Sck1 Negatively Regulates Gpa2-Mediated Glucose Signaling in Schizosaccharomyces pombe

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Schizosaccharomyces pombe detects extracellular glucose via a G protein-mediated cyclic AMP (cAMP)-signaling pathway activating protein kinase A (PKA) and regulating transcription of genes involved in metabolism and sexual development. In this pathway, Gpa2 Gα binds to and activates adenyl cyclase in response to glucose detection by the Git3 G protein-coupled receptor. Using a two-hybrid screen to identify extrinsic regulators of Gpa2, we isolated a clone that expresses codons 471 to 696 of the Sck1 kinase, which appears to display a higher affinity for Gpa2K270E-activated Gα relative to Gpa2+ Gα. Deletion of sck1+ or mutational inactivation of the Sck1 kinase produces phenotypes reflecting increased PKA activity in strains expressing Gpa2+ or Gpa2K270E, suggesting that Sck1 negatively regulates PKA activation through Gpa2. In contrast to the Gpa2K270E GDP-GTP exchange rate mutant, GTPase-defective Gpa2R176T weakly binds Sck1 in the two-hybrid screen and a deletion of sck1+ in a Gpa2R176H strain confers phenotypes consistent with a slight reduction in PKA activity. Finally, deleting sck1+ in a gpa2Δ strain results in phenotypes consistent with a second role for Sck1 acting in parallel with PKA. In addition to this parallel role with PKA, our data suggest that Sck1 negatively regulates Gpa2, possibly targeting the nucleotide-free form of the protein that may expose the one and only AKT/PKB consensus site in Gpa2 for Sck1 to bind. This dual role for Sck1 may allow S. pombe to produce distinct biological responses to glucose and nitrogen starvation signals that both activate the Wis1-Spc1/Sty1 stress-activated protein kinase A (SAPK) pathway.

Eukaryotic cells utilize heterotrimeric guanine nucleotide-binding protein (G protein) signaling pathways to sense their extracellular environment (1). Both the fission yeast Schizosaccharomyces pombe and the budding yeast Saccharomyces cerevisiae possess two G protein pathways that are used to sense glucose and pheromones (2). In all four pathways, the G protein is activated by a seven-transmembrane G protein-coupled receptor protein (GPCR), leading to the activation of adenyl cyclase in the glucose signaling pathways or a mitogen-activated protein kinase (MAPK) in the pheromone signaling pathways (2).

Upon binding of an agonist, GPCRs activate G proteins by triggering the release of the GDP nucleotide bond to the Gα subunit, thus allowing GTP binding that leads to a conformational change in Gα (3). This change results in the dissociation of Goα-GTP from its Gβγ dimeric partner (although some G protein pathways lack a canonical Gβγ [2]) that allows both Gα GTp and Gβγ to bind and regulate downstream effectors. Negative regulation of G protein signaling to restore Gα to its inactive, GDP-bound state occurs through both intrinsic and extrinsic mechanisms. Gα possesses a GTPase domain that converts bound GTP to GDP. In addition, regulators of G protein signaling (RGS) proteins bind to activated Gα subunits to stimulate GTPase activity, thus serving as negative regulators of signaling (4, 5). Such RGS proteins have been identified in the S. pombe pheromone pathway and in both the glucose and pheromone pathways of S. cerevisiae but not in the S. pombe glucose/cAMP (cyclic AMP) pathway (6–8). In this pathway, glucose is detected by the Git3 GPCR that leads to the activation of Gpa2 Gα, which forms a heterotrimer with the Git5 Gβ and Git1 Gγ subunits (9, 10). The activated Gpa2 protein then binds and activates the Git2/Cyr1 adenyl cyclase protein to produce a transient cAMP signal that activates the cAMP-dependent protein kinase A (PKA) Pka1 (11).

Previously, we identified a large collection of mutationally activated alleles of the S. pombe gpa2 gene that allowed PKA activation to repress transcription of the flb1 gene in cells lacking the Git3 GPCR, the Git5 Gβ subunit, or the G11 Gγ subunit (12). In this study, we set out to identify regulators of S. pombe Gpa2 Gα by looking for proteins that preferentially bind mutationally activated Gpa2. Using a two-hybrid screen with a Gpa2K270E bait protein, we identified two clones that displayed a stronger interaction with this bait relative to a Gpa2+ wild-type bait. These clones are identical and express a portion of Sck1, an AGC family protein kinase that we previously identified as a multicopy suppressor of mutations that reduce PKA activity (13). Using two functionally distinct biological assays that reflect PKA activity, we obtained data supporting a model in which Sck1 plays two distinct and opposing roles in nutrient sensing, serving as a negative regulator of PKA through the regulation of Gpa2 activity and a positive regulator of nutrient sensing acting in parallel with PKA.

MATERIALS AND METHODS

S. pombe strains and media. S. pombe strains used in this study are listed in Table 1. Yeasts were grown at 30°C in yeast extract medium with supplements (YES) or Edinburgh minimal medium (EMM) containing 3% glucose and supplemented with required nutrients (at 75 mg/liter, except for leucine, which was present at 150 mg/liter) as previously described (14). Crosses were performed on malt extract agar plates (MEA) containing 0.4% glucose (14), and tetrad dissection on YES plates was used for...
### RESULTS

Two-hybrid screen for proteins that preferentially bind Gpa2<sup>ΔH11001</sup> relative to Gpa2<sup>ΔT325A</sup>. To identify extrinsic regulators of S. pombe Gpa2<sub>G</sub>, we screened a two-hybrid library for clones whose products interact with a Gpa2<sup>ΔH11001</sup> bait, as this mutation was previously shown to confer a defect in GDP binding and thus activate Gpa2 in the absence of signaling from the Git3 GPCR (11). Of ~1 × 10<sup>6</sup> Trp<sup>+</sup> Leu<sup>+</sup> transformants, 41 grew on SC-Trp-Leu-His medium containing 5 mM 3-aminotriazole due to transcription of the his3 reporter and tested positive in an X-Gal filter lift due to transcription of the lacZ reporter. (The Gpa2 bait plasmids confer weak His<sup>+</sup> growth on their own, requiring the use of 3AT to detect protein–protein interactions (11).) After single colony purification, 27 transformants remained 3AT resistant (3AT<sup>+</sup>) and X-Gal blue. DNA sequence analysis of the prey plasmid inserts showed that they represented eight genes. Upon rescue to E. coli and transformation of strains expressing either a wild-type Gpa2<sup>+</sup> bait or an activated Gpa2<sup>ΔH11001</sup> bait, only two clones, which both express residues 471 to 696 of the Sck1 kinase (13), showed a preferential interaction with the activated Gpa2<sup>ΔH11001</sup> bait (Fig. 1A). Surprisingly, the Sck1 prey display a reduced interaction with a Gpa2<sup>R176H</sup> bait that is activated due to a loss of GTPase activity rather than a reduction in GDP binding relative to the Gpa2<sup>+</sup> bait (Fig. 1A, X-Gal filter lift). β-Galactosidase assays of transformants expressing Sck1 prey plasmids confirm the qualitative results from the X-Gal filter lift. An empty bait plasmid produces little activity (1.1 ± 0.3 units [specific activity]). The relative strength of the interaction with Gpa2 baits is Gpa2<sup>ΔT325A</sup> (16.6 ± 1.3 units) > Gpa2<sup>+</sup> (5.6 ± 2.9 units) > Gpa2<sup>R176H</sup> (3.6 ± 1.6 units). This also contrasts with the relative strength of the interactions between these three Gpa2 baits and a Git2 adenyl cyclase prey for which both activated Gpa2 baits show a stronger interaction than the Gpa2<sup>+</sup> bait (Fig. 1A, X-Gal filter lift), consistent with our previous observation of an enhanced interaction between Gpa2<sup>R176H</sup> and Git2 relative to Gpa2<sup>+</sup> and Git2 in a two-hybrid assay (11). Furthermore, Western blots of the two-hybrid trans-
formants indicate that the weak interaction between Gpa2R176H and Sck1 is not due to low abundance of the Gpa2R176H bait (Fig. 1B). In fact, it is the Gpa2R176H bait that is least abundant; thus, it may be that the apparent increased affinity for Sck1 by Gpa2R176H is actually a reflection of various levels of these bait proteins.

**Sck1 negatively regulates PKA activity in cells expressing Gpa2K270E.** Sck1 was previously identified as a multicopy suppressor of mutations that reduce or eliminate PKA signaling (13). We therefore considered that the binding of Sck1 to Gpa2K270E might mean that Sck1 is either a downstream effector of Gpa2 or a negative regulator of activated Gpa2. To distinguish between these two models, we examined the effect of deleting sck1+ or expressing a second copy of sck1+ on cells expressing Gpa2K270E. PKA activity was assessed using two distinct assays: expression of a PPA-repressed fbp1-lacZ reporter (17) and measurement of the length of cells displaying septa (22). Low PKA activity results in elevated fbp1-lacZ expression and decreased cell length as seen for these strains in Fig. 2 and summarized as a bar graph in Fig. 3. If Sck1 is a downstream effector of Gpa2, we would expect that the sck1− deletion would produce a low PKA phenotype. However, if Sck1 is a negative regulator of Gpa2, the sck1− deletion would produce an elevated PKA phenotype. To enhance our ability to assess changes in Gpa2-mediated signaling, we examined these interactions in the context of a deletion of the git3+ GPCR gene, as mutational activation of Gpa2 restores PKA activation in cells lacking Git3 (12). As seen in Fig. 2 and Fig. 3, relative to wild-type cells (Fig. 2A), git3α cells display “low PKA” phenotypes of increased fbp1-lacZ expression under glucose-rich (repressed) conditions and decreased cell length during cytokinesis (Fig. 2B). The gpa2ΔK270E allele suppresses the loss of Git3 as it reduces fbp1-lacZ expression under both glucose-rich and glucose-starved conditions and increases the cell length at cytokinesis (Fig. 2C), consistent with an increase in PKA activity. Sck1 appears to be a negative regulator of Gpa2K270E, as a deletion of sck1− enhances PKA-mediated repression of fbp1-lacZ expression and increases the length of dividing cells (Fig. 2D), while introducing a second copy of sck1+ elevates fbp1-lacZ expression and reduces the length of dividing cells (Fig. 2E). Statistical significance of cell length changes was determined by two-tailed Student’s t test (Fig. 2A versus B, P = 5.7 × 10−7; Fig. 2B versus C, P = 3.4 × 10−5; Fig. 2C versus D, P = 1.2 × 10−12; and Fig. 2E versus F, P = 4.9 × 10−4).

**Sck1 does not regulate PKA expression in cells expressing Gpa2R176H.** Unlike the GDP-binding mutant Gpa2K270E, the GTPase-defective Gpa2R176H displays reduced binding to Sck1 in the two-hybrid assay (Fig. 1A). As previously shown (10), the gpa2R176H mutation suppresses the loss of the Git3 GPCR (compare Fig. 2B and F; see also Fig. 3), increasing the length in septated cells (P = 1.2 × 10−10) and reducing fbp1-lacZ expression in both glucose-rich and glucose-starved cells. However, unlike the effect observed in cells expressing Gpa2K270E, deletion of sck1− has no effect on the cell length of a gpa2ΔR176H mutant (for cell length comparison of Fig. 2F to G, P = 0.51) and confers a modest increase in fbp1-lacZ expression under glucose-starved conditions (Fig. 2G). The latter effect suggests a slight reduction in PKA activity, consistent with our previous observation that Sck1 acts in parallel with PKA in nutrient sensing to regulate spore germination and exit from stationary phase (13). It is possible that since Gpa2R176H appears to be more active than Gpa2K270E, it is harder to detect further activation due to the loss of Sck1. However, it may be that the loss of the interaction with Sck1, as suggested by the two-hybrid assay data, is the reason that cells expressing Gpa2R176H are longer than cells expressing Gpa2K270E (compare Fig. 2C and F) and display lower levels of fbp1-lacZ expression under glucose starvation conditions (Fig. 2 and 3). The effect of deleting sck1− on the activity of other mutationally activated gpa2 alleles is shown in Table 2. The three alleles, gpa2ΔK90E, gpa2ΔK270E, and gpa2ΔK270EΔK176, that appear to be further activated by the loss of Sck1 are the ones that display the least activity in the presence of Sck1, which may support the idea either that the Sck1-insensitive alleles are more active due to the loss of Sck1 regulation or that the more active alleles have already reached the detectable limit of activity such that loss of Sck1 does not confer a measurable increase.

**Sck1 negatively regulates PKA activity via Gpa2.** The effect of the sck1− deletion varies as a function of the gpa2 allele, suggesting that Sck1 acts via its interaction with Gpa2. We therefore determined the effect of deleting sck1− in a strain lacking Gpa2 rather than the Git3 GPCR. Consistent with our previous studies of Gpa2 (11, 21), loss of Gpa2 confers a low PKA phenotype of elevated fbp1-lacZ expression in cells grown in glucose-rich medium and a reduction in the length of septated cells (compare Fig. 2H and A; P = 3.2 × 10−9). However, while deleting sck1− in a strain expressing Gpa2K270E increases the length of septated cells, suggesting an elevation of PKA activity (compare Fig. 2C and D), deleting sck1− in a strain lacking Gpa2 confers a dramatic reduction in cell length, consistent with a role for Sck1 acting in parallel to PKA and the absence of a Gpa2-mediated increase of PKA activity (compare Fig. 2H and I; P = 1.7 × 10−10).

**Sck1 kinase activity is required for regulation of Gpa2.** To determine whether Sck1 acts in a structural manner or via its kinase activity, we constructed an unmarked, kinase-dead

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**FIG 1** Two-hybrid interactions reveal preferential binding of Sck1 to Gpa2R176H-activated Gnu. (A) Various combinations of Gpa2 bait proteins were coexpressed in YRG2 cells with the indicated preys. Transformants were replica plated to media lacking histidine and containing either 0 mM, 20 mM, or 50 mM 3-aminitotriazole as indicated and were grown for 3 days before being photographed. X-Gal filter lift was performed as previously described (17) from transformants grown on the 0 mM 3AT plate. Transformants expressing the Snf1 bait and Snf4 prey were included as controls for specificity (18). (B) Western blot detecting HA-tagged Gpa2-Gal4-binding domain (GBD) bait and Sck1-Gal4 activation domain (GAD) prey proteins. Extracts contain similar levels of Sck1-GAD, while the Gpa2−GBD (wt) protein is of lower abundance than either mutant Gpa2-GBD protein.
sck1^K331A allele by first disrupting sck1 with a ura5^-lys7^+ cassette and then replacing the cassette with the mutated sequence (selecting for 5-fluoroorotic acid-resistant [5-FOAr] colonies and screening for loss of lys7/H11001 [16]). Cells that coexpress the kinase-dead Sck1K331A (Sck1-KD) with Gpa2K270E (Fig. 2J) display the same elevated PKA phenotype as seen in sck1/H9004 gpa2K270E cells (Fig. 2D), indicating that Sck1 kinase activity is required for its role in regulating Gpa2 activity. Finally, we examined the effect of the sck1KD allele on PKA activity in a strain lacking the Git3 GPCR but expressing wild-type Gpa2/H11001G/H9251. Similar to that of strains expressing Gpa2K270E, both Gpa2^+ strains displayed elevated PKA phenotypes, as seen by an increase in cell length and a reduction in fbp1-lacZ expression (Fig. 2K and L) compared to a git3Δ sck1^+ strain (Fig. 2B; P = 1.8 × 10^-11 and 3.0 × 10^-10, respectively). However, as these strains display a lower cell length and a higher level of β-galactosidase activity than do git3Δ gpa2K270E sck1Δ (Fig. 2D) or git3Δ gpa2K270E sck1-KD (Fig. 2I) strains, Gpa2^+ remains less active than Gpa2^K270E in cells lacking Sck1 (for cell length, Fig. 2D versus K, P = 2.5 × 10^-6; Fig. 2J versus L, P = 9 × 10^-4).

DISCUSSION

In this study, we identified the Sck1 kinase in a two-hybrid screen for proteins that preferentially bind a GDP-GTP exchange mutant form of Gpa2 G/H9251 (Gpa2K270E) relative to the wild-type Gpa2/H11001 protein (Fig. 1). While this may be due to the lower abundance of the Gpa2/H11001 bait as seen by Western blotting (Fig. 1B), Sck1 was the only one of eight Gpa2-binding preys that showed such a bias. Thus, the Gpa2^+ bait may be at a high enough level that further increases in abundance do not lead to a greater occupancy of the Gal4-binding sites in the host strain. We should also note that this screen was not exhaustive, as it failed to detect the Gpa2-binding domain of the Git2/Cyr1 adenylyl cyclase, which is a downstream effector of the activated Gpa2 [11]. However, unlike Git2, the Sck1 prey shows a lower affinity for the GTPase-defective Gpa2^R176H prey than it does for the Gpa2^+ prey (Fig. 1A), indicating that the
FIG 3 Bar graph of data from cell length assays together with β-galactosidase assays under glucose-repressed and -derepressed conditions from Fig. 2. The graph shows the correlation between increased cell length and reduced fbp1-lacZ expression resulting from increased PKA activity.

R176H substitution alters the Sck1-binding site and/or that Sck1 does not preferentially bind the GTP-bound form of Gpa2. Based on its similarity to S. cerevisiae Sch9p, one might expect Sck1 to phosphorylate proteins on AKT/PKB sites (RXRXX[S/T]) or PKA sites [(R/K)X(S/T)], of which there is one of the former and none of the latter sites in Gpa2. Remarkably, R176 is the residue in the −3 position of the only AKT consensus site, and the loss of this consensus site may explain the insensitivity of the Gpa2R176H protein to Sck1. However, other mutant forms of Gpa2, such as Gpa2T49A, Gpa2L57P, and Gpa2F62S, show weak interactions with Sck1 in the two-hybrid assay (data not shown) and are unaffected by the loss of Sck1 as judged by expression of the fbp1-lacZ reporter (Table 2). Therefore, the conformation of Gpa2, along with its primary sequence, may regulate the interaction with Sck1. Structural studies of other G proteins show that the helical domain containing this AKT site undergoes a dramatic change in conformation and accessibility as a function of the presence or absence of a bound guanine nucleotide, with the nucleotide-free state showing the greatest accessibility (23). In fact, a crystal structure of a rat G, bound to GTPγS (PDB identifier [ID]: 1AS0) shows that the entire length of this sequence (RTRVKT) contacts the guanine nucleotide, with the final threonine directly interacting with the gamma-phosphate of the bound GTP molecule (24), supporting the idea that GTP binding could make this site inaccessible to a kinase. In addition, Gpa2R176 maps only five residues from a glycine that is equivalent to the amino acid altered by a G302S substitution that activates the S. cerevisiae Gpa1p protein by disrupting an interaction with the Sst2p RGS (25); thus, there is precedent for this region of the Gα serving as the target for regulation by extrinsic factors. Finally, the proposed nucleotide-dependent binding specificity (nucleotide-free > GDP-bound > GTP-bound) is identical to that seen for the Dbl/Mcf2 guanine nucleotide exchange factor in its binding to both the Cdc42 and RhoA G proteins (26). We therefore suggest that the Gpa2K270E mutant protein shows an enhanced sensitivity to Sck1 due to the fact that its defect in guanine nucleotide binding causes more of the protein to be in the nucleotide-free state. While the interaction between Sck1 and Gpa2K270E could be an artifact of the gpa2 mutation, the fact that Sck1 affects PKA activity in a Gpa2− strain (Fig. 2K and L and Fig. 3) shows that Sck1 plays a normal role in regulating Gpa2−-mediated signaling.

Sck1 was originally identified as a multicopy suppressor of mutations that lower or eliminate PKA activity, and Sck1 appears to play a role in parallel to PKA in sensing nutrients to regulate spore germination and exit from stationary phase (13). We show here that deletion of sck1 in a gpa2Δ background results in a further shortening of cell length, consistent with a role for Sck1 acting in parallel to PKA, leading to a model in which Sck1 carries out two contradictory roles as a negative regulator of Gpa2 and a nutrient-sensing kinase that acts in parallel to PKA (Fig. 4). Further support for a downstream role in nutrient signaling by Sck1 and its budding yeast homolog Sch9p comes from studies of trehalose mobilization as a function of nitrogen signaling (27, 28). The study of Sch9p identified it as a negative regulator of PKA and the likely target of activation by nitrogen via the fermentable-growth-medium-induced pathway (27). In addition, S. pombe TORC1-mediated phosphorylation of Sck1 in response to nitrogen in the growth medium has been observed (29). While the biological effect of Sck1 phosphorylation in S. pombe has yet to be determined, it has been shown that the S. cerevisiae Sch9p kinase is phosphorylated and activated by TORC1 (30). Finally, the S. cerevisiae

TABLE 2 Effect of deleting sck1+ upon fbp1-lacZ expression in glucose-starved git3Δ gpa2 mutants

| gpa2 allele | sckl+ | scklΔ |
|------------|-------|-------|
| T49A       | 630 ± 308 | 643 ± 354 |
| L57P       | 666 ± 56  | 533 ± 52  |
| F62S       | 741 ± 186 | 815 ± 98  |
| V90A       | 947 ± 292 | 465 ± 38  |
| R176H      | 626 ± 264 | 1,161 ± 459 |
| K270E      | 942 ± 268 | 553 ± 106 |
| T325A      | 1,633 ± 99 | 1,211 ± 222 |

*β-Galactosidase activity expressed from the fbp1-lacZ reporter was determined from three independent S. pombe cultures as described in Materials and Methods. The values are averages ± standard deviations and are given as specific activity per mg of soluble protein.*
FIG 4 Pathway model showing two distinct roles for Sck1 in nutrient signaling. Work described here and elsewhere suggests that Sck1 is both a negative regulator of CAMP signaling via Gpa2 regulation and a kinase that acts in parallel to PKA, possibly as a sensor of nitrogen levels. Gpa2 activation of PKA is via the activation of adenyl cyclase (Git2/Cyr1; not shown).

Sch9p kinase that was also discovered as a multicopy suppressor of defects in the PKA pathway (31) acts as a negative regulator of the budding yeast Gpa2 Gα. Zaman et al. found that a hypomorphic allele of SCH9 enhanced Gpa2-mediated transcriptional repression (32), similar to what we observe in strains that express Gpa2K270E or Gpa2C270E (compare Fig. 2B and K and L or Fig. 2C and D and J).

It is not immediately obvious what benefit there is to S. pombe cells by having Sck1 carry out two seemingly opposing roles in nutrient signaling, i.e., negatively regulating Gpa2-mediated activation of PKA versus acting in a partially redundant manner to PKA. This may allow cells to better distinguish their responses to nitrogen starvation versus glucose starvation. For example, S. pombe cells enter stationary phase from G2 upon nitrogen starvation (33), while glucose starvation does not have a substantial effect on cell length or spore germination (34). In addition, nitrogen starvation does not have a substantial effect on fbp1 transcription, while glucose starvation does (34), even though both starvation signals act through the Wsi1-Spc1/Sty1 signaling pathway, which acts antagonistically to PKA (35–37). Thus, as a negative regulator of PKA activity via Gpa2, Sck1 may allow cells to respond differently to glucose starvation versus nitrogen starvation or starvation for both nutrients.

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