Phospholipase C Binds to the Receptor-like GPR1 Protein and Controls Pseudohyphal Differentiation in Saccharomyces cerevisiae*

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The hormone receptor-like protein Gpr1p physically interacts with phosphatidylinositol-specific phospholipase C (Plc1p) and with the Go protein Gpa2p, as shown by two-hybrid assays and co-immune precipitation of epitope-tagged proteins. Plc1p binds to Gpr1p in either the presence or absence of Gpa2, whereas the Gpr1p/Gpa2p association depends on the presence of Plc1p. Genetic interactions between the null mutations plclΔ, gpr1Δ, gpa2Δ, and ras2Δ suggest that Plc1p acts together with Gpr1p and Gpa2p in a growth control pathway operating in parallel to the Ras2p function. Diploid cells lacking Gpr1p, Plc1p, or Gpa2p fail to form pseudohyphae upon nitrogen depletion, and the filamentation defect of gpr1Δ and plc1Δ strains is rescued by activating a mitogen-activated protein kinase pathway via STE11-4 or by activating a CAMP pathway via overexpressed Tpk2p. Plc1p is also required for efficient expression of the FG(TyA)::lacZ reporter gene under nitrogen depletion.

In conclusion, we have identified two physically interacting proteins, Gpr1p and Plc1p, as novel components of a nitrogen signaling pathway controlling the developmental switch from yeast-like to pseudohyphal growth. Our data suggest that phospholipase C modulates the interaction of the putative nutrient sensor Gpr1p with the Go protein Gpa2p as a downstream effector of filamentation control.

Phosphatidylinositol-specific phospholipase C (PI-PLC), which hydrolyzes the membrane phospholipid phosphatidylinositol 4,5-bisphosphate (PIP2) to produce inositol 1,4,5-trisphosphate and diacylglycerol. In animal cells, these cleavage products serve as important second messengers; 1,4,5-trisphosphate triggers an intracellular Ca2+ release, whereas diacylglycerol activates protein kinase C. Furthermore, the PI-PLC substrate PIP2 itself is an important signal modulating the activity of membrane-bound proteins. The 10 known mammalian PI-PLC isoforms can be divided into three subtypes β, γ, and δ, differing in their structural organization and in their mode of activation by heterotrimeric G proteins and G protein-coupled hormone receptors (3).

The budding yeast Saccharomyces cerevisiae contains a single phospholipase C gene (PLC1) encoding a δ-type PIP2-specific enzyme (4–6). In most yeast strains the PLC1 gene is not essential for viability at 25 °C, but Plc1p-deficient mutants arrest at temperatures above 35 °C as multibudded enlarged cells unable to complete cytokinesis, they are sensitive against osmotic stress and nitrogen starvation, they do not sporulate as homozygous diploids, and they are defective in the utilization of nonfermentable carbon sources, suggesting that the hydrolysis of PIP2 is required for a number of nutritional and stress-related responses (4, 7). The Plc1p-catalyzed formation of 1,4,5-trisphosphate is stimulated by nitrogen feeding of starved cells; this response depends on a functional Ras GDP-GTP exchange factor, Cdc25p (8).

To learn more about specific PI-PLC functions in yeast, we have performed a two-hybrid screen with Plc1p as bait and identified several different prey peptides physically interacting with Plc1p (9). One of the prey peptides was detected in three independent clones and identified as a carboxy-terminal region of the GPR1 gene product, a hormone receptor-like plasma membrane protein that was previously found in a similar screen using the Go protein Gpa2p as bait (10). A mutational analysis has demonstrated that Gpr1p acts upstream of Gpa2p, an activator of adenylate cyclase, in a Ras-independent growth control chain (10). The deletion of GPR1 abolishes the hyperactivation of adenylate cyclase upon glucose feeding, suggesting a glucose-sensing function of Gpr1p (11). The activation of adenylate cyclase by Gpa2p is required for another nutrient signaling pathway, the induction of pseudohyphal growth in diploid cells upon nitrogen depletion (12–15). Here we report the physical interactions between Gpr1p, Plc1p, and Gpa2p, and we demonstrate important functions of Gpr1p and Plc1p in nitrogen-controlled signaling pathways leading to pseudohyphal growth.

EXPERIMENTAL PROCEDURES

Yeast Strains, Media, and Growth Conditions—Yeast strains used in this study (Table I) are congenic to the CEN.PK2 (EUROFAN) or the Σ1278b (16) background. HMC372 was obtained from E. Kübler, and YEK107 is a HMC072 derivative that has acquired a spontaneous mutation partially suppressing the slow growth phenotype of plclΔ null mutants. Gene replacements were performed as described (17) using PCR-derived cassettes containing the kanamycin resistance gene loxP-kanMX4-loxP or the Schizosaccharomyces pombe his3 gene. The following primers were used to replace PLC1 codons 6–834 (4–6), GPR1 codons 36–937 (10), and RAS2 codons 18–304 (13): CGTAAGCTCTTACCCGAGTGATAGATAGAATGACTGAAAGTGC and CGGAATGTGGCATCAGTATCCACCTACCTAAGTTCGATGTTAAGGC (PLC1); GAGAGGTGATTCTCTCCGACAGCTAACTACACTACGGGTACACCAGATATCCATCTCCAAATATGGTCAGTTAAAGGC (GPR1); CTTTTGAAACACGGAATTAGAGGATCAGATGACGTGACGTGAGCAGCAGAGGTGACGAGGATCTGAGTATGACGTGAAGGATTCGAGCTTTCTGACACGACCAAGAGCTAGAGAGGATGACGTGACGTGACGGGTACACCAGATATCCATCTCCAAATATGGTCAGTTAAAGGC (RAS2). PCR-derived cassettes containing the loxP-kanMX4-loxP gene in the vector pUG6 (19), followed by the GAL1 promoter and three copies of either the HA or Myc epitope, were inserted at the transcriptional starts of genes by using the following primers: CCTCTCTTCCACCTCCTCATATCCATAGAATGTTAATACCTGTAG and CTTTTGAAACACGGAATTAGAGGATCAGATGACGTGACGTGAGCAGCAGAGGTGACGAGGATCTGAGTATGACGTGAAGGATTCGAGCTTTCTGACACGACCAAGAGCTAGAGAGGATGACGTGACGTGACGGGTACACCAGATATCCATCTCCAAATATGGTCAGTTAAAGGC.

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TABLE I
Yeast strains

| Yeast strains | Constructs | Plasmids |
|---------------|------------|----------|

A. CEN.PK2 background

| Yeast strains | Constructs | Plasmids |
|---------------|------------|----------|

CEN.PK2

- MATa/MATa ura3-52/ura3-52 his3-11 his3-1 trp1-289/trp1-289 leu2-3/leu2-3

SYM246

- MATa/MATa gpr1::kanMX4-GAL1p-3xHA-GPR1/GPR1

FMY525

- MATa/MATa plcl1::kanMX4-GAL1p-3xMyc-PLC1/PLC1

KAY370

- MATa/MATa gpr1::kanMX4-GAL1p-3xHA-GPR1/GPR1 plcl1::kanMX4-GAL1p-3xMyc-PLC1/PLC1

KAY370

- MATa/MATa gpr1::kanMX4-GAL1p-3xHA-GPR1/GPR1 plcl1::kanMX4-GAL1p-3xMyc-PLC1/PLC1 gpa2::HIS3/gpa2::HIS3

SMY193

- MATa/MATa gpa2::kanMX4-GAL1p-3xMyc-GPA2/GPA2

SMY294

- MATa/MATa gpr1::kanMX4-GAL1p-3xHA-GPR1/GPR1 gpa2::kanMX4-GAL1p-3xMyc-GPA2/GPA2

SMY296

- MATa/MATa gpr1::kanMX4-GAL1p-3xHA-GPR1/GPR1 gpa2::kanMX4-GAL1p-3xMyc-GPA2/GPA2 p1c1::kanMX4/G1::HIS3

FMY559

- MATa/MATa plcl1::kanMX4-GAL1p-3xHA-PLC1/PLC1

SMY302

- MATa/MATa gpr1::kanMX4-GAL1p-3xHA-GPR1/GPR1 plcl1::kanMX4-GAL1p-3xMyc-PLC1/PLC1 gpa2::HIS3/gpa2::HIS3

SMY306

- MATa/MATa plcl1::kanMX4-GAL1p-3xHA-PLC1/PLC1 plcl1::kanMX4-GAL1p-3xMyc-GPA2/GPA2 gpr1::HIS3/gpr1::HIS3

B. Σ1278b background

HMC372

- MATa/MATa ura3-52/ura3-52 his3::hisG his3::hisG trp1::hisG TRP1 leu2::hisG LEU2

KAY322

- MATa/MATa gpr1::kanMX4/gpr1::kanMX4

YEK44

- MATa/MATa ras2::kanMX4/ras2::kanMX4

YEK107

- MATa/MATa ura3-52/ura3-52 his3::hisG his3::hisG leu2::hisG leu2::hisG

YEK111a

- MATa gpr1::kanMX4

YEK114

- MATa/MATa gpr1::kanMX4/gpr1::kanMX4

YEK110a

- MATa plcl1::kanMX4/plcl1::kanMX4

YEK115a

- MATa gpa2::HIS3

KAY332

- MATa plcl1::kanMX4/p1c1::kanMX4

YEK118a

- MATa gpr1::HIS3

KAY305

- MATa/MATa gpa2::LEU2/gpa2::LEU2

YEK112a

- MATa ras2::HIS3

YEK134a

- MATa plcl1::HIS3 gpr1::kanMX4

YEK120a

- MATa plcl1::kanMX4 gpa2::HIS3

YEK135a

- MATa plcl1::kanMX4 ras2::HIS3

TABLE II
Plasmids

| Code | Coding region* | Vector |
|------|----------------|--------|

pKA42 | PLC1 | pRS416 |
pKA43 | PLC1 | YEp24 |
pKA47 | GPR1 | pRS416 |
pKA49 | GPR1 | YEp24 |
pEH4 | GPA2 | YEp24 |
pEH31 | TPK2 | YEp24 |
pMS58 | AD-P1c1p, 79–896 | pG4–5 |
pMS61 | DB-P1c1p, 79–896 | pEG202 |
pMS72 | AD-Gpr1p, 821–961 | pG4–5 |
pEH21 | AD-Gpr1p, 671–961 | pG4–5 |
pKA50 | AD-Gpa2p, 1–448 | pG4–5 |
pMS81 | AD-Gpa2p, 1–448 | pG4–5 |
pMS82 | DB-Gpa2p, 1–448 | pEG202 |
pFM224 | kanMX4-GAL1p-3xHA | pUG6 |
pFM225 | kanMX4-GAL1p-3xmyc | pUG6 |

* AD, activating domain (acid blob B42); DB, DNA-binding domain (LexA).

TAGTCTCGAG, to obtain pEH2. A 1.8-kb EcoRI/SalI fragment containing the PLC1 coding region of pMS56 was converted to an EcoRI site by introducing the tag adaptor TAGTCTCGAG to obtain pMS57. A 2.4-kb EcoRI/BamHI fragment containing the PLC1 coding region of pMS58 was converted to an EcoRI site by introducing the tag adaptor TAGTCTCGAG to obtain pMS57. A 2.4-kb EcoRI/SalI fragment containing the PLC1 coding region of pMS58 was converted to an EcoRI site by introducing the tag adaptor TAGTCTCGAG to obtain pMS57.

Yeast Two-hybrid Methods—A two-hybrid screen with Plc1p as bait was performed as described (22, 23) by using a yeast genomic (S288c) library in the pEG202 vector to obtain PKA42 and PLC1, respectively. The same BamHI PLC1 fragment was subcloned in a pUC19 derivative lacking a BamHI site to obtain pMS56. The NdeI site within the PLC1 coding region of pMS56 was converted to an EcoRI site by introducing the tag adaptor TAGTCTCGAG to obtain pMS57. A 2.4-kb EcoRI/SalI fragment containing the PLC1 coding region of pMS58 was converted to an EcoRI site by introducing the tag adaptor TAGTCTCGAG to obtain pMS57. A 2.4-kb EcoRI/SalI fragment containing the PLC1 coding region of pMS58 was converted to an EcoRI site by introducing the tag adaptor TAGTCTCGAG to obtain pMS57. A 2.4-kb EcoRI/SalI fragment containing the PLC1 coding region of pMS58 was converted to an EcoRI site by introducing the tag adaptor TAGTCTCGAG to obtain pMS57. A 2.4-kb EcoRI/SalI fragment containing the PLC1 coding region of pMS58 was converted to an EcoRI site by introducing the tag adaptor TAGTCTCGAG to obtain pMS57. A 2.4-kb EcoRI/SalI fragment containing the PLC1 coding region of pMS58 was converted to an EcoRI site by introducing the tag adaptor TAGTCTCGAG to obtain pMS57. A 2.4-kb EcoRI/SalI fragment containing the PLC1 coding region of pMS58 was converted to an EcoRI site by introducing the tag adaptor TAGTCTCGAG to obtain pMS57. A 2.4-kb EcoRI/SalI fragment containing the PLC1 coding region of pMS58 was converted to an EcoRI site by introducing the tag adaptor TAGTCTCGAG to obtain pMS57.
centrifugation, washed twice with YP buffer, resuspended in YPGal and grown to an OD600 = 1.5. All subsequent steps were performed at 4 °C. Cells were collected, washed twice with IP buffer (50 mM HEPES, pH 8, 50 mM NaCl, 2 mM EDTA, 1 mM Na2SO4, 5% glycerol, 1 mM phenylmethylsulfonyl fluoride, 1 tablet of Roche Molecular Biochemicals protease inhibitor mixture Complete25 μl), and resuspended in an equal volume of IP buffer. The cell suspension was homogenized with 1 volume of acid-washed, sterilized, and chilled glass beads (0.45 mm) by vortexing at maximum speed for 30 s, followed by chilling on ice for 60 s. This step was repeated 10 times. Supernatants were then removed by centrifugation and pooled together with supernatants obtained after washing glass beads twice with 2 to 4 volumes of IP buffer containing 0.1% Triton X-100. The lysate was then centrifuged for 10 min at 10,000 × g to remove cell debris. Protein concentration in the supernatant was determined by a Bio-Rad (Bradford) protein assay.

Immunoprecipitation was performed in 450 μl of IP buffer containing 0.1% Triton X-100 and 2 mg of total protein by adding 3 μg of purified monoclonal anti-HA antibodies (BABC0, catalog no. 16B12) or anti-Myc antibodies (Santa Cruz Biotechnology, catalog no. 9E10). After incubation for 1 h at 4 °C, a suspension of protein A-conjugated Sepharose beads (Amersham Pharmacia Biotech) in equal volume of IP buffer containing 2% bovine serum albumin (40 μl final volume) was added, and the mixture was incubated for 2 h at 4 °C with constant mixing. The beads were pelleted at 1000 × g and washed four times with 700 μl of IP buffer for 5 min at 4 °C under constant mixing. The washed beads were resuspended in 20 μl of 2× Laemmli sample buffer and incubated for 5 min at 100 °C. The supernatant (15 μl) was analyzed by SDS-PAGE and blotted onto Hybond ECL membranes (Amersham Pharmacia Biotech) for 24 h at 4 °C in transfer buffer (25 mM Tris, 195 mM glycine, 0.1% SDS, 10% methanol). Nonspecific binding sites were blocked by incubating the membrane for 1 h at room temperature in PBST (2.7 mM KCl, 1.5 mM KH2PO4, 137 mM NaCl, Na2HPO4, 0.1% Tween 20, pH 7.5) containing 5% nonfat dry milk. Immunodetection and the mixture was incubated for 2 h at 4 °C with constant mixing. The washed beads were resuspended in 20 μl of 2× Laemmli sample buffer and incubated for 5 min at 100 °C. The supernatant (15 μl) was analyzed by SDS-PAGE and blotted onto Hybond ECL membranes (Amersham Pharmacia Biotech) for 24 h at 4 °C in transfer buffer (25 mM Tris, 195 mM glycine, 0.1% SDS, 10% methanol). Nonspecific binding sites were blocked by incubating the membrane for 1 h at room temperature in PBST (2.7 mM KCl, 1.5 mM KH2PO4, 137 mM NaCl, Na2HPO4, 0.1% Tween 20, pH 7.5) containing 5% nonfat dry milk. Immunodetection was accomplished by using biotinylated anti-mouse antibodies (1:2000 dilution) and streptavidin-biotinylated horseradish peroxidase complex (1:3000 dilution) with the enhanced chemiluminescence Western blotting system (ECL, Amersham Pharmacia Biotech).

RESULTS

Two-hybrid Interactions between Gpr1p, Plc1p, and Gpa2p—We have used a two-hybrid screen (22, 23) to identify proteins physically interacting with phospholipase C. A functional Plc1 protein lacking only the dispensable first 78 residues (5) was fused to the DNA-binding LexA protein, and the resulting plasmid pMS61 (see Table I) was co-transformed as bait together with a genomic yeast library in the activating domain fusion vector pJG4–5. Three independent prey clones giving strong signals (expression of LEU2 and lacZ reporter genes) were found to contain the carboxyl-terminal residues 821–961 of the G protein-coupled receptor (GPR1) gene product (9). Almost identical Gpr1p regions (residues 863–961 and 839–961) were previously identified as preys by using the G protein Gpa2p as bait (10). As shown in Fig. 1A, the GPR1 gene product exhibits seven membrane-spanning domains, a feature predicted from its sequence. Two largest Gpr1p domains predicted to face the cytosolic side of the plasma membrane are the third intracellular loop (residues 273–621) and the carboxyl-terminal domain (residues 671–961).

The two-hybrid interactions of three Gpr1p peptides (Fig. 1A) with almost intact Plc1p (residues 79–869) and intact Gpa2p (Fig. 1, B and C) are summarized in Table III. Both Plc1p and Gpa2p interact strongly with the two minor Gpr1p regions and, to a much lesser extent, with the third intracellular loop. We note that the longer carboxyl-terminal domain of Gpr1p is more efficient than the short one, if combined with Plc1p, whereas Gpa2p interacts more efficiently with the short C terminus of Gpr1p. There is also a weak but significant interaction between Plc1p and Gpa2p.

Co-immune Precipitation of Gpr1p, Plc1p, and Gpa2p—To confirm a physical association of Gpr1p with Plc1p and Gpa2p by co-immune precipitation, we have inserted a PCR-based cassette containing the kanamycin resistance gene kanMX4, the GAL1 promoter, and the epitope tags 3xMyc or 3xHA at the translational starts of the genes GPR1, PLC1, and GAPA2. Heterozygous diploid strains co-expressing HA-Gpr1p and Myc-Plc1p (KAY320), HA-Gpr1p, and Myc-Gpa2p (SMY294) or HA-Plc1p and Myc-Gpa2p (SMY302) were grown in galactose media and analyzed by immunoblotting of crude extracts, using monoclonal antibodies against HA and c-Myc.

Fig. 2 demonstrates that galactose-induced cells produce immunoreactive proteins corresponding in size to the calculated molecular mass of the tagged proteins. Myc-Gpa2p (55 kDa, lanes B and C) and HA-Gpr1p (110 kDa, lanes D and F) migrate as single bands, whereas Myc-Plc1p or HA-Plc1p form a minor 92-kDa band in addition to the major 105-kDa species (lanes A and E), probably reflecting some proteolysis.

Fig. 3 shows the results of co-immunoprecipitation experiments using heterozygous diploid strains expressing either HA-Gpr1p (SMY246), Myc-Plc1p (FMY525), or both tagged proteins (KAY20). Crude lysates of cells grown in galactose medium were treated with purified monoclonal anti-Myc antibodies and the immunoprecipitated material was absorbed to protein A-conjugated Sepharose beads. The immunoprecipitate was then probed by Western blotting using the two specific antibodies.

Fig. 3, lane A, demonstrates that HA-Gpr1p co-precipitates with Myc-Plc1p from lysates of strain KAY320, whereas HA-Gpr1p is absent from anti-Myc precipitates of cells expressing
null mutations was tested on solid SC medium at 25 and 37 °C, as shown in Fig. 6. The deletion of PLC1 in the YEK107 background only slightly impairs growth at 25 °C, but it leads to an arrest at 37 °C, as observed in several other backgrounds (4–6), whereas strains lacking GPRI, GPA2, or RAS2 remain viable at both temperatures. The reduced growth rate of these mutants at 37 °C appears to be a property of the 1278b background (HMC372 and YEK107). The double mutant gpr1Δ gpa2Δ has previously been shown to grow as efficiently as the single mutants, whereas combinations with the ras2Δ mutation (gpr1Δ ras2Δ and gpa2Δ ras2Δ) lead to a synthetic slow growth phenotype (10, 14). Fig. 6 demonstrates that combinations with the plc1Δ null mutation lead to similar results; the double mutants plc1Δ gpr1Δ and plc1Δ gpa2Δ have the growth phenotype of plc1Δ (slightly impaired growth at 25 °C, temperature sensitivity), whereas the plc1Δ ras2Δ double mutant exhibits extremely slow growth at 25 °C. The growth defects of all PLC1-deficient strains could be rescued by adding the PLC1-containing plasmids pK4A2 and pK4A3 (data not shown). We have also confirmed the synthetic slow growth phenotype of gpr1Δ ras2Δ and gpa2Δ ras2Δ double mutants in the 1278b background (data not shown). Our findings suggest that Plc1p acts in the same growth control pathway as Gpr1p and Gpa2p (no synthetic growth defects of double mutants) and in parallel with a Ras2p-controlled pathway (synthetic growth defects of plc1Δ). Gpr1p and Plc1p Are Required for Pseudohyphal Growth under Nitrogen Depletion—Gpa2p has previously been shown to control the diploid-specific dimorphic transition from yeast-like to pseudohyphal growth in response to nitrogen starvation (13, 14). This observation has prompted us to test a possible role of the Gpa2p-interacting proteins Gpr1p and Plc1p in the same nutrient-dependent signaling process.

Figs. 7 and 8 show the colony morphology of diploid cells

null mutations were replaced either by the Kanamycin resistance gene (congenic to the

were introduced into strain YEK107, a derivative of HMC372

Deletions of either

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FIG. 2. Immunoblot analysis of epitope-tagged Gpr1p, Plc1p, and Gpa2p. Total protein of strains KAY320 (lanes A and D), SMY302 (lanes B and E), and SMY294 (lanes C and F) was obtained by lysis in 2 N NaOH containing 5% mercaptoethanol and trichloroacetic acid precipitation. Protein was analyzed by SDS-PAGE and immunoblotting using anti-Myc (lanes A–C) or anti-HA (lanes D–F). Protein size standards (β-galactosidase, bovine serum albumin, and carbonic anhydrase) was from Bio-Rad.

FIG. 3. Co-immune precipitation of Myc-Plc1p and HA-Gpr1p in the presence and absence of Gpa2p. Anti-Myc immunoprecipitates obtained from strains KAY320 (lanes A and D), SMY246 (lanes B and F), FMY525 (lanes C and G), and KAY70 (lanes D and H) were analyzed by SDS-PAGE and immunoblotting using anti-HA (lanes A–D) or anti-Myc (lanes E–H) antibodies.

only HA-Gpr1p (lane B) or Myc-Plc1p (lane C). Lanes E–G confirm the presence or absence of Myc-Plc1p in the anti-Myc precipitates. As a further negative control we have co-expressed HA-Kex2p and Myc-Plc1p (data not shown).

The complex formation between HA-Gpr1p and Myc-Plc1p may depend on the presence of Gpa2p. To test this possibility, we have deleted the GPA2 gene from cells co-expressing HA-Gpr1p and Myc-Plc1p (KAY370). Fig. 3, lane D, shows the presence of HA-Gpr1p in the anti-Myc precipitate derived from KAY370, indicating that the Gpr1p/Plc1p association does not depend on the presence of Gpa2p.

Similar co-immunoprecipitation experiments were performed with strains expressing HA-Gpr1p and/or Myc-Gpa2p in the presence or absence of the PLC1 gene (strains SMY294 and SMY296). Fig. 4 demonstrates that HA-Gpr1p co-purifies with Myc-Gpa2p using a PLC1-wt strain lysate (lane A), whereas the Gpr1p band is absent from the immunoprecipitate of a plc1Δ deletant strain (lane D), suggesting that the Gpr1p/Gpa2p association depends on the presence of Plc1p.

Finally, the Plc1p/Gpa2p association was tested in the presence or absence of Gpr1p. According to Fig. 5 Myc-Gpa2p co-purifies with HA-Plc1p both in the presence (lane A) and absence (lane D) of Gpr1p.

Genetic Interactions of gpr1Δ, plc1Δ, gpa2Δ, and ras2Δ Null Mutations—Deletions of either PLC1, GPRI, GPA2, or RAS2 were introduced into strain YEK107, a derivative of HMC372 (congenic to the 1278b genetic background) (12). The above genes were replaced either by the kanamycin resistance gene kanMX4 or by the HIS3 gene of S. pombe, using PCR-derived cassettes (17).

Growth of haploid strains containing either single or double
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...after 5 days incubation at 30 °C on plates containing nitrogen-starved synthetic low ammonia dextrose medium (12). Fig. 7A demonstrates the formation of agar-invading pseudohyphal filaments at the periphery of colonies from strain HMC372 (containing the "empty" plasmid YeEp24). This filamentation effect is completely abolished by the deletion of the GPR1 gene, as seen in Fig. 7B (strain KAY232 carrying YeEp24). The filamentation defect of the gpr1Δ strain is rescued if the "empty" plasmid YeEp24 is replaced by pKA49, a YeEp24 derivative containing the wt GPR1 gene (Fig. 7C). Furthermore, filamentation is also restored by the dominant-active RASgVol-19 allele (Fig. 7D) by increasing the dosage of the TPK2 gene, which encodes one of the three catalytic subunits of cAMP-dependent protein kinase (26, 27), or by introducing the dominant-active STE11-4 allele (29), a component of the MAPK cascade involved in filamentation control (13, 14, 16), as seen in Fig. 7, panels E and F, respectively.

Similar results were obtained with homozygous diploid strains lacking the PLC1 gene, as shown in Fig. 8. The plc1Δ strain KAY322 exhibits a somewhat ragged colony morphology but does not form filaments (Fig. 8B). The filamentation defect of plc1Δ cells is rescued by a multicopy TPK2 plasmid (Fig. 8E) or a centromeric STE11-4 plasmid (Fig. 8F) but not by the RASgVol-19 allele (Fig. 8D), suggesting a role of Plc1p downstream of Ras2p (see “Discussion”). We do not have an explanation why the filamentation defect of plc1Δ cells is only partially restored by the multicopy PLC1 plasmid pKA43 (Fig. 8C) or by the centromeric PLC1 plasmid pKA42 (data not shown).

Differential Effects of GPR1 and PLC1 Deletions on the Transcription of the Reporter Gene FG(TyA)::lacZ—The expression of the reporter FG(TyA)::lacZ, a fusion of the Ty1 transposon to the β-galactosidase gene, depends on the Ste12p transcription factor (31) and correlates well with pseudohyphal growth induced by nitrogen starvation, mainly reflecting the activity of the Ste20p-MAPK cascade involved in the filamentation process (14–16, 32). We have therefore used this reporter to test the role of Gpr1p and Plc1p in filamentation control by an alternative approach.

The data of Table IV indicate a strong influence of Plc1p on the reporter gene expression; a 5-fold reduction of lacZ activity is observed upon the plc1Δ deletion, and the activity is almost completely recovered by adding back a plasmid-borne PLC1 gene. The 2-fold reduction of lacZ activity in the rasΔ2 strain YEK44 corresponds to the effects of deleting components of the MAPK pathway (e.g. ste20Δ, ste7Δ, ste12Δ), as observed by others (15). In contrast, the lacZ activity remains relatively high upon the GPR1 deletion (1.2-fold reduction), suggesting that Plc1p is more important than Gpr1p for controlling the MAPK pathway (see “Discussion”).
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**TABLE IV**

Expression of FG(TyA)::lacZ in homozygous diploid strains under nitrogen starvation

| Strain       | Relevant genotype | Relative β-galactosidase activity |
|--------------|------------------|---------------------------------|
| YEK107       | wt               | 1.0                             |
| YEK114       | gpr1             | 0.90                            |
| YEK114/pKA49 | gpr1, GPR1       | 0.96                            |
| KAY322       | plc1             | 0.18                            |
| KAY322/pKA43 | plc1, PLC1       | 0.91                            |
| YEK44        | ras2             | 0.5                             |

**DISCUSSION**

We have shown that the hormone receptor-like plasma membrane protein Gpr1p (10) interacts physically with both Plc1p (4–6) and with the Gα protein Gpa2p (25, 10), using two independent methods: two-hybrid interactions of fusion proteins and co-immune precipitation of epitope-tagged proteins. Furthermore, we demonstrate that all three proteins play important roles in controlling the switch between yeast-like and pseudohyphal growth of diploid cells upon nitrogen depletion (12–16).

The simplified model shown in Fig. 9 may help to discuss some details of these findings. The GPR1 gene sequence predicts the intracellular orientation of two large protein regions (see Fig. 1A): the loop between the membrane-spanning domains 5 and 6 (349 residues) and the carboxyl-terminal 291 residues. A shorter carboxyl-terminal region (141 residues), which was identified as prey during two-hybrid screens with intact Gpa2p (10) or near-intact Plc1p (9) as bait, interacts more efficiently with Gpa2p (relative activity 1334 units, see Table III) than the long carboxyl terminus (919 units), whereas the long carboxyl terminus is more efficient in binding Plc1p (340 units) than the short one (Ref. 28.7 units). Gpa2p and Plc1p also interact with each other (12 units) and with the Gpr1p loop region (7 and 9 units, respectively) but with lower efficiency.

Although two-hybrid data do not necessarily reflect in vivo interactions, we tentatively conclude that Plc1p and Gpa2p associate preferentially with the carboxyl-terminal Gpr1p domain, whereby the upstream region of the long carboxyl terminus is more important for Plc1p binding than for Gpa2p binding. We have confirmed and extended the physical interaction studies by co-immune precipitation of Myc- or HA-tagged proteins, carefully ruling out nonspecific interactions. It turned out that the Gpr1p/Plc1p complex is formed either in the presence or absence of Gpa2p, whereas Gpa2p associates with Gpr1p only in the presence of Plc1p but not in its absence.

These findings may suggest that PIP₂-specific phospholipase C is required to expose a Gpa2p binding site at the carboxyl-terminal Gpr1p domain, perhaps by modulating the interaction between basic residues of the Gpr1p polypeptide and the acidic PIP₂ head groups at the inner side of the plasma membrane. Such interactions have been shown to be critically important in exposing an ATP binding site at the carboxyl-terminal domain of a mammalian ATP-sensitive potassium channel (34). Gpa2p is known to be required for the induction of pseudohyphal growth in diploid cells upon nitrogen starvation (13, 14, 25). Here we show that Gpr1p and Plc1p act upstream of Gpa2p in the same process: filamentation is prevented by deleting either GPR1 or PLC1 (in homozygous diploids), and is restored by the corresponding wt genes.

Furthermore, the filamentation defect of the null mutations gpr1Δ and plc1Δ is suppressed by activating the cAMP pathway via overexpression of the cAMP-dependent protein kinase Tpk2 or by activating the MAPK pathway via the dominant active STE11-4 allele. Both signaling pathways have previously been shown to control pseudohyphal growth upon nitrogen starvation (13–16, 27, 35, 36), converging in the transcriptional control of filamentation genes such as FLO11 (35).

The unusually large FLO11 promoter (spanning at least 2.8 kb) is regulated by the cAMP-dependent pathway via the transcription factor Flo8p and by the MAPK pathway via the transcription factors Ste12p and Tec1p. Both filamentation pathways can replace each other by overexpression or constitutive activation of single components (e.g. overexpressed Flo8p suppresses the loss of Ste12p and vice versa) (35).

The MAPK filamentation pathway can be monitored in a more specific way by the transcriptional reporter FO(TyA)::lacZ, which responds much more to dominant-activated STE11-4 (8-fold stimulation of lacZ activity) than to the activation of the cAMP pathway (less than 2-fold stimulation) (15). Using this reporter we find that the lacZ activity strongly depends on the Plc1p function (5-fold reduction in plc1Δ extracts), but it is less dependent on the Gpr1p function (1.2-fold reduction in gpr1Δ extracts). In comparison, the lacZ activity is reduced 2-fold in extracts of ras2Δ (see Table IV) and ste20Δ mutants (15).

This somewhat unexpected result appears to indicate that the MAPK filamentation pathway requires the Plc1p function, whereas Gpr1p may operate mainly through the Gpa2p/cAMP pathway. In addition, we find that the dominant-active RAS2Val-19 mutation suppresses the filamentation defects of gpr1Δ (Fig. 7D) and gpa2Δ strains (data not shown) but does not rescue the plc1Δ defect (Fig. 8D). This observation suggests that Plc1p has a second function downstream of Ras2p, perhaps that of controlling the activation of the MAPK pathway via Cdc42p (15) in addition to activating the cAMP pathway by modulating the Gpr1p/Gpa2p interaction. According to the model of Fig. 9, the G protein-coupled receptor-like Gpr1 pro-
tein functions as a nitrogen sensor, which activates a cAMP-dependent filamentation signaling pathway by subsequently binding Plc1p and Gpa2p. A similar nutrient-sensing function has been suggested for Mep2p, a high affinity ammonium permease required for filamentation control (36). Although the filamentation defect of mep2Δ/mep2Δ diploids is suppressed by dominant active GPA2 or RAS2 mutations, there is no evidence directly linking Mep2p and Gpa2p, and it is possible that Mep2p functions in a signaling pathway separate from either the Gpa2p/cAMP or MAPK pathway (36). The relationship between the Gpr1p- and Mep2p-controlled filamentation routes thus remains to be established.

Previous studies have implied a general role for yeast Plc1p in regulatory pathways necessary for adaptation to changing nutrient and temperature conditions (4, 6, 7), and some phenotypic properties of plc1Δ mutants (loss of viability upon nitrogen starvation, sporulation defect of homozygous diploids) point to the role of Plc1p in nitrogen-controlled signaling pathways (4). Our data are in agreement with this view by placing the Plc1p function within a well defined nitrogen signaling pathway involved in filamentation control. The interaction of Plc1p with a receptor-like protein (Gpr1p) and a Gα protein (Gpa2p) is reminiscent of the association of mammalian PLC-δ with agonist-bound α1-adrenergic receptors and with receptor-coupled Gα, a multifunctional GTP-binding protein having transglutaminase activity (3, 37).

Other phenotypic properties of yeast plc1Δ strains such as cytokinesis defects (4) or aberrant chromosome segregation (6) point to multiple functions of Plc1p. Indeed, we observe a physical interaction of Plc1p with Num1p, a cortical protein controlling nutrient-dependent nuclear migration (38, 39), with Bni4p, a bud neck protein controlling septum formation and cytokinesis (40), as well as with a few functionally unknown transmembrane proteins (9). Our data support the view that yeast Plc1p has multiple roles in modulating membrane/protein interactions by cleavage of the lipidPIP2.

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