Phosphorylation of the Ga protein Gpa2 promotes protein kinase A signaling in yeast

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Running title: Function and Regulation of Gpa2 Phosphorylation

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ABSTRACT

Heterotrimeric G proteins are important molecular switches that facilitate the transmission of a variety of signals from outside to the inside of the cells. G proteins are highly conserved, enabling studies of their regulatory mechanisms in model organisms such as the budding yeast Saccharomyces cerevisiae. Gpa2 is a yeast G alpha protein that functions in the nutrient signaling pathway. Using phos-tag, a highly specific phosphate binding tag for separating phosphorylated proteins, we found that Gpa2 undergoes phosphorylation and that its level of phosphorylation is markedly increased upon nitrogen starvation. We also observed that phosphorylation of Gpa2 is dependent on glycogen synthase kinase (GSK). Disrupting GSK activity diminishes Gpa2 phosphorylation levels in vivo, and purified GSK isoforms Mck1 and Ygk3 are capable of phosphorylating Gpa2 in vitro. Functionally, phosphorylation enhanced plasma membrane localization of Gpa2 and promoted nitrogen starvation-induced activation of protein kinase A. Together, the findings of our study reveal a mechanism by which GSK-and nutrient-dependent phosphorylation regulates subcellular localization of Gpa2 and its ability in activating downstream signaling.

Heterotrimeric G proteins are highly conserved molecular switches that regulate a variety of cellular processes (1-3). The strength and duration of G protein signaling have to be tightly regulated, and their alteration can have significant impact on both pathophysiology and pharmacology (4-6). Our goal is to elucidate molecular mechanisms that regulate G proteins, with a focus on the role of post-translational modifications, especially those that are responsive to environmental signals.

Gpa2 is a Ga protein in the budding yeast Saccharomyces cerevisiae, and it primarily functions in nutrient signaling pathway (7,8). In the presence of glucose, Gpr1, a G protein-coupled receptor, becomes activated, which in turn facilitates guanine nucleotide exchange of Gpa2 from GDP-bound form to GTP-bound form (8,9). GTP-bound Gpa2 then activates adenylate cyclase Cyr1 to increase the production of cAMP (7,10), an important second messenger that activates protein kinase A (PKA) to regulate cell growth and proliferation. Given the pivotal role of Gpa2 in the nutrient signaling pathway, its activity has to be tightly regulated. One well-established mechanism for Gpa2 regulation is via the action of RGS protein Rgs2 (11), which accelerates the GTPase activity of Gpa2 and thus keeps its signaling in check. Other mechanisms for Gpa2 regulation likely exist. An earlier study implied that Gpa2 may be phosphorylated when diploid cells undergo...
nitrogen deprivation-induced sporulation and meiosis (12). More recently, global phosphoproteomics studies also revealed Gpa2 as a potential phospho-protein (13). Thus, it appears that Gpa2 and Gpa2-mediated signaling may be regulated via phosphorylation.

In this study, we find that Gpa2 protein indeed undergoes phosphorylation. Interestingly, nitrogen starvation markedly elevates the level of Gpa2 phosphorylation and enhances its plasma membrane localization. We also find that preventing Gpa2 phosphorylation via mutating its candidate phosphorylation sites diminishes the level of nitrogen starvation-induced activation of protein kinase A. Our results suggest that nitrogen starvation-induced phosphorylation of Gpa2 regulates subcellular localization of Gpa2 and enhances its ability in activating downstream protein kinase A signaling.

RESULTS

Gpa2 undergoes phosphorylation. To examine whether Gpa2 is phosphorylated, we made use of a yeast strain that expresses GFP tag at the C-terminal end of Gpa2 at its genomic locus (14). Cell extracts from Gpa2-GFP cells treated or not-treated with nitrogen starvation condition were analyzed on a regular gel as well as a gel containing phos-tag. The nitrogen starvation condition was chosen because an earlier work has implied that Gpa2 may undergo phosphorylation under the sporulation-inducing condition (12). Phos-tag is a small chemical compound that forms a specific binding site for phosphate group in the presence of divalent metal ions such as Zn$^{++}$, thus SDS-PAGE using the acrylamide gel that contains phos-tag can specifically retard the migration of phosphorylated protein species (15,16). As shown in Fig. 1A, on a regular gel, Gpa2-GFP ran as a single band. Interestingly, on a phos-tag gel, extra and slower migrating bands were visible for Gpa2-GFP, especially in samples prepared from cells that were subject to nitrogen starvation.

Since the C-terminus of Gp protein is critical for its functions that include coupling to receptors (17-19), we wish to examine whether the position of GFP tag matters to the observed phosphorylation. For this purpose, we made a construct that expresses N-terminally tagged Gpa2. Since the first few residues in the N-terminal region of Gpa2 are important for its myristoylation and membrane localization (20-22), the GFP tag was inserted after the 10th residue of the Gpa2 protein as described earlier (20). The resulting Gpa2$^{1-10}$-GFP-Gpa2$^{4-449}$ protein has been previously demonstrated as fully functional and can replace wild type Gpa2 (20). To verify this, we examined the ability of Gpa2$^{1-10}$-GFP-Gpa2$^{4-449}$ in supporting invasive growth, a phenomenon that occurs in haploid yeast under nutrient depletion condition and requires a functional Gpa2 (23). As shown in Fig. 1B, disrupting Gpa2 severely impairs invasive growth as expected, and expressing Gpa2$^{1-10}$-GFP-Gpa2$^{4-449}$ restored the ability of the gpa2Δ cells in promoting invasive growth, indicating the fusion protein is functional. We then examined phosphorylation of this N-terminally GFP-tagged Gpa2. As shown in Fig. 1C, phosphorylation of Gpa2$^{1-10}$-GFP-Gpa2$^{4-449}$ was readily detectable, and once again, the level of phosphorylation was markedly elevated upon nitrogen starvation. Given that nitrogen starvation condition can be mimicked by rapamycin treatment, we also examined if rapamycin treatment can likewise promote Gpa2 phosphorylation. As shown in Fig. 1D, rapamycin treatment can indeed stimulate Gpa2 phosphorylation, even though to a somewhat lesser extent than nitrogen starvation.

Next, we sought to examine whether phosphorylation of Gpa2 is sensitive to the availability of other nutrients such as glucose. For this purpose, cells expressing Gpa2$^{1-10}$-GFP-Gpa2$^{4-449}$ protein were grown to mid-log phase, shifted to medium without glucose for indicated time (glucose starvation), and then shifted back to medium with glucose (glucose re-addition). As shown in Fig. 1E, altering the level of glucose (either removal or re-addition) has very little effect on the extent of Gpa2 phosphorylation. Together, these results indicate that Gpa2 protein undergoes phosphorylation and its phosphorylation is sensitive to the availability of nitrogen.

Phosphorylation-null mutant of Gpa2. To start characterizing Gpa2 phosphorylation, we sought to identify the regions on Gpa2 that are required for its phosphorylation. Global
phospho-proteomics studies indicated that several residues in the N-terminal region of Gpa2 may undergo phosphorylation (Fig. 2A) (13). Thus, we first examined whether the N-terminal region of Gpa2 is critical for its phosphorylation. To this end, we generated a truncated version of Gpa2 that lacks a stretch of 120 residues from the N-terminal region of the protein. As shown in Fig. 2B, nitrogen starvation-induced phosphorylation was absent in this N-terminal truncated version of Gpa2, suggesting that sites of Gpa2 phosphorylation may indeed reside in the N-terminal region of the protein. To further test this, we constructed a Gpa2-10SA mutant, in which a total of 10 serine residues (S12, S20, S23, S61, S90, S111, S113, S116, S117, and S119) present in the N-terminal region of the protein were replaced with alanine. Those residues were chosen because they either represent phosphorylation sites revealed by phosphor-proteomics studies (13) or are adjacent to those sites. As shown in Fig. 2C, the Gpa2-10SA mutant also displayed a much diminished level of phosphorylation induced by nitrogen starvation, especially the species that run very slow and presumably represent multiply phosphorylated species.

**Glycogen synthase kinases are required for Gpa2 phosphorylation.** Next, we sought to identify the kinase that is responsible for Gpa2 phosphorylation. One candidate is glycogen synthase kinase (GSK), because GSK is known to be active under the condition of nitrogen starvation and there are predicted GSK phosphorylation sites on Gpa2 (24, 25). There are a total of four GSK isoforms, i.e., Mrk1, Mck1, Rim11, and Ygk3 (YOL128C), in yeast. These GSK isoforms have partially redundant functions in phosphorylating substrates (24, 26). To determine if GSK activity is required for Gpa2 phosphorylation, we made use of a strain that lacks three GSK isoforms (Mck1, Rim11, and Ygk3) and has been used as a gsk-null strain (26). As shown in Fig. 3A, the level of Gpa2 phosphorylation is substantially diminished in the gsk-null strain, indicating that GSK activity is required for full phosphorylation of Gpa2. To determine if the requirement of GSK on Gpa2 phosphorylation is direct, we first examined whether Gpa2 interacts with any of the GSK isoforms. For this purpose, we made a Flag-tagged version of Mck1, Rim11 and Ygk3, and examined the interaction between Gpa2 and Mck1/Rim11/Ygk3 using co-immunoprecipitation. As shown in Fig. 3B, we were able to detect interaction between Mck1 and Gpa2 as well as Ygk3 and Gpa2. Next, we tested whether Mck1 and Ygk3 are capable of phosphorylating Gpa2. For this purpose, we purified Mck1-Flag and Ygk3-Flag from cells treated with nitrogen starvation condition (to maximize its kinase activity) and GFP-tagged Gpa2 from normally growing cells and conducted an in vitro kinase assay. As shown in Fig. 3C, in the presence of both ATP and Mck1-Flag, an increase in phosphorylated Gpa2 was observed. Likewise, the presence of both ATP and Ygk3-Flag also led to a subtle increase in the level of Gpa2 phosphorylation. For reasons not clear, the expression level of Ygk3 is much lower than that of Mck1, which may explain the lesser extent of Gpa2 phosphorylation caused by Ygk3. From these data, we conclude that Mck1 and Ygk3 are capable of phosphorylating Gpa2. Together, these findings support that glycogen synthase kinases are responsible for Gpa2 phosphorylation.

**Subcellular localization of Gpa2.** Accumulating evidence indicates that G proteins can function in both plasma membrane and other subcellular compartments that include endosome and Golgi (27, 28). In principle, altering subcellular localization of G proteins could either enable or impair their ability in regulating specific effectors by either facilitating G protein/effecter interaction or sequestering G protein away from its effector. To investigate if Gpa2 may be regulated via this mechanism, we examined the effect of nitrogen starvation as well as phosphorylation on subcellular localization of GFP-tagged Gpa2, i.e., Gpa2\(^{1-10}\)-GFP-Gpa2\(^{4-449}\). As shown in Fig. 4A, in cells grown in normal medium, fluorescence signal representing GFP-tagged Gpa2 can be found in both plasma membrane and cytoplasm. Upon nitrogen starvation, a clear concentration of Gpa2 signal on plasma membrane was observed, indicating that nitrogen starvation enhances plasma membrane localization of Gpa2. In addition, clusters of fluorescent signals are present in starved cells, which presumably represent free GFP or remnant of GFP-Gpa2 in
the vacuole as a consequence of nitrogen starvation-induced autophagy. Interestingly, nitrogen starvation has less effect on the localization of Gpa2-10SA mutant, suggesting that nitrogen starvation-induced alteration of Gpa2 localization is phosphorylation-dependent. To confirm the effect of nitrogen starvation and phosphorylation on Gpa2 subcellular localization, we also subjected cells expressing Gpa2-GFP-Gpa2 to sucrose density gradient fractionation analysis (29). Consistent with our fluorescence imaging results, a substantial fraction of GFP-tagged Gpa2 is present in both the cytosolic fraction and the plasma membrane fractions (Fig. 4B). Nitrogen starvation clearly induced a shift of GFP-tagged Gpa2 from cytosolic fractions to plasma membrane fractions, and this effect was not apparent in the GFP-tagged Gpa2-10SA mutant (Fig. 4B).

To determine if nitrogen starvation induces a general enrichment of protein to plasma membrane, we also examined the subcellular localization of Gpa1, the only other Gα protein in yeast. A yeast strain expressing a CFP-tagged Gpa1 was used for this purpose. Note this strain was created for the FRET analysis of G protein activation (30), and presumably the CFP-tagged Gpa1 is fully functional. As shown in Fig. 4C, in cells grown in normal medium, CFP-Gpa1 is primarily on the plasma membrane, with some cytoplasmic localization; when treated with nitrogen starvation, CFP-Gpa1 signal is clustered inside of the cells and no enrichment on plasma membrane was observed, which is very different from what was found in Gpa2.

It is possible that the apparent enrichment of Gpa2 from cytoplasm to plasma membrane in response to nitrogen starvation requires the presence of its interacting proteins in plasma membrane. Gpr1 and Plc1 are two plasma membrane-associated proteins that are known to interact with Gpa2 (31,32). Gpr1 acts as a receptor exchange factor for Gpa2, while Plc1 interacts with both Gpr1 and Gpa2 and serves to enhance the interaction between Gpr1 and Gpa2 by exposing a Gpa2 binding site at the carboxyl-terminal region of Gpr1 (32). To determine if these two proteins are required for Gpa2 localization to plasma membrane, we examined the effect of disrupting their genes. As shown in Fig. 4D, disrupting Gpr1 has no effect on subcellular localization of Gpa2. However, in the plc1Δ mutants, the nitrogen starvation-induced plasma membrane enrichment of Gpa2 is much less pronounced, suggesting a potential role of Plc1 in anchoring Gpa2 on plasma membrane upon nitrogen starvation.

Functional consequences of Gpa2 phosphorylation. Finally, we wished to investigate the impact of phosphorylation on Gpa2-mediated signaling outputs. To this end, we first examined whether wild type Gpa2 and the Gpa2-10SA mutant show any difference in their behavior in promoting invasive growth. As shown in Fig. 5A, disrupting Gpa2 severely impairs invasive growth as expected. Compared to cells expressing wild type Gpa2, cells that express Gpa2-10SA mutant display a similar level of invasive growth, suggesting phosphorylation is not important to promote haploid invasive growth. This result also indicates that the Gpa2-10SA protein is functional, as it is clearly able to fully support the invasive growth.

Next, we examined if phosphorylation of Gpa2 has any effect on the level of protein kinase A (PKA) activation, as one important downstream signaling component of Gpa2 is protein kinase A (33). For this purpose, we utilized Myc-Cki, a well-established PKA activity reporter, whose level of phosphorylation is directly proportional to the activity of PKA (34). As shown in Fig. 5B, basal PKA activity is very similar between Gpa2 and Gpa2-10SA. As reported previously, nitrogen starvation induces a clear elevation of PKA activity (34), and this effect is more prominent in cells expressing Gpa2 than those expressing Gpa2-10SA, suggesting phosphorylation is required for full activation of PKA induced by nitrogen starvation (Fig. 5B). No difference was observed between Gpa2 and Gpa2-10SA with regarding to the level of MAP kinase activation, as measured by the extent of phospho-Mpk1 and phospho-Kss1 (Fig. 5B). This is expected, as MAP kinases are not downstream components of Gpa2 pathway like protein kinase A (35,36).

Lastly, we examined if phosphorylation of Gpa2 has any impact on sporulation. This is possible because Gpa2 is known to interact with
Ime2, a kinase that promotes sporulation of diploid yeast in response to nitrogen starvation and the presence of non-fermentable carbon such as acetate (12). It was suggested that in nutrient rich conditions Gpa2 is capable of inhibiting Ime2, and thereby preventing the inappropriate sporulation (12). Presumably, this inhibitory effect of Gpa2 has to be relieved when cells undergo sporulation. Phosphorylation of Gpa2 could potentially serve as a mechanism to relieve the inhibitory effect of Gpa2 on cytoplasmic Ime2 by enhancing the plasma membrane localization of Gpa2. To test this, we first examined whether nitrogen starvation also induces phosphorylation and enhances plasma membrane localization of Gpa2 in the diploid cells. Diploid cells expressing Gpa2\textsuperscript{1-10}-GFP-Gpa2\textsuperscript{4-449} were grown into mid-log phase and subjected to nitrogen starvation. As shown in Fig. 5C, nitrogen starvation similarly induces Gpa2 phosphorylation and plasma membrane localization. We then examined the sporulation efficiency of diploid cells that express either wild type Gpa2 or the Gpa2-10SA mutant. Cells with a clear sign of the presence of spores (Fig. 5D) were counted under the microscope. As a result, we find that about 20.1% of cells (out of 1005 counted cells) expressing wild type Gpa2 underwent sporulation and about 16.9% of cells (out of 706 counted cells) expressing Gpa2-10SA mutant underwent sporulation. Thus, diminishing the level of Gpa2 phosphorylation had a modest inhibitory effect on the efficiency of sporulation.

**DISCUSSION**

Gpa2 is a G\textalpha protein that regulates nutrient signaling in yeast. In this work, we report that Gpa2 protein undergoes nutrient-regulated phosphorylation. Specifically, nitrogen starvation induces Gpa2 phosphorylation and enhances its localization on plasma membrane. Gpa2 mutant with decreased phosphorylation shows a reduced level of PKA activation induced by nitrogen starvation and a lower efficiency in sporulation. Our results suggest phosphorylation as a mechanism in regulating subcellular localization and signaling of Gpa2.

One interesting observation from our study is that nitrogen starvation induces enhanced plasma membrane localization of Gpa2 (Fig. 4). The difference of Gpa2 localization with and without nitrogen starvation is very clear: a substantial portion of Gpa2 is present in the cytoplasm in cells grown in normal medium, but in nitrogen starved cells, there exists a substantially lower level of cytoplasmic Gpa2 and a higher level of plasma membrane Gpa2. Thus, nitrogen starvation clearly induces a translocation of Gpa2 from cytoplasm to plasma membrane. Notably, this effect is fairly specific to Gpa2, as nitrogen starvation does not increase plasma membrane localization of Gpa1, the other G\textalpha protein in yeast. As Gpa2 has effector proteins both on plasma membrane, i.e., adenylate cyclase Cyr1, and cytoplasm, i.e., Ime2, such an alteration in Gpa2 localization can in principle impact the interactions between Gpa2 and its effectors, which in turn modulates Gpa2-mediated signaling (Fig. 5E). Specifically, enhanced plasma membrane localization will facilitate the interaction between Gpa2 and adenylate cyclase Cyr1, which could lead to an increased activity of the downstream protein kinase A. Likewise, diminished cytoplasmic localization of Gpa2 will reduce the interaction between Gpa2 to Ime2, a cytoplasmic localized protein kinase important for sporulation (37,38), thereby relieving the inhibitory effect of Gpa2 on Ime2 and resulting in an elevated sporulation (Fig. 5E). The signaling behavior of the Gpa2-10SA mutant is consistent with this model. This mutant displays a diminished level of phosphorylation, a reduced translocation to plasma membrane in response to nitrogen starvation, a decreased activation of protein kinase A, and a decreased efficiency in sporulation. A role of GSKs in promoting sporulation in yeast has been reported (39) and our findings here suggest Gpa2 may be one of the targets involved.

It was demonstrated previously that inhibiting the target of rapamycin pathway either through nitrogen starvation or rapamycin treatment leads to an enhanced activation of protein kinase A (34). Conversely, inhibiting the protein kinase A pathway also elevates the activation level of the target of rapamycin pathway. Thus, there appears to have an antagonistic relationship between these two major nutrient signaling pathways. However, the
target that is involved is not known (34). The behavior of Gpa2-10SA suggests that nitrogen starvation-induced phosphorylation of Gpa2 might be one of the underlying mechanisms that allow the antagonistic relationship between the target of rapamycin and protein kinase A pathways. Notably, a previous research has indicated that the expression level of Gpr1 is elevated by nitrogen starvation (23), which presumably could contribute to the activation of plasma membrane-enriched phosphorylated Gpa2.

While nitrogen starvation leads to robust phosphorylation of Gpa2, glucose deprivation has very little effect on Gpa2 phosphorylation. A reason for that is likely due to the different impacts of these two nutrient deprivation conditions on the activity of GSKs, as nitrogen starvation but not glucose deprivation can lead to an increased activation of GSKs (24,36). What could be the benefit of phosphorylating Gpa2 under nitrogen-starvation condition but not in response to glucose deprivation? One possibility is that this provides a mechanism that allows cells to distinguish the different nutrient deprivation conditions and modulate the level of protein kinase A activation accordingly. One key function of protein kinase A in yeast is to phosphorylate and stimulate the activity of pyruvate kinase, leading to enhanced glycolysis in the presence of glucose (40). Thus, under the condition of glucose deprivation, probably there is no need for cells to phosphorylate Gpa2 to boost the activity of protein kinase A. Under nitrogen starvation condition, however, Gpa2 phosphorylation and the resulting enhanced activation of protein kinase A could allow cells to make better use of glucose, preventing premature cell growth arrest and gaining some advantages.

It remains to be understood how nitrogen starvation and/or phosphorylation promotes plasma membrane enrichment of Gpa2. Our analysis revealed that Plc1 is required for the re-localization of Gpa2, suggesting Plc1 as a potential factor that helps retaining Gpa2 on plasma membrane. Notably, similarly to the Gpa2-10SA mutant, the plc1Δ mutant also displays a decreased level of sporulation efficiency (41) and protein kinase A activation (42), consisting with our overall model that plasma membrane enrichment of Gpa2 serves as a mechanism in promoting sporulation and protein kinase A activation. It is highly likely that there exist other factors that are important for the enrichment of Gpa2 on plasma membrane. One potential way to identify those factors is isolating proteins that interact with Gpa2, and those that differentially interact with Gpa2 in response to nitrogen starvation would be a good candidate. Future studies will focus on identifying novel Gpa2 interacting proteins and investigating their roles in regulating Gpa2 localization and signaling.

**EXPERIMENTAL PROCEDURES**

**Strains and Plasmids**-- Standard methods for the growth, maintenance, and transformation of yeast and bacteria and for the manipulation of DNA were used throughout. The yeast *S. cerevisiae* strains used in this study are BY4741 (MATα leu2Δ met15Δ his3Δ ura3Δ), BY4741-derived mutant lacking GPA2 (Research Genetics, Huntsville, AL), BY4743 (MATα his3Δ1/hi3Δ1 leu2Δ0/leu2Δ0 LYS2/lys2Δ0 met15Δ0/MET15 ura3Δ0/ura3Δ0 ), BY4743-derived mutant lacking GPA2 (Dharmacon), a triple-gsk mutant (GSK3-mck1 rim11 yol128c, MATα his3Δ1 leu2Δ0 ura3Δ0 trpl1 ade2 mck1::TRP1 rim11:HIS3 yol128c::LEU2, generously provided by Dr. Claudina Rodrigues-Pousada, ITQB-UNL, Portugal) (26), a pka-null mutant GG104 (MATα pde2:::TRP1 cdc35::KanMX2 msn2::HIS3 msn4::TRP1, generously provided by Dr. Joris Winderickx, KU LEUVEN, Belgium), TMY102 (gpa1::[CFP-GPA1, KAN], generously provided by Dr. Tau-Mu Yi) (30). The invasive growth assay was performed in the Σ1278-based invasive strain MLY218a (MATα leu2Δ ura3Δ, from J. Heitman) and MLY218a-gpa2 (MLY218a, gpa2Δ::KanMX2) (this study).

The expression plasmids pYES-Gpa2-FLAG, pYES-Gpa2<sup>1-110</sup>-FLAG, pYES-Mck1-FLAG, pYES-Rim11-FLAG, and pYES-Ygk3-FLAG were constructed by amplifying the appropriate DNA fragment using yeast genomic DNA as a template. The individual PCR product was subcloned by digestion with HindIII and ligation to pYES2.1/V5-His-TOPO that has been engineered to have a HindIII site and a C-terminal FLAG tag. The plasmids expressing
Gpa2-10SA and Gpa2\textsuperscript{1-10}-GFP-Gpa2\textsuperscript{1-10} were constructed by subcloning appropriate DNA fragments synthesized by IDT (Integrated DNA Technology) to either pYES vector, or pRS315 vector that contains GPA2 promoter. The plasmid that expresses RFP-Ime2 was constructed by two steps. First, the DNA fragment encoding RFP was cloned to pYES2.1 and restriction enzyme sites (HindIII and XhoI) were added after the last codon for RFP, which allowed the ligation of Ime2 open reading frame to the resulting plasmid.

**Immunoprecipitation and in vitro kinase assay.** The interaction between Flag-tagged GSK isoforms and Gpa2 was examined using the procedure as described in our previous studies (43). Flag-tagged GSK isoforms were purified from yeast cells treated with nitrogen starvation condition for 12 hours to enrich activated version of GSK. Cells were lysed in the lysis buffer (50 mM NaPO\textsubscript{4} pH 7.5, 400 mM NaCl, 0.1% Triton X-100, 10% glycerol, 0.5 mM dithiothreitol, 25 mM NaF, 25 mM glycerophosphate, 1 mM sodium orthovanadate, 10 mM N-ethylmaleimide, 5 mM phenylmethylsulfonyl fluoride, and 1 pellet of complete EDTA-free protease inhibitor mixture (Roche) for every 50 ml of buffer), and the cleared whole cell lysate was incubated with anti-FLAG M2 affinity resin (Sigma) for 2 h. After extensive washing with lysis buffer (three times) and kinase buffer (#9802, Cell Signaling Technology, twice), the resin was incubated with Flag-peptide at 30°C for 30 min to elute the purified Flag-Mck1 and Flag-Ygk3. GFP-tagged Gpa2 was isolated from cells in mid-log phase. Cells were lysed using the same lysis buffer as above, and the cleared whole cell lysate was incubated with anti-FLAG M2 affinity resin (Sigma) for 2 h. After extensive washing with lysis buffer (three times) and kinase buffer (twice), an aliquot of purified Flag-Mck1 or Flag-Ygk3 was added to the beads, and ATP (or not) was added subsequently to initiate the kinase reaction. The reaction took place at 30°C for 3 h, and sample buffer was added to stop the reaction. The kinase reaction was analyzed on 8% phos-tag SDS-PAGE, and phosphorylated GFP-Gpa2 was detected using anti-GFP (abcam).

**Sporulation Bioassay.** BY4743-derived gpa2\textsuperscript{Δ} cells were transformed with pRS315-Gpa2\textsuperscript{1-10}-GFP-Gpa2\textsuperscript{1-10} or pRS315-Gpa2\textsuperscript{1-10}-GFP-Gpa2\textsuperscript{1-10} -10SA plasmids and were grown up in SCD media at 30°C overnight. Cell cultures were diluted to OD\textsubscript{600} about 0.1 and then grown to OD\textsubscript{600} about 1.8. Cells were collected, washed with sterile water, and resuspended in the sporulation media (2% Potassium acetate, 0.005% Zinc Acetate, 0.02g/L uracil, 0.02g/L L-histidine, 0.02g/L L-leucine, pH 7.0) to a density of OD\textsubscript{600} about 1.2.

A volume of 2.5 ml of cultures were gently mixed on a nutator at 23°C for 7 days to allow sporulation. The occurrence of sporulation as indicated by asci appearance was assessed by phase-contrast microscopy.

**Phosphorylation and Immunoblotting Bioassays.** Gpa2 phosphorylation was analyzed using phos-tag SDS-PAGE of whole cell lysates, followed by immunoblotting, using antibodies that recognize epitope-tagged Gpa2. For all the immunoblotting analysis, mid-log phase cells were grown on appropriate medium, treated or not treated with nitrogen starvation or other conditions as indicated. Proteins were extracted via trichloroacetic precipitation, following procedures described previously (27). Whole cell extracts were resuspended in boiling SDS-PAGE sample buffer (62.5 mM Tris-HCl, pH 6.8, 10% glycerol, 2% SDS, 1% 2-mercaptoethanol, 0.0005% bromphenol blue) for 5 min. Following either regular SDS-PAGE or phos-tag SDS-PAGE and transfer to nitrocellulose, the membrane was probed with antibodies to GFP at 1:5,000 (from Abcam, ab13970), and Flag at 1:1,000 (from Sigma). Immunoreactive species were visualized by enhanced chemiluminescence detection (Pierce) of horseradish peroxidase-conjugated anti-rabbit IgG (Bio-Rad), anti-mouse IgG (Santa Cruz), or anti-Chicken IgY (Abcam). Specificity of detection was established using cell extracts without tagged proteins as negative controls. All experiments have been repeated at least three times. Immunoblotting signals were quantified with ImageJ software, and the dot bar graphs were generated using Interactive Dotplot (44), and the bars represent standard deviations. Where indicated, the data were statistically analyzed.
analyzed by t test, with $p < 0.050$ considered significant.

**Microscopy Analysis.** Cells expressing GFP-tagged Gpa2 or CFP-tagged Gpa1 were grown to either mid-log phase or treated with nitrogen starvation condition for the indicated times. Cells were concentrated and 10 μl of concentrated cell suspensions were placed on a slide with a thin-layer of 0.5% agar and visualized by fluorescence microscopy using an Olympus FV1000 laser scanning confocal microscope. Fluorescence images were analyzed and quantified with ImageJ software.

**Sucrose Gradient Fractionation.** Cells expressing GFP-tagged Gpa2 was subjected to sucrose gradient fractionation, using a protocol as described previously (29). Spheroplasts were prepared by incubation with 20 μg/ml of zymoylase in SK buffer (1.2 M sorbitol, 0.1 M KPO₄, pH 7.5) for 45 minutes at 30°C. All subsequent steps were carried out at 4°C. The resulting spheroplasts were washed once with ice cold buffer SK and resuspended in lysis buffer C (0.8 M sucrose, 20 mM triethanolamine hydrochloride [pH 7.2], 1 mM EDTA, 1 mM dithiothreitol, 0.2 mM phenylmethylsulfonylfluoride, 1 roche protease inhibitor cocktail tablet per 50 mL) and disrupted by 15 strokes in a motorized homogenizer. The lysate was cleared of unbroken cells with 15 minutes of 500 x g centrifugation. Powdered sucrose was added to each sample to make 70% total sucrose, and mixed on a stir plate at 4°C for 1 h. They were then carefully overlaid with 60, 50, 40, and 30% sucrose solutions, and subjected to ultracentrifugation at 190,000 x g for 19 h. Fifteen equal portions (300 μl) were drawn from the top of each tube, mixed with 2 x SDS sample buffer, and boiled at 100°C for 5 min, cooled, and resolved on 10% SDS-PAGE. Blots were probed with anti-GFP for examining GFP-Gpa2 localization. Membrane fraction markers were used to establish which fractions corresponded to which membranes, anti-Ste4 (from Duane Jenness, University of Massachusetts) for plasma membrane, and anti-Pgk1 (from Jeremy Thorner, University of California) for cytosolic fractions.

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**Author contributions:** SH performed most phos-tag gel analysis of Gpa2, made the constructs that express Gpa2 under its own promoter, and conducted sporulation assay. AB, RG, SK, BJ, NC, and GL conducted protein extractions and constructed plasmids. YW and SH designed the experiments and wrote the manuscript.

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FOOTNOTES

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The abbreviations used are: GSK, glycogen synthase kinase; PKA, protein kinase A

FIGURE LEGENDS

Figure 1. Phosphorylation of Gpa2 and its regulation by nutrient status.

A, Gpa2-GFP cells with GFP tagged at the C-terminus of Gpa2 at its genomic locus were grown to mid-log phase and shifted to
nitrogen starvation condition for the indicated time. Whole cell extracts were resolved on 8% regular (top panel) or phos-tag containing (lower panel) SDS-PAGE and probed with anti-GFP antibodies (ab13970, Abcam). p-Gpa2, phosphorylated Gpa2. B, Σ1278-based invasive strain lacking GPA2 was transformed with either empty vector, or plasmids that express Gpa2^{1-10}–GFP-Gpa2$_{4-449}$ under the control of GPA2 promoter. Overnight cultures were spotted onto YPD plate, grown at 30°C for 2 d, and gently washed with water to reveal the invasive growth. The plate before and after wash were photographed. C, gpa2Δ cells transformed with either empty vector (EV) or plasmids expressing Gpa2^{1-10}–GFP-Gpa2$_{4-449}$ were grown to mid-log phase and shifted to nitrogen starvation condition for the indicated time. Whole cell extracts were resolved on 8% regular (top panel) or phos-tag containing (lower panel) SDS-PAGE and probed with anti-GFP antibodies. Quantification of immunoblots by densitometry from three independent experiments is shown in the right panel. The difference between each time point and time 0 was statistically analyzed (*, p < 0.050). D, the same cells as in panel C were grown to mid-log phase and treated with rapamycin (0.2 μg/ml) for 2 h. Whole cell extracts were resolved on either regular or phos-tag containing SDS-PAGE and probed with anti-GFP antibodies. Quantification of immunoblots by densitometry from three independent experiments is shown in the right panel. The difference between each time point and time 0 was statistically analyzed (*, p < 0.050). E, gpa2Δ cells transformed with plasmids expressing Gpa2^{1-10}–GFP-Gpa2$_{4-449}$ were grown to mid-log phase, switched to medium without glucose (glucose starvation) for the indicated time, and then shifted to medium with glucose (glucose re-addition). Whole cell extracts were resolved on either regular or phos-tag containing SDS-PAGE and probed with anti-GFP antibodies. Quantification of immunoblots by densitometry from three independent experiments is shown in the right panel.

**Figure 2. Phosphorylation of Gpa2 requires its N-terminal region.** A, The amino acid sequence of Gpa2 with phosphorylation sites revealed by phosphor-proteomics studies highlighted as red. The region highlighted as blue was removed in generating the Gpa2-ΔN-Flag construct. B, Whole cell extracts from cells expressing either full-length Gpa2 (Gpa2-Flag) or a Gpa2 variant lacking the first 120 amino acid residues (Gpa2-ΔN-Flag) were separated on phos-tag containing SDS-PAGE and immunoblotted to reveal Gpa2. The data shown are representative of three independent experiments. C, Whole cell extracts from cells expressing either wild type Gpa2 or a Gpa2-10SA mutant were separated on phos-tag containing SDS-PAGE and immunoblotted to reveal Gpa2. Quantification of immunoblots by densitometry from three independent experiments is shown in the right panel. The difference between Gpa2 and Gpa2-10SA at time 5 h was statistically analyzed (*, p < 0.050).

**Figure 3. Phosphorylation of Gpa2 is GSK-dependent.** A, cells lacking three GSK isoforms (Mck1, Rim11, and Ygk3) and its isogenic wild type were grown to mid-log phase, and treated or not treated with nitrogen starvation condition. Whole cell extracts were separated on phos-tag containing SDS-PAGE gel and Gpa2 was detected via immunoblotting. Quantification of immunoblots by densitometry from three independent experiments is shown in the right panel. The difference between gsk-null and wild type at time 5 h was statistically analyzed (*, p < 0.050). B, Interaction between Flag-tagged Mck1, Rim11, or Ygk3 with GFP-tagged Gpa2 was analyzed by immunoprecipitation of Flag-tagged protein and immunoblotting of GFP-tagged Gpa2. The data shown are representative of three independent experiments. C, Flag-tagged Mck1 and Ygk3 were immune-purified from cells that were nitrogen starved for 12 h. The purified proteins were mixed with immunopurified GFP-tagged Gpa2 in the presence or absence of ATP, and the resulting phosphorylated Gpa2 was monitored using phos-tag SDS-PAGE. The data shown are representative of three independent experiments.

**Figure 4. Subcellular localization of Gpa2.** A, the gpa2Δ mutant cells expressing either Gpa2^{1-10}–GFP-Gpa2$_{4-449}$ or Gpa2^{1-10}–GFP-Gpa2$_{4-449}$-10SA were grown in normal media to mid-log phase, and the localization of GFP-tagged Gpa2 was visualized using confocal microscopy. A portion of mid-log phase cells were switched to nitrogen starvation media, incubated for 6 h, and imaged similarly. Quantification of immunofluorescence signals was conducted using ImageJ. A small section from plasma membrane and
Function and regulation of Gpa2 phosphorylation

from cytoplasm from each cell was selected for density measurement, and their ratio was calculated. Data from 100 cells is shown in the right panel. m-Gpa2: density of plasma membrane localized Gpa2; c-Gpa2: density of cytoplasmic localized Gpa2. B, cells expressing either Gpa2\(^{1-10}\)-GFP-Gpa2\(^{4-449}\) or Gpa2\(^{1-10}\)-GFP-Gpa2\(^{4-449}\)-10SA were grown to mid-log phase and then subjected to nitrogen starvation treatment for 6 h. Whole cell extracts were prepared, separated by sucrose gradient fractionation, resolved by 8% SDS-PAGE, and probed with anti-GFP, anti-Ste4, or anti-Pgk1 antibodies. C, cells expressing CFP-Gpa1 were grown in normal media to mid-log phase, and the localization of CFP-tagged Gpa1 was visualized using confocal microscopy. D, wild type (WT) or mutants lacking GPR1 or PLC1 were transformed with plasmids expressing Gpa2\(^{1-10}\)-GFP-Gpa2\(^{4-449}\) were imaged similarly as described in panel A. The data shown are representative of three independent experiments.

**Figure 5. Functional consequences of Gpa2 phosphorylation.** A, Σ1278-based invasive strain lacking GPA2 was transformed with either empty vector, or plasmids that express wild type Gpa2 or Gpa2-10SA under the control of GPA2 promoter. Overnight cultures were spotted onto YPD plate, grown at 30°C for 2 d, and gently washed with water to reveal the invasive growth. The plate before and after wash were photographed. The data shown are representative of three independent experiments. B, BY4741-derived gpa2Δ mutant cells expressing either empty vector (EV), wild type Gpa2 or Gpa2-10SA under the control of GPA2 promoter, were grown to mid-log phase, treated or not treated with nitrogen starvation. Whole cell extracts were separated by 8% SDS-PAGE and probed for myc-Cki and phospho-MAPKs. Quantification of immunoblots by densitometry from three independent experiments is shown in the bottom panel. The difference between Gpa2 at time 0 h and 4 h was statistically analyzed (*, p < 0.050). C, BY4743-derived gpa2Δ mutant cells (diploid) expressing Gpa2\(^{1-10}\)-GFP-Gpa2\(^{4-449}\) were grown to mid-log phase and subjected to nitrogen starvation for 12 h. Phosphorylation of Gpa2\(^{1-10}\)-GFP-Gpa2\(^{4-449}\) as well as the localization of the protein was analyzed. The data shown are representative of three independent experiments. D, BY4743-derived gpa2Δ mutant cells expressing either empty vector (EV), wild type Gpa2 or Gpa2-10SA under the control of GPA2 promoter, were subject to sporulation assay. Cells with clear sign of sporulation as marked with an arrow were counted. The difference between Gpa2 and Gpa2-10SA was statistically analyzed with a p value of 0.10. E, Model of Gpa2 regulation by phosphorylation. Gpa2 is present in both cytoplasm and plasma membrane. In cytoplasm, Gpa2 binds and inhibits Ime2, a kinase critical for sporulation; on plasma membrane, Gpa2 binds and activates adenylate cyclase, leading to PKA activation. Upon nitrogen starvation, activated glycogen synthase kinases (GSKs) phosphorylate Gpa2 and enhance plasma membrane localization of Gpa2. As a result, nitrogen starvation-induced phosphorylation of Gpa2 diminishes its inhibition of cytoplasmic effector Ime2 and increases its activation of plasma membrane localized effector adenylate cyclase, promoting sporulation and PKA activation. The red arrows indicate the steps that are enhanced by nitrogen starvation.
Figure 2

A

MGLCASSEKNGSTPDQTASAGSDNVGKAKVPKFQEPQKTVR
TVNTANQEQEKKORQQSSPHNVRKDRKEQONGSINNAISPAT
ANTSSQQINIDSAIRDRSSNVAAQPSLSDSSGNDELKV
LLLGASESKSTVLQQLKILHNGFSEQIEKEYIPPIYQNL
EIGNLiQARTRFVNLPEECELTQQDLSRTMSYEMPNNYTG
QFPEDIAVGVIITLWALPSQDQTVGPNASKFYMMDTPYFME
NFTRITSNYPRTQDIILSRQMTSGIFDVTIDMGSDIKMHI
YDVGQGQRSERKKWIFDNVTLVIFCVCVSLSEYDQTLMEDKNGQ
NRFQESLVIFDNIVSVSFARTSVVLFINKDILFAEKL3KVP
MENYFPDYGSDINKAAKYLWRFVQLNRANLSIYPHTVTA
TDTSNIRLVLVFAAIKTILENTLKDGSVLQ

B

w/o nitrogen

|          | Gpa2-ΔN-Flag | Gpa2-Flag |
|----------|--------------|-----------|
| 0 h      | 150 kD       | 75 kD     |
| 5 h      |              |           |

p-Gpa2 / Gpa2 Ratio

|          | Gpa2-ΔN-Flag | Gpa2-Flag |
|----------|--------------|-----------|
| 0 h      | 1.0          | 1.0       |
| 5 h      | *            | 1.0       |

C

w/o nitrogen

|          | Gpa2-Flag | Gpa2-10SA-Flag |
|----------|-----------|---------------|
| 0 h      | 150 kD    | 75 kD         |
| 5 h      |           |               |

p-Gpa2 / Gpa2 Ratio

|          | Gpa2 | Gpa2-10SA |
|----------|------|----------|
| 0 h      | 1.0  | 1.0      |
| 5 h      | *    | 1.0      |
Figure 3

A

B

C

1 mM ATP

-  
-  
-  
-  

+  
+  
+  
+  

-  
-  
-  
-  

150 kD

-  
-  
-  
-  

-  
-  
-  
-  

75 kD

-  
-  
-  
-  

50 kD

-  
-  
-  
-  

37 kD

-  
-  
-  
-  

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**Figure 4**

**A**

- Gpa2
  - normal medium
  - w/o nitrogen
- Gpa2-10SA
  - normal medium
  - w/o nitrogen

**B**

| Protein      | Ratio of m-Gpa2 to c-Gpa2 |
|--------------|---------------------------|
| Gpa2         | 30%                       |
| Gpa2-10SA    | 30%                       |
| Gpa2         | 70%                       |
| Gpa2-10SA    | 70%                       |

**C**

- normal medium
- w/o nitrogen

**D**

- WT
  - normal medium
  - w/o nitrogen
- gpr1Δ
  - normal medium
  - w/o nitrogen
- plc1Δ
  - normal medium
  - w/o nitrogen
**Figure 5**

A. Wild type and gpa2Δ strains before and after wash.

B. Western blot analysis showing the expression of p-myc-cki, myc-cki, PMpk1, and pKss1 under w/o nitrogen conditions with EV, Gpa2, and Gpa2-10SA at 0, 4 h.

C. Western blot analysis for p-Gpa2 and Gpa2 under normal medium and w/o nitrogen conditions at 0 h and 20 h.

D. Images showing the percentage of Nitrogen depletion with Gpa2 and Gpa2-10SA at 20.1% and 16.9% respectively.

E. Diagram illustrating the relationship between Adenylate Cyclase, Gsk1, Sporulation, cAMP, and PKA.

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**Legend:**
- **EV**: Empty vector
- **Gpa2**: Gpa2 gene
- **Gpa2-10SA**: Gpa2 with a mutation at position 10SA
- **p-Gpa2**: Phosphorylated Gpa2
- **p-Mpk1**: Phosphorylated Mpk1
- **p-Kss1**: Phosphorylated Kss1
Phosphorylation of the Gα protein Gpa2 promotes protein kinase A signaling in yeast
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