Regulators of G protein signaling (RGS proteins) are well known to accelerate G protein GTPase activity in vitro and to promote G protein desensitization in vivo. Less is known about how RGS proteins are themselves regulated. To address this question we purified the RGS proteins in yeast, Sst2, and used electrospray ionization mass spectrometry to identify post-translational modifications. This analysis revealed that Sst2 is phosphorylated at Ser-539 and that phosphorylation occurs in response to pheromone stimulation. Ser-539 lies within a consensus mitogen-activated protein (MAP) kinase phosphorylation site, Pro-X-Ser-Pro. Phosphorylation is blocked by mutations in the MAP kinase genes (FUS3, KSS1), as well as by mutations in components needed for MAP kinase activation (STE11, STE7, STE4, STE18). Phosphorylation is also blocked by replacing Ser-539 with Ala, Asp, or Glu (but not Thr). These point mutations do not alter pheromone sensitivity, as determined by growth arrest and reporter transcription assays. However, phosphorylation appears to slow the rate of Sst2 degradation. These findings indicate that the G protein-regulated MAP kinase in yeast can act as a feedback regulator of Sst2, itself a regulator of G protein signaling.

All eukaryotic cells respond to external signals through cell surface receptors linked to heterotrimeric G proteins. In humans, G protein-linked receptors can detect hormones, neurotransmitters, and sensory stimuli (odors, taste, light). In the yeast Saccharomyces cerevisiae, G protein linked pheromone receptors mediate events required for mating and cell fusion. Upon receptor stimulation, the G protein binds GTP and undergoes subunit dissociation. The Go subunit or the Gβγ subunit dimer can then propagate the signal through a variety of effector enzymes or ion channels. Upon GTP hydrolysis, the G protein subunits reassociate and signaling stops. Additional proteins can modulate this cycle of G protein activation and inactivation. For instance, G protein-coupled receptor kinases (GRKs)\(^1\) and arrestins promote desensitization through phosphorylation, uncoupling, and internalization of the receptor (1).

It is now evident that G proteins are also subject to desensitization and that this process involves members of the RGS protein family. The contribution of RGS proteins in vivo has been established through genetic analysis in yeast and nematodes. In yeast, disruption of the RGS gene SST2 can increase pheromone sensitivity by 100–300-fold (2). The mechanism of RGS action has also been well characterized through detailed biochemical and x-ray crystallographic analysis of purified components (3, 4). Stop flow fluorescence measurements reveal that RGS proteins can accelerate Gα GTPase activity by up to 1000-fold (5).

How are RGS proteins themselves regulated? Some RGS genes, including SST2, are transcriptionally induced by G protein activation (6). Virtually nothing is known about how these proteins are regulated post-translationally, however. In particular, it is not known if RGS proteins are phosphorylated or otherwise modified in response to cell stimulation. To address this question, we examined whether Sst2 undergoes any chemical modifications in vivo. Our analysis reveals that Sst2 is stoichiometrically phosphorylated at Ser-539, in response to pheromone stimulation, and in a Fus3-dependent manner. Phosphorylation appears to stabilize the protein, thereby augmenting the transcriptional induction that occurs in response to pheromone stimulation.

EXPERIMENTAL PROCEDURES

Strains and Plasmids—S. cerevisiae stains used in this study are BJ2168 (MATα ura3–52 leu2–1 Δ1 trpl–1 Δ63 prb1–1122 pcr1–1 407p4–3), BJ53 (sst2–Δ2, derived from BJ2168) (7), YPH 499 (MATα ura3–52 lys2–801am ade2–101 trpl–1 Δ63 his3–Δ200 leu2–Δ1), and MPY576c (MATα sst2–Δ2:GUS1-HIS3 fus2–Δ2:CAN1 far1–1LYS2 ura3 his3 leu2 trpl lys2 ade2 can1). Signaling mutants were derived from YPH499 and are designated YDK101 (ste11::HIS3, from J. Thorner, University of California), YSB1 (gpa1Δ trpl1Δ, from S. Burchett and H. G. D.) (8), YDM400 (sst2–Δ2) (7), YTG4 (ste4::hisG, this laboratory), MPH16 (ste18::LEU2) (9), YTD20 (ste20::LEU2) (10), YTG11 (ste11::hisG) (9), JTY2556 (ste7::ADE2, from J. Thorner), YDM600 (kss1::hisG) (11), YDM200 (fus3::LEU2) (11), YM300 (kss1::hisG, fus3::LEU2) (11), YDK12/JDY3 (ste12::LEU2) (12). Plasmid pAD4M-SST2-his was constructed by replacing the BsoHI-HindIII fragment of SST2 in pAD4M (13) with the corresponding sequence from pQE60-SST2-his (14). Plasmid pRS316-ADH-SST2 contains SST2 under the control of a constitutive promoter from ADH1 and is described in Ref. 14. pRS315-SST2 contains SST2 under the control of its own promoter (14). Plasmid pGA1905 contains a myc epitope-tagged Fus1::HIS3 protein.}

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\(^1\) The abbreviations used are: GRK, G-protein coupled receptor kinase; MAP, mitogen-activated protein; RGS, regulator of G protein signaling; SPE, solid phase extraction; Ni\(^2+\)-NTA, nickel nitrilotriacetic acid; MS, mass spectrometry; PAGE, polyacrylamide gel electrophoresis.

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nase dead" mutant, as described in Ref. 15. SST2 mutants were obtained using the QuickChange mutagenesis kit (Stratagene) and confirmed by DNA sequencing.

**Purification**—BJ2168 cells transformed with pAD4M-SST2-his were grown to an optical density at 600 nm of 0.8 and treated with 2.5 μM α-factor, as indicated. Cells were chilled, harvested by centrifugation, rapidly frozen in liquid nitrogen, and thawed in urea buffer (6 M urea, 100 mM Na2HPO4, 10 mM Tris, 10 mM 2-mercaptoethanol, pH 8.0), 250 mM NaCl, 15 mM imidazole (at 250 ml/6 liter of medium) at room temperature. Cells were further disrupted using a stainless steel bead beater (Biospec) packed in ice and salt, with 10 × 30-s pulses, once every 90 s. The remaining procedures were carried out at room temperature. The disrupted cells were rocked for 60–90 min, then purified by centrifugation 3840 × g, 15 min and paper filtration (Whatman No. 1). The soluble material was mixed with 3 ml of equilibrated Superflow Ni2+-NTA resin (Qiagen) for 60–90 min, then packed into a HR 10/10 (Amersham Pharmacia Biotech) column and washed with 10 column volumes (CV) of urea buffer, 250 mM NaCl, 15 mM imidazole at 1.5 ml/min, followed by 10 CV urea buffer at 1.0 ml/min. Sst2 was eluted in 10 CV of urea buffer, 75 mM imidazole at 1.0 ml/min. The eluate was mixed with 2 ml of equilibrated Mono Q resin (Amersham Pharmacia Biotech) for 60 min, then packed into a HR 10/10 column and washed with 5 CV of urea buffer at 1.0 ml/min, then 7.5 CV of urea buffer and a linear gradient (0–60 mM) of NaCl at 0.5 ml/min and 7.5 CV 60 mM NaCl urea buffer at 0.5 ml/min. Sst2 was eluted with 15 CV urea buffer, 1 mM NaCl at 0.5 ml/min. Peak fractions were pooled, concentrated, and desalted using an Ultrafree-30 (Millipore) filter. The final purified product (500 μl) was resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and silver staining (Silver Stain Plus, Bio-Rad). Except where indicated, 16-cm 8% acrylamide gels were used to resolve the 82- and 84-kDa species of Sst2. Antibodies and conditions for immunoblot detection of Sst2 are described elsewhere (7).

**Sample Preparation**—Each silver-stained protein band (~1 μg) was excised (20-cm gel) and transferred to a 0.5-ml microcentrifuge tube and destained with a 1:1 solution of 30 mM potassium ferricyanide and 100 mM sodium thiosulfate for 2 min. The gel was washed four times with Milli-Q water, cut into small pieces, washed three times for 5 min each with 12.5 mM ammonium bicarbonate, 50% acetonitrile and Speedvac-dried. Samples were reconstituted with 40 μl of 12.5 ng/ml endo-protease Asp-N or pepsin (Roche Molecular Biochemicals), incubated on ice for 40 min, then at 37 °C overnight. Peptides were extracted three times with 40 μl of 5% acetic acid, 50% acetonitrile. All four supernatants were pooled, and the solution was dried under vacuum and reconstituted in 10 μl of capillary electrophoresis buffer (10 mM sodium thiosulfate, 10% MeOH).

**Mass Spectrometry**—Solid phase extraction (SPE) capillary electrophoresis electrospray ionization tandem mass spectrometry was performed essentially as described (16). The SPE cartridge consisted of Spherisorb C-18 material between two Teflon membranes inside a Teflon tube (17). The reconstituted peptide mixture was loaded on the SPE cartridge at 1 μl/min. The concentrated peptides were washed for 10 min and eluted by applying a small plug of 67% acetonitrile, 3 mM TFA. All four supernatants were pooled, and the solution was dried under vacuum and reconstituted in 10 μl of capillary electrophoresis buffer (10 mM sodium thiosulfate, 10% MeOH).

**RESULTS**

To determine whether Sst2 undergoes post-translational modification, we isolated a His-tagged version of the protein from both pheromone-stimulated and -unstimulated cells. Cells were disrupted in a denaturing buffer containing 6 M urea, so as to fully solubilize Sst2 and to preserve any modifications. Sst2 was then purified by sequential Ni2+-NTA affinity and Mono Q-Sepharose ion exchange chromatography, and resolved by gel electrophoresis. As shown in Figs. 1 and 2, Sst2 from control (unstimulated) cells migrates at the predicted size of ~82 kDa, while Sst2 from pheromone-stimulated cells migrates as a doublet of ~82 and 84 kDa (top panel, "silver stain"). An identical gel was immunoblotted using anti-Sst2 antibodies, to confirm that the two bands represent Sst2 (bottom panel, "Sst2 Ab"). B, purified protein was excised from a 20-cm silver-stained gel, subjected to limited proteolysis, and analyzed by SPE capillary electrophoresis electrospray ionization tandem mass spectrometry. Using either Asp-N (shown) or pepsin (not shown), the pheromone-treated 84-kDa band yielded a phospho-Ser that was absent in the treated and untreated 82-kDa bands. MS analysis also revealed a second phospho-Ser present in both the 82- and 84-kDa bands, indicating that Sst2 undergoes constitutive and pheromone-dependent phosphorylation.

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Phosphorylation requires Fus3 and Ser-539. A, to determine whether Ser-539 is phosphorylated in vivo, this residue was replaced with Ala (A), Glu (E), Asp (D), or Thr (T). BJ2168 cells expressing wild-type or mutant Sst2 (pAD4M-SST2-His) were treated with α-factor for 1 h (+ +). Cells were lysed in SDS-PAGE sample buffer, and the conversion of Sst2 to the phosphorylated species was monitored by 8% SDS-PAGE and immunoblotting, using anti-Sst2 antibodies (7). B, to determine the time course of Sst2 phosphorylation in vivo, BJSSST2 cells containing pRS316-ADH-SST2 were exposed to 2.5 μM α-factor for the indicated times, lysed in SDS-PAGE sample buffer, and monitored by immunoblotting. C, to determine which signaling components are needed for Sst2 phosphorylation, extracts were prepared from YPH499 cells containing pRS316-ADH-SST2 were exposed to 2.5 μM α-factor for the indicated times, lysed in SDS-PAGE sample buffer, and monitored by immunoblotting.

Discussion

One of the defining characteristics of desensitization is feedback inhibition. One way this is accomplished is through increased expression of factors that can attenuate the signal. Indeed it is well known that increased expression of Sst2 will inhibit the pheromone response (7). It is also known that pheromone stimulation leads to increased transcription of SST2 (6). Our findings indicate that pheromone stimulation promotes increased stabilization of the protein, and this is achieved in part through phosphorylation at Ser-539. The combined effect of transcriptional induction and protein stabilization should allow for sustained expression of Sst2 and at a lower energy expenditure than transcriptional induction alone. Consistent with this interpretation, we have found that expression of endogenous Sst2 is induced by pheromone (7), and the induced post-translational modification. To characterize this further, each band was excised from the gel, subjected to limited proteolysis, and analyzed by electrospray ionization tandem mass spectrometry. As indicated in Fig. 1B, a phosho-Ser at position 539 was present in the 84-kDa band. The corresponding nonphosphorylated peptide was obtained from the 82-kDa bands, from either stimulated or unstimulated cells (data not shown).

To confirm that phosphorylation occurs on Ser-539, we replaced this amino acid with Ala, Glu, Asp, or Thr. With the exception of Thr, these residues cannot be phosphorylated by protein kinases. The acidic residues Asp and Glu can often substitute for the negatively charged phospho-Ser (19). As expected, the Ser-539 → Thr mutation preserved the pheromone-dependent mobility shift, while the Ala, Asp, and Glu mutations blocked the shift completely (Fig. 2A). These data indicate that Sst2 undergoes a stimulus-dependent phosphorylation at Ser-539 and that Ser-539 phosphorylation causes the gel mobility shift.

Ser-539 lies within an ideal consensus sequence (Pro-X-Ser-Pro) for phosphorylation by MAP kinases (19). Indeed, the pheromone signaling pathway involves at least one MAP kinase family member, Fus3 (20). To determine whether Sst2 is a substrate for Fus3 in vivo, we examined whether Sst2 phosphorylation requires Fus3 expression or activity. We first established the time course of Sst2 phosphorylation (Fig. 2B). We then tested whether Sst2 is phosphorylated in cells lacking the Fus3 gene or any of the other known components of the signaling cascade (Fig. 2C). As predicted, Sst2 failed to undergo phosphorylation in cells lacking Fus3, provided that KSS1 (another MAP kinase that can partially rescue a fus3 mutant) was also deleted (21, 22). Phosphorylation was not restored by a Fus3 mutant (Lys-42 → Arg) that lacks kinase activity. Phosphorylation was also abolished by mutations that block expression of the upstream MAP kinase kinase (MAPKK, Ste7) or MAPKK kinase (Ste11). This was expected, since both Ste7 and Ste11 are required for Fus3 activity (23). Phosphorylation was preserved in a Gpa1Gly-302 → Ser mutant that cannot bind Sst2 (24), as well as in mutants lacking the MAPKKK kinase Ste20 or the transcription factor (and Fus3 substrate) Ste12. Deletion of the pheromone receptor Ste2 blocks the pheromone-mediated response, but also leads to elevated basal signaling (9) and Sst2 phosphorylation (Fig. 2C). Thus, the only components of the pathway necessary for Sst2 phosphorylation are those needed to activate MAP kinase and the MAP kinase itself. Taken together, these findings indicate that Sst2 is phosphorylated in vivo by Fus3.

Loss-of-function mutations in Sst2 are well known to increase pheromone sensitivity (25). To determine whether MAP kinase phosphorylation has any effect on pheromone signaling in vivo, we tested the ability of the Ser-539 mutants to regulate the pheromone response pathway, using a reporter transcription assay. As shown in Fig. 3A, pheromone-induced gene transcription (using the fus1 promoter linked to the essential HIS3 gene) was unaffected by any of the Ser-539 substitutions. Likewise, no difference was seen using an alternative pheromone bioassay, the growth inhibition plate assay (halo assay, Fig. 3B) (26, 27).

Like many cell regulators, Sst2 has a short half-life in vivo (7, 28). Some regulatory factors (e.g., cyclins, cyclin inhibitors) are targeted for destruction by phosphorylation and, in some cases, by ubiquitination (28). Thus, we considered whether phosphorylation of Ser-539 contributes to Sst2 proteolysis. Cells were transformed with plasmids containing wild-type or the 539 mutant forms of Sst2, under the control of a constitutive promoter (ADH1, to prevent pheromone-mediated changes in SST2 transcription). Cells were treated with cycloheximide to block new protein synthesis and sampled at various times. As shown in Fig. 3C, the overall levels of Sst2 drop rapidly when translation is blocked, but the decrease is most pronounced for the nonphosphorylated (82-kDa) species. An alternative explanation, that loss of the 82-kDa species is due to increased phosphorylation, is improbable, since there is no concomitant increase in the 84-kDa form of the protein. Surprisingly, all of the 539 mutants degraded at rates comparable with the phosphorylated form of Sst2 (data not shown). These data suggest that Sst2 is rapidly degraded, except when Ser-539 is phosphorylated or replaced with another amino acid.
structure-function relationships? The crystal structure determination of rat G_{i1}\alpha complexed with RGS4 provides some important insights (3). The RGS core region contains nine α-helices that form two subdomains. The majority of residues that contact G_{α} are on the “bottom” of the subdomain comprised of helices 4, 5, 6, and 7. A structurally based alignment of RGS4 and Sst2 reveals that Ser-539 lies between helices 6 and 7, on “top” of this subdomain. Thus it appears that Sst2 is phosphorylated at a site distal from the G protein binding interface and therefore would not be expected to have any direct effect on Sst2-Gpa1 interaction. Correspondingly, Sst2 could undergo phosphorylation even in the Gpa1-bound state. This model is consistent with the lack of any G protein modulatory effects of the Ser-539 mutants.

There is also the possibility that phosphorylation modulates some other (G protein-independent) function of Sst2. The adjoining region of Sst2 has a 118-amino acid insert (residues 539–657). The function of this region is unknown, but it is probably dispensable for GTPase accelerating activity, since it is absent in all other RGS proteins. Interestingly, the same insert contains at least two PEST motifs (544–581 and 606–626) (7), which are often found in proteins that are rapidly degraded (29). Perhaps this insert serves as a proteolytic signal, but one which is regulated by pheromone-dependent MAP kinase activity.

How do these findings fit with our understanding of MAP kinase function? To our knowledge, this work represents the first direct identification of a MAP kinase phosphorylation site in yeast. It is also a rare example of a MAP kinase substrate outside of the nucleus (30–32). In yeast, Fus3 is known to phosphorylate a transcriptional activator (Ste12), transcriptional repressors (Dig1, Dig2), and cyclin inhibitors (30, 33, 34). Even in mammalian cells only a handful of non-nuclear substrates for MAP kinases have been identified. However, this list includes members of the GRK and arrestin families, two proteins that promote desensitization of G protein coupled receptors (35). Thus the regulation of receptors and G proteins may be coordinated in part by the ability of MAP kinases to phosphorylate a variety of desensitization factors acting throughout the signaling pathway.

In summary, RGS proteins are well known to promote G protein GTPase activity and desensitization. Our objective was to determine whether RGS proteins are themselves regulated, through post-translational modification. Our demonstration that Sst2 is phosphorylated in response to pheromone stimulation reveals one way that G protein signaling pathways might undergo feedback regulation in vivo. Given the striking similarities in yeast and human RGS proteins, it is likely that the regulatory mechanisms described here will also be recapitulated in more complex organisms.

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