinal cGMP phosphodiesterase γ subunit (for $G_t\alpha$) (*51*, *52*). More recently, RGS proteins have been shown to act as GAPs for members of the G_i and G_q subfamilies (*14*, *19*, *20*, *26*−*32*). Here we have shown that Sst2 is a GAP for its cognate Gα protein, Gpa1. Sst2 is the only RGS protein known to regulate G protein signaling in vivo and to promote G protein GTPase activity in vitro.

The amino acid sequence of Sst2 is weakly related to the other RGS proteins. Among the mammalian RGS proteins,

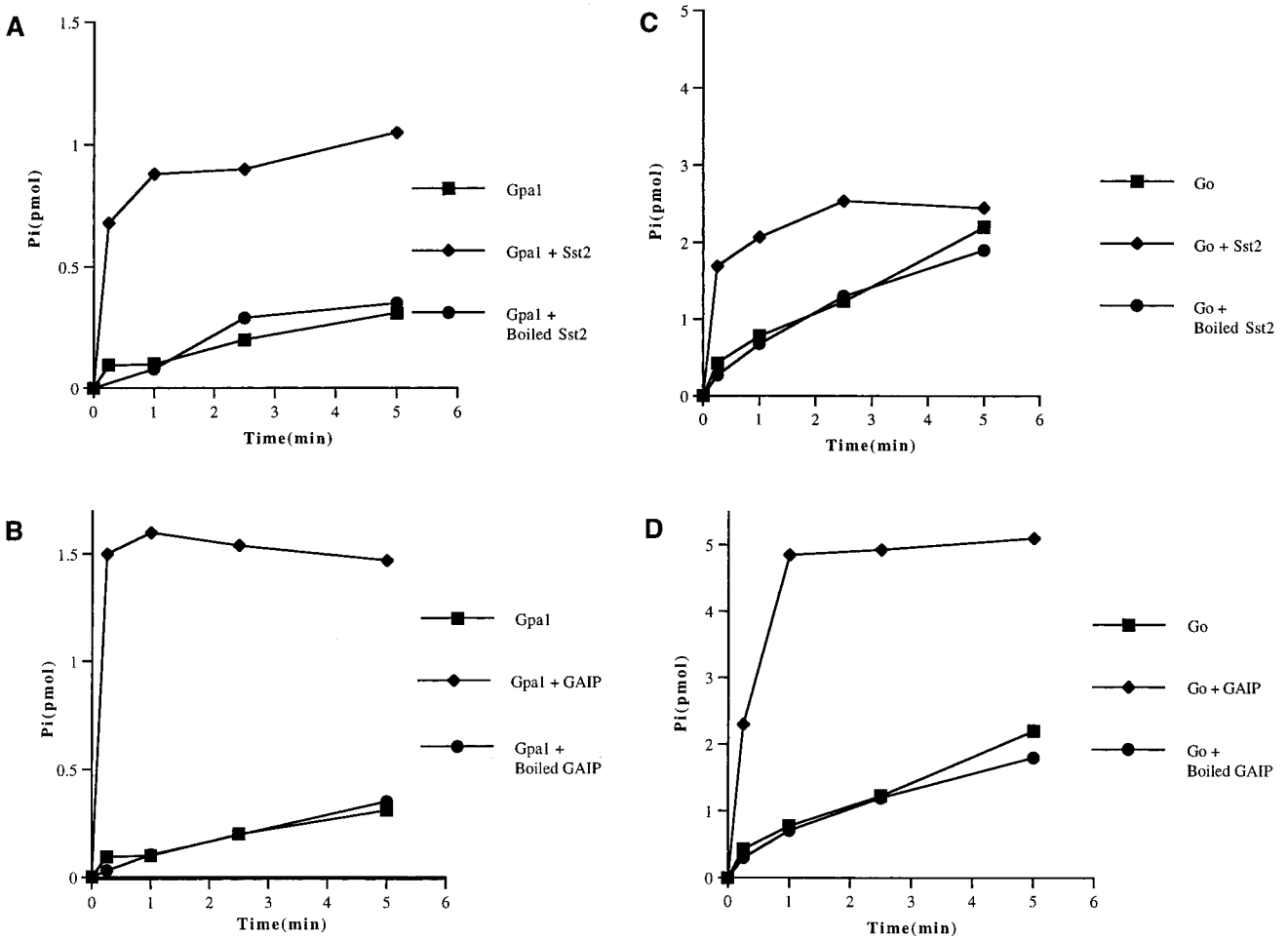

FIGURE 4: Sst2 accelerates GTP hydrolysis by Gpa1: single-turnover GTPase activity. Gpa1 or $G_o\alpha$ (250 nM) was incubated with [γ-^{32}P]-GTP (1 μM) at 30 °C for 15 min. Samples were placed on ice for 5 min prior to the addition of $MgCl_2$ (15 mM), in the absence or presence of Sst2 (2 μM) or GAIP (20 μM), to initiate the reaction. At the indicated times, duplicate aliquots were withdrawn, charcoal-extracted, and counted as described under Experimental Procedures. Data shown are the average of duplicate measurements, and are representative of at least three independent experiments.

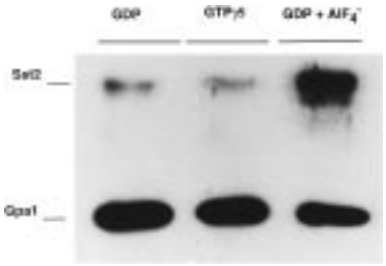

FIGURE 5: Sst2 binds preferentially to the transition state of Gpa1. Gpa1 (5 μg) was incubated at 20 °C for 30 min in the presence of either GDP (10 μM), GTPγS (10 μM), or GDP (10 μM) and AlF_4^- (10 μM). Solubilized cell lysates containing non-His-tagged Sst2 were then added; the complex was allowed to form for 30 min at 4 °C and then immobilized on Ni−NTA−agarose for an additional 30 min. After extensive washing, the bound proteins were eluted in SDS−PAGE sample buffer and resolved by immunoblotting with Gpa1 and Sst2 antibodies, as detailed under Experimental Procedures.

the highest sequence similarity is found within the 125 residue "RGS core domain", whereas in yeast the corresponding domain of Sst2 is much larger and bears only modest sequence similarity to its mammalian counterparts (*5*, *12*). Despite limited sequence similarity, yeast and mammalian RGS proteins share remarkable functional similarity. We have shown that Sst2 does not alter the rate-limiting guanine nucleotide exchange step (Figures 2 and 3), but is a potent activator of the GTP hydrolytic step for Gpa1 (Figure 4). This is consistent with similar studies using mammalian Gα and RGS proteins (Figures 2−4) (*14*, *19*, *20*, *26*−*32*). We have also shown that Sst2 binds with highest affinity to the presumed transition state mimic, Gpa1·GDP−AlF_4^-, and with much lower affinity to the inactive (GDP-bound) and active (GTPγS-bound) conformations (Figure 5). This is consistent with the studies of Berman et al., who found that RGS4 binds preferentially to the GDP−AlF_4^--bound form of $G_{i1}\alpha$ (*32*). They inferred from this result that RGS4 promotes GTP hydrolysis by stabilizing the transition state of the reaction, rather than elevating the energy level of the enzyme−substrate complex. This supposition is supported by the recent crystal structure determination of $G_{i1}\alpha$·GDP−AlF_4^- complexed with RGS4 (*42*), as well as of Ras·GDP−AlF_3 complexed with Ras-GAP (*53*). Ras and the GTPase domain of all Gα subunits exhibit extensive sequence similarity, and have overlapping three-dimensional structures (*44*). Ras-GAP and RGS proteins are not homologous, are structurally distinct, and do not act on one another's substrates. Nevertheless, both proteins are similar in that they form most of their contacts with the "switch" regions of their target GTPase. Both RGS and Ras-GAP bind stably to the GDP−AlF_4^--complex of

the target GTPase (*32*, *54*), and both promote GTP hydrolysis in part by stabilizing the transition state of the reaction (*42*, *53*).

Preferential binding to the transition state of Gα is not a universal property of all RGS proteins, however. RGS10 also binds well to G_{i3}α and G_zα in the active GTP-bound state (*14*) while other RGS proteins can bind to certain Gα subtypes in the inactive GDP-bound state; examples include binding of GAIP to G_{i3}α, RGS1 to G_{i3}α, RGS2 to G_{i3}α, RGS5 to G_{i2}α, RGS5 to G_oα, and RGS16 to G_oα (*13*, *18*, *28*). The ability of some RGS proteins to recognize multiple conformational states of Gα could reflect some additional functions besides GAP activity. For instance, RGS binding to Gα·GDP would block subunit reassociation, thereby prolonging G$\beta\gamma$-mediated (but not Gα-mediated) signaling. Binding of RGS to Gα·GTP could block coupling to effectors, thereby attenuating Gα-mediated (but not G$\beta\gamma$-mediated) signaling. Alternatively, some RGS proteins that bind Gα·GTP could act as effectors, just as phospholipase Cβ functions both as a GAP and as an effector for G_qα. Our finding that Sst2 binds preferentially to Gpa1·GDP−AlF_4^- suggests that its primary role is to promote GTP hydrolysis, rather than to regulate Gα protein−protein interactions. This is significant because these proteins represent the only RGS−Gα pair known to interact in vivo, as well as in vitro.

Although Sst2 does not exhibit the properties of an effector, it almost certainly has additional functions besides acting as a GAP for Gpa1. Indeed, Sst2 is known to bind Mpt5, a protein implicated in cell cycle regulation (*55*). In addition, several RGS proteins (e.g., Sst2, EGL-10, RGS3, RET-RGS) have large N-terminal extensions that bear little similarity to one another, or to any other known proteins. In the case of Sst2, the N-terminal domain is clearly required for in vivo function, since both activating and inactivating mutations have been mapped to this region (*9*, *10*).

Yeast has long been recognized as a premier model system for in vivo genetic analysis of signaling and regulation. The ability to purify Sst2 and Gpa1 will now permit us to determine how these proteins are regulated biochemically, as well as genetically.

ACKNOWLEDGMENT

We are very grateful to Pat Casey and Rick Neubig for their advice and for providing samples of purified G_oα, and to members of the Dohlman and Sigler laboratories for their comments and support.

REFERENCES

1. Dohlman, H. G., Thorner, J., Caron, M. G., and Lefkowitz, R. J. (1991) *Annu. Rev. Biochem. 60*, 653−688.
2. Neer, E. J. (1995) *Cell 80*, 249−257.
3. Premont, R. T., Inglese, J., and Lefkowitz, R. J. (1995) *FASEB J. 9*, 175−182.
4. Iyengar, R. (1997) *Science 275*, 42−43.
5. Dohlman, H. G., and Thorner, J. (1997) *J. Biol. Chem. 272*, 3871−3874.
6. Chan, R. K., and Otte, C. A. (1982) *Mol. Cell. Biol. 2*, 21−29.
7. Chan, R. K., and Otte, C. A. (1982) *Mol. Cell. Biol. 2*, 11−20.
8. Dietzel, C., and Kurjan, J. (1987) *Mol. Cell. Biol. 7*, 4169−4177.
9. Dohlman, H. G., Apaniesk, D., Chen, Y., Song, J., and Nusskern, D. (1995) *Mol. Cell. Biol. 15*, 3635−3643.
10. Dohlman, H. G., Song, J., Ma, D., Courchesne, W. E., and Thorner, J. (1996) *Mol. Cell. Biol. 16*, 5194−5209.
11. Lee, B. N., and Adams, T. H. (1994) *Mol. Microbiol. 14*, 323−334.
12. Koelle, M. R., and Horvitz, H. R. (1996) *Cell 84*, 115−125.
13. DeVries, L., Mousli, M., Wurmser, A., and Farquhar, M. G. (1995) *Proc. Natl. Acad. Sci. U.S.A. 92*, 11916−11920.
14. Hunt, T. W., Fields, T. A., Casey, P. J., and Peralta, E. G. (1996) *Nature 383*, 175−177.
15. Druey, K. M., Blumer, K. J., Kang, V. H., and Kehrl, J. H. (1996) *Nature 379*, 742−746.
16. Hong, J. X., Wilson, G. L., Fox, C. H., and Kehrl, J. H. (1993) *J. Immunol. 150*, 3895−904.
17. Wu, H. K., Heng, H. H., Shi, X. M., Forsdyke, D. R., Tsui, L. C., Mak, T. W., Minden, M. D., and Siderovski, D. P. (1995) *Leukemia 9*, 1291−1298.
18. Chen, C., Zheng, B., Han, J., and Lin, S. C. (1997) *J. Biol. Chem. 272*, 8679−8685.
19. Chen, C. K., Wieland, T., and Simon, M. I. (1996) *Proc. Natl. Acad. Sci. U.S.A. 93*, 12885−12889.
20. Faurobert, E., and Hurley, J. B. (1997) *Proc. Natl. Acad. Sci. U.S.A. 94*, 2945−2950.
21. Huang, C., Hepler, J. R., Gilman, A. G., and Mumby, S. M. (1997) *Proc. Natl. Acad. Sci. U.S.A. 94*, 6159−6163.
22. Yan, Y., Chi, P. P., and Bourne, H. R. (1997) *J. Biol. Chem. 272*, 11924−11927.
23. Chatterjee, T. K., Eapen, A. K., and Fisher, R. A. (1997) *J. Biol. Chem. 272*, 15481−15487.
24. Neill, J. D., Duck, L. W., Sellers, J. C., Musgrove, L. C., Scheschonka, A., Druey, K. M., and Kehrl, J. H. (1997) *Endocrinology 138*, 843−846.
25. Siderovski, D. P., Hessel, A., Chung, S., Mak, T. W., and Tyers, M. (1996) *Curr. Biol. 6*, 211−212.
26. Berman, D. M., Wilkie, T. M., and Gilman, A. G. (1996) *Cell 86*, 445−452.
27. Hepler, J. R., Berman, D. M., Gilman, A. G., and Kozasa, T. (1997) *Proc. Natl. Acad. Sci. U.S.A. 94*, 428−432.
28. Watson, N., Linder, M. E., Druey, K. M., Kehrl, J. H., and Blumer, K. J. (1996) *Nature 383*, 172−175.
29. Natochin, M., Granovsky, A. E., and Artemyev, N. O. (1997) *J. Biol. Chem. 272*, 17444−17449.
30. Nekrasova, E. R., Berman, D. M., Rustandi, R. R., Hamm, H. E., Gilman, A. G., and Arshavsky, V. Y. (1997) *Biochemistry 36*, 7638−7643.
31. Wieland, T., Chen, C. K., and Simon, M. I. (1997) *J. Biol. Chem. 272*, 8853−8856.
32. Berman, D. M., Kozasa, T., and Gilman, A. G. (1996) *J. Biol. Chem. 271*, 27209−27212.
33. Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A., and Struhl, K. (1987) *Current Protocols in Molecular Biology*, Wiley-Interscience, New York.
34. DiBello, P., Garrison, T. R., Apanovitch, D. M., Hoffman, G., Shuey, D. J., Mason, K., Cockett, M. I., and Dohlman, H. G. (1998) *J. Biol Chem. 273*, 5780−5784.
35. Lee, E., Linder, M. E., and Gilman, A. G. (1994) *Methods Enzymol. 237*, 146−164.
36. Dohlman, H. G., Goldsmith, P., Spiegel, A. M., and Thorner, J. (1993) *Proc. Natl. Acad. Sci. U.S.A. 90*, 9688−9692.
37. Laemmli, U. K. (1970) *Nature 227*, 680−685.
38. Masters, S. B., Miller, R. T., Chi, M. H., Chang, F. H., Beiderman, B., Lopez, N. G., and Bourne, H. R. (1989) *J. Biol. Chem. 264*, 15467−15474.
39. Coleman, D. E., Berghuis, A. M., Lee, E., Linder, M. E., Gilman, A. G., and Sprang, S. R. (1994) *Science 265*, 1405−1412.
40. Sondek, J., Lambright, D. G., Noel, J. P., Hamm, H. E., and Sigler, P. B. (1994) *Nature 372*, 276−279.
41. Linder, M. E., Ewald, D. A., Miller, R. J., and Gilman, A. G. (1990) *J. Biol. Chem. 265*, 8243−8251.
42. Tesmer, J. J., Berman, D. M., Gilman, A. G., and Sprang, S. R. (1997) *Cell 89*, 251−261.
43. Lambright, D. G., Noel, J. P., Hamm, H. E., and Sigler, P. B. (1994) *Nature 369*, 621−628.

44. Noel, J. P., Hamm, H. E., and Sigler, P. B. (1993) *Nature 366*, 654−663.
45. Lambright, D. G., Sondek, J., Bohm, A., Skiba, N. P., Hamm, H. E., and Sigler, P. B. (1996) *Nature 379*, 311−319.
46. Mixon, M. B., Lee, E., Coleman, D. E., Berghuis, A. M., Gilman, A. G., and Sprang, S. R. (1995) *Science 270*, 954−960.
47. Wall, M. A., Coleman, D. E., Lee, E., Iniguez, L. J., Posner, B. A., Gilman, A. G., and Sprang, S. R. (1995) *Cell 83*, 1047−1058.
48. McCormick, F. (1989) *Cell 56*, 5−8.
49. Berstein, G., Blank, J. L., Jhon, D. Y., Exton, J. H., Rhee, S. G., and Ross, E. M. (1992) *Cell 70*, 411−418.
50. Biddlecome, G. H., Berstein, G., and Ross, E. M. (1996) *J. Biol. Chem. 271*, 7999−8007.
51. Arshavsky, V. Y., and Bownds, M. D. (1992) *Nature 357*, 416−417.
52. Angleson, J. K., and Wensel, T. G. (1993) *Neuron 11*, 939−949.
53. Scheffzek, K., Ahmadian, M. R., Kabsch, W., Wiesmüller, L., Lautwein, A., Schmitz, F., and Wittinghofer, A. (1997) *Science 277*, 333−338.
54. Mittal, R., Ahmadian, M. R., Goody, R. S., and Wittinghofer, A. (1996) *Science 273*, 115−117.
55. Chen, T., and Kurjan, J. (1997) *Mol. Cell Biol. 17*, 3429−3439.

BI9729965