Isolation of a Novel Gene from *Schizosaccharomyces pombe*: **stm1**

**Encoding a Seven-transmembrane Loop Protein That May Couple with the Heterotrimeric Gα2 Protein, Gpa2**

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A putative seven transmembrane protein gene, **stm1**

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which is required for proper recognition of nitrogen starvation signals, was isolated as a multicopy suppressor of a ras1 synthetic lethal mutant in *Schizosaccharomyces pombe*. Under nitrogen-deficient conditions, transcription of the **stm1** gene was induced; deletion of **stm1** was associated with early entry into GI arrest. Under nutritionally sufficient conditions, overexpression of **stm1** inhibited vegetative cell growth, resulted in decreased intracellular cAMP levels, increased the expression of the meiosis-specific genes **ste11**, **mei2**, and **mam2**, and facilitated sexual development in homothallic cells. However, inhibition of vegetative cell growth and reduction of cAMP levels were not observed in a deletion mutant of the heterotrimeric G protein Go2 gene, **gpa2**, that is responsible for regulating intracellular cAMP levels, a key factor in determining the sexual development in *S. pombe*. **Stm1** protein was shown to interact with Gpa2 through its C-terminal transmembrane domains 5–7. Mutation at Lys199 in the C-terminal domain (**stm1**K199A) abolished the **Stm1** overexpression effect on lowering cAMP levels. Induction of **ste11**, a meiosis-specific gene transcription factor, by **Stm1** overexpression was enhanced in **gpa2**-deleted cells but was absent in a deletion mutant of **sty1**, a key protein kinase that links mitotic control with environmental signals and induces stress-responsive genes. Moreover, deletion of both **stm1** and **ras1** caused delayed entry into GI arrest in *S. pombe* when the cells were grown in a nitrogen-deficient medium. Thus we consider that the **stm1** gene function through Gpa2-dependent and/or -independent pathways and may play a role in providing the prerequisite state for entering the pheromone-dependent differentiation cycle in which heterotrimeric Go1 protein, Gpa1, and Ras1 play major roles. **Stm1** could function as a sentinel molecule sensing the nutritional state of the cells, stopping the proliferative cell cycle, and preparing the cell to enter meiosis under nutritionally deficient conditions.

Many of the cell membrane G protein-coupled hormone receptors relay signals to the interior of the cell using several different classes of heterotrimeric G proteins: Gs, Gi, Go, Gq, G12, and G13. These heterotrimeric G protein molecules specify intracellular signal pathways by either activating or inactivating different effector systems such as adenylyl cyclase or phospholipase C, which in turn initiate signals via second messengers such as cAMP or calcium (1–8). On the other hand small G proteins, including Ras, modulate effector elements in response to external signaling, which can influence cell growth and differentiation (9–11). Ras is capable of binding to different effector proteins such as Raf (12, 13), phosphatidylinositol 3-kinase (14), and Ral (15, 16). The interactions of different effectors with Ras can activate different downstream signaling pathways, including those that stimulate the cell division cycle, alter cell shape, or induce cellular differentiation (17). Thus signals emanating from the cell surface are delivered to diverse downstream effectors by switching G protein functions on and off. The integrated G protein signals are responsible for orchestrating a coherent specific biological response.

Fission yeast, *Schizosaccharomyces pombe*, possesses two genes encoding heterotrimeric G protein α-subunits, **gpa1** and **gpa2** (18, 19), and one gene, **gpb1**, encoding a β-subunit (20). Gpa1 is responsible for pheromone-responsive sexual development, and Gpa2 relays the nutritional status information necessary to initiate the sexual differentiation of *S. pombe*. The Gβ subunit, Gpb1, functions as a negative factor in sexual development (20). Whereas only one ras gene, **ras1**+, has been identified in *S. pombe*, it is required for at least two distinct cellular functions: sexual differentiation, including conjugation and sporulation, and the control of cell morphology (21). Ras1 plays a major role in the initiation of meiotic differentiation when cells endure nutritionally unfavorable conditions (22). Unlike *Saccharomyces cerevisiae*, in which ras genes are essential for cell growth and modulate adenylate cyclase activity (16, 23–26), *S. pombe* cells carrying a null mutation in the **ras1** gene proliferate normally. These cells are deficient, however, in terms of sexual differentiation in that haploid cells are completely sterile and homozygous diploid cells have a very low frequency of sporulation. Mutated Ras1 does not affect the intracellular cAMP levels in *S. pombe*, indicating that Ras1 does not act primarily by modulating adenylate cyclase activity. Instead, when a mating pheromone binds to its serpentine receptor (Mam2 or Map3), Ras1 activates a mitogen-activated protein (MAP)1 kinase module composed of Byr2, Byr1, and

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1 The abbreviations used are: MAP, mitogen-activated protein; DAPI, 4,6-diamidino-2-phenylindole; EMM, Edinburgh minimal medium; HA, hemagglutinin; PAGE, polyacrylamide gel electrophoresis; GFP, green fluorescent protein.
Spk1 protein kinases in concert with a heterotrimeric G protein α-subunit, Gap1, Gpa1 (18, 21, 27–29). This leads to the initiation of conjugation and sporulation. However, the activation of MAP kinases by Ras1 should be preceded by cell cycle arrest at G1. It has been observed that in S. pombe, when the nitrogen source is depleted, cell growth is arrested at G1 first (30–33), and then the heterotrimeric G protein α-subunit, Gap2, which regulates adenylyl cyclase in accordance with nutritional state of the cells (19), lowers the intracellular CAMP levels. This in turn triggers the induction of expression of genes such as mamm2, ste11, and mei2, which are required in the initial stage of meiosis (22). Thus the decision to exit the cell cycle and initiate mating requires the integration of signal information concerning both the nutrient status and the presence of mating partners. The ras1− gene is considered to be involved in integrating these two signals by increasing the sensitivity to pheromone in the appropriate pathways when nutrients are in short supply (22). However, the link between the acceptance of the nutritional starvation signal and the activation of the differentiation cycle is poorly understood. In this report, we describe the isolation of the stm1+ gene encoding a putative seven-loop transmembrane protein, which may function as a receptor that couples with the heterotrimeric Gα2 protein, Gap2. Our studies indicate that Stm1 can interact with Gap2 through the C-terminal domain. This interaction may provide the initiating signal that links signals triggered by nutritional deficiency and pheromone-dependent sexual differentiation, to allow Ras1 to activate sexual differentiation in S. pombe.

**EXPERIMENTAL PROCEDURES**

**Strain Manipulation and Media**—The S. pombe strains used in this study are listed in Table I. The newly isolated synthetic lethal mutant of ras1, KSC3, was derived from the KSC1 strain. S. pombe cells were grown in YEPD (0.5% yeast extract, 0.5% peptone, and 3% glucose), YES (0.5% yeast extract and 3% glucose with supplement), EMM (Edinburgh minimal medium) (34), EMM2 (EMM without supplements, CLONTECH), and ME (0.3% malt extract) media supplemented with adenine, uracil, and/or leucine as described previously (35, 36). For detection of dead cells, phloxine B (Sigma) was added to the YES and EMM plates at a concentration of 20 mg/liter (37). Prior to analyzing the effects of nitrogen starvation on transcript levels of the genes of S. pombe, cells were grown in EMM overnight and resuspended at 10^7 cells/mL in EMM containing different nitrogen sources (0.5% NH4Cl, 0.5% proline, or no NH4Cl). For flow cytometry, cells were grown in EMM to log phase, harvested, washed, and then transferred to EMM devoid of a nitrogen source. Transformation of S. pombe cells was carried out using the lithium acetate method and standard yeast genetic techniques as described by Moreno et al. (38). Escherichia coli DH5α was used for subcloning.

* Isolation of the stm1 Gene by Functional Complementation of a Synthetic Lethal Mutant of the ras1 Gene—To isolate a gene that may function in association with Ras1 in sexual differentiation of S. pombe, we used a mutant, KSC3, that exhibits a lethal phenotype when the ras1 gene under the nmt1 promoter is deleted. The nmt1 promoter was excised from the nmt1a::URA4 plasmid by PstI digestion and inserted into the nmt1 promoter-regulated selectable marker, respectively.

**TABLE I**

| Strains | Genotypes |
|---------|------------|
| 972     | h-         |
| JY4     | h manoe-261 leu1-32 ura4-D18 |
| ED665   | h manoe-261 leu1-32 ura4-D18 |
| KSC1    | h manoe-261 leu1-32 ura4-D18 ras1::nmt1 ras1::ura4 |
| HD1     | h manoe-261 leu1-32 ura4-D18 stm1::ura4 |
| HD3     | h manoe-261 leu1-32 ura4-D18 gpa2::ura4 |
| HD8     | h manoe-261 leu1-32 ura4-D18 ras1::ura4 |
| HD9     | h manoe-261 leu1-32 ura4-D18 gpa2::ura4 pem1::LEU2 |
| HD11    | h manoe-261 leu1-32 ura4-D18 gpa2::ura4 pem1::LEU2 |
| HD13    | h manoe-261 leu1-32 gpa2::a-stm1 |
| HD15    | h manoe-261 leu1-32 ura4-D18 sty1::ura4 |
| ED19    | h manoe-261 leu1-32 ras1::ura4 |
| ED29    | h manoe-261 leu1-32 ras1::leu2 |
| ED39    | h manoe-261 gpa2::ura4 |
| ED49    | h manoe-261 leu1-32 ura4-D18 sta1::ura4, ras1::leu2 |
| KSC1    | h manoe-261 leu1-32 ura4-D18 / pmnt1::sta1 |
| KSC2    | h manoe-261 leu1-32 ura4-D18 / pmnt1::sta1 |
| KSC3    | h manoe-261 leu1-32 ura4-D18 gpa2::ura4 / pmnt1::sta1 |
| KSC4    | h manoe-261 leu1-32 ura4-D18 / pmnt1::sta1 |
| KSC5    | h manoe-261 leu1-32 ura4-D18 ras1::ura4 / pmnt1::sta1 |
| KSC6    | h manoe-261 leu1-32 ura4-D18 gpa2::ura4 / pmnt1::sta1 |
| KSC7    | h manoe-261 leu1-32 ura4-D18 pem1::LEU2 |
| KSC8    | h manoe-261 leu1-32 gpa2::a-stm1 |
| KSC9    | h manoe-261 leu1-32 ras1::ura4 |
| KSC11   | h manoe-261 ura4-D18 leu1-32 sta1::ura4 / pmnt1::sta1 |
| KSC12   | h manoe-261 ura4-D18 leu1-32 sta1::ura4 / pmnt1::sta1U1PA |
| KSC13   | h manoe-261 ura4-D18 leu1-32 sta1::ura4 / pmnt1::sta1UKPA |
| KSC14   | h manoe-261 ura4-D18 leu1-32 sta1::ura4 / pmnt1::sta1U1PAKPA |
| KSC15   | h manoe-261 ura4-D18 leu1-32 sta1::ura4 / pmnt1::sta1UKPAKPA |
| KSC16   | h manoe-261 ura4-D18 leu1-32 sta1::ura4 / pmnt1::sta1UKPA |

h manoe-261 adenine, uracil, and/or leucine as described previously (35, 36). For detection of dead cells, phloxine B (Sigma) was added to the YES and EMM plates at a concentration of 20 mg/liter (37). Prior to analyzing the effects of nitrogen starvation on transcript levels of the genes of S. pombe, cells were grown in EMM overnight and resuspended at 10^7 cells/mL in EMM containing different nitrogen sources (0.5% NH4Cl, 0.5% proline, or no NH4Cl). For flow cytometry, cells were grown in EMM to log phase, harvested, washed, and then transferred to EMM devoid of a nitrogen source. Transformation of S. pombe cells was carried out using the lithium acetate method and standard yeast genetic techniques as described by Moreno et al. (38). Escherichia coli DH5α was used for subcloning.

**Isolation of the stm1 Gene by Functional Complementation of a Synthetic Lethal Mutant of the ras1 Gene**—To isolate a gene that may function in association with Ras1 in sexual differentiation of S. pombe, we used a mutant, KSC3, that exhibits a lethal phenotype when the ras1 gene under the nmt1 promoter is deleted. This synthetic lethal mutant of Ras1 was isolated by UV mutagenesis from the strain KSC1, which contains the ras1 gene under the nmt1 promoter regulated by thiamine (nmt1::ras1− ura4−) (35, 36). Approximately 2.5 × 10^5 cells on EMM plates were treated with UV (dose: 254 nm, 115 V, 60 Hz, 0.16 A), which empirically is known to generate on average one point mutation/chromosome and leads to 20% survival of S. pombe cells. The mutated cells were incubated at 30 °C for 6 days. The resulting 5 × 10^4 colonies that survived were patched onto EMM plates and then replica- plated on EMM plates containing phloxine B (20 μg/ml) with or without thiamine. KSC3 was selected from 50 candidate colonies that grew on EMM plates but did not grow on EMM containing thiamine. This mutant grew normally only when the ras1 gene under the nmt1 promoter was expressed in the absence of thiamine.

To clone a gene complementing this lethal phenotype, the mutant KSC3 strain was cultured in EMM in the absence of thiamine and was transformed with the genomic library constructed by Chung et al. (36). The transformed cells were plated on EMM plates and incubated at 30 °C for 4 days. The transformants grown in EMM were replica-plated onto EMM containing phloxine B and thiamine. Approximately 1 × 10^6 transformants were screened, and 18 independent colonies that grew on EMM containing thiamine where the Ras1 function was turned off, were selected. DNAs were recovered from these transformants, and the insert DNAs were subcloned and tested for complementation of the mutant phenotype of KSC3. The shortest insert DNA showing complementation was used for Northern blot and sequencing analysis.

**Northern Blot Analysis**—Total RNAs were prepared from S. pombe cells grown to log phase in different growth media as reported previously (20) and resolved on a 1% agarose gel containing 2.2 M formalde-
The 1.06-kb fragment of the G protein-coupled receptor for nitrogen starvation signal (stm1) gene was amplified from genomic DNA with the primers 5'-CCGGATCCGATTTTTAATGGA-3' and 5'-CCGGATCCCAACAGCAAACTACTGTGTC-3' (genomic and cDNA sequences are underlined in all primers). The 1.06-kb fragment of the gpa2 gene was amplified from the cDNA using the primers 5'-CCGGATCCGATTTTTAATGGA-3' and 5'-CCGGATCCCAACAGCAAACTACTGTGTC-3'. These amplified fragments were cloned into the BamHI site of pGBT9 (CLONTECH) or pGAD424 to generate pGBT-Gpa2 and pGTP-Gpa2 or pGAD-Gpa2 and pGAD-Gpa2. The 0.96-kb fragment of the G protein β-subunit gene, gpb1, was amplified from the genomic DNA with the primers 5'-CCGGATCCTTGCAGTATCCTGCTTGCCTC-3' and 5'-CCGGATCCCTGCTTGCAGTATCCTGCTTGCCTC-3' and cloned into the EcoRI and SalI sites of pGBT9 to generate pGBT-Gpb1. The 0.8-kb fragment of the stm1 gene was amplified from genomic DNA with the primers 5'-CCGGATCCTTGCAGTATCCTGCTTGCCTC-3' and 5'-CCGGATCCATGATATTAGACGGAATACT-3' and cloned into the BamHI site of pGAD424 or pGBT9 to produce pGAD-STM1 or pGBT-STM1, respectively. The expression of GST and other proteins of E. coli cell extract expressing GST-Stm1 fusion protein in vitro binding experiments was selected.

In vitro binding assay—To investigate whether Stm1 binds to Gpa2 directly in vitro, GST or maltose-binding protein (MBP) fusion proteins were prepared by cloning stm1 into an E. coli expression vector, pGEX-3X, encoding isopropyl-β-thiogalactosidase-inducible glutathione S-transferase (GST) and by cloning gpa2 into pMAL-c1, containing the MBF gene. The coding region DNA or cDNA was amplified by PCR under denaturing conditions of 95 °C for 1 min, annealing at 55 °C for 1 min, and poly-merizing at 72 °C for 2 min. The resulting DNAs were fused at the 3′-end of either the GST or MAL gene. The clones expressing proteins of the correct size were selected and used for in vitro binding experiments. Expression of GST-Stm1 or MBP-Gpa2 fusion proteins was induced in the presence of 0.5 mM isopropyl-β-thiogalactosidase at 25 °C as described previously (46).

In vitro binding of GST-Stm1 to MBP-Gpa2 was performed as follows. Harvested E. coli cells expressing GST-Stm1 or MBP-Gpa2 were suspended in NETN buffer (20 mM Tris-HCl, pH 8.0, 5 mM Na2EDTA, 100 mM NaCl, and 0.5% Nonidet P-40) containing 5 mM phenylmethylsulfonyl fluoride, sonicated, and centrifuged as described previously. The supernatant was treated with 2% Triton X-100 and 1.5% Sarcosyl for 10 min (41). The supernatant was centrifuged again at 100,000 × g for 3 min (41). The supernatant was used as a cytosolic fraction. The pellet fraction was treated with 1.06 M NaCl, 1 mM dithiothreitol, 2 mM MgCl2, 1 mM phenylmethylsulfonyl fluoride, and 1 mg/ml each of aprotinin, leupeptin, and pepstatin). The cells were broken by a bead beater and centrifuged at 5,000 × g for 3 min (41). The supernatant was centrifuged again at 100,000 × g for 10 min. The clarified supernatant was used as a membrane fraction. Proteins (40 µg from each fraction) were resolved on 12% SDS-polyacrylamide electrophoresis gels (PAGE), and electrotransferred to a nitrocellulose membrane (Schleicher & Schull). The proteins on the membrane were treated first with HA. The cells containing the plasmid pREP1 vector (45). The resulting plasmid, pREP1-stm1-1-GFP, were grown in minimal medium containing 2 mM thiamine, and Stm1-GFP fusion protein was detected by immunofluorescent microscopy.

Disruption and overexpression of the STM1 gene—The STM1 gene was disrupted using the one-step gene disruption method (38). The 0.9-kb HindIII-HpaI fragment containing the entire stm1 coding region of genomic clone pWH031 (Fig. 2A) was replaced with the ura4 gene. The resulting plasmid was treated with XbaI and HindIII, and the 3.2-kb XbaI-HindIII fragment so obtained was isolated and then used to replace one chromosomal copy of the STM1 gene in a diploid strain, SP286. After obtaining stable Ura4 transformants, genomic DNA was prepared from each transcript (38), and these were analyzed by Southern blotting with the probes of STM1 and URA4. The diploid strain containing one wild-type and one disrupted copy of STM1 was selected. After transformation of the resulting tetrad analysis, the pGBT-Stm1 and pGAD424 fusion proteins were mixed with amylase-agarose beads, and the beads were then washed with NETN buffer. The purified MBP-Gpa2 on agarose beads was incubated with crude E. coli cell extract expressing GST-Stm1 fusion protein in NETN buffer for 1 h. After washing the agarose beads with NETN buffer, the proteins on the beads were subjected to electrophoresis on a 12% SDS-polyacrylamide gel. The proteins on the gel were transferred to a nitrocellulose membrane. The membrane was analyzed by Western blotting with anti-GST (α-GST) and anti-MBP (α-MBP) antibodies.

Construction of mutant alleles of Gpa2 and stm1—To generate a mutation in the GTase domain of the gpa2 gene (gpa2ΔGTTился), we used a double-stranded site-directed mutagenesis kit (Strategene) and followed the protocol described by the supplier. The sequences of the oligonucleotides used for construction of the R176H mutation in gpa2 were 5′-ACCCAGATCTACTTTGTTGACGGAGAAGTTC-3′. The changed sequences are underlined, and this caused the elimination of 1 XbaI recognition site at amino acid position 176. After confirming the successful construction of the mutation in gpa2 by sequence analysis, we replaced the mutant allele in the chromosome by the method of Moreno et al. (38). A PUC19-based plasmid that contained the 0.85-kb EcoRI-Stm1 fragment carrying the 5′-upstream element of gpa2, a 1.1-kb BamHI fragment of gpa2 coding region carrying the mutant allele, a 1.8-kb ura4+ cassette with blunt ends, and an 0.6-kb SalI-HindIII fragment carrying the immediate 3′-downstream elements to the gpa2 open reading frame, was digested with EcoRI and HindIII. The 1.4-kb fragment that spans the entire gpa2 region with the mutant and ura4+ gene was replaced with the chromosomal copy of wild type gpa2, using the homologous recombination event and by PCR. The oligonucleotides used for generating mutations at amino acid residues 197, 199, or both in stm1 were 5′-CGTATTTCAAGCGCATCAAAGCCCAAACTAGTGTCC-3′, 5′-ATTATCCTCAAAACTGGAGAATCAGCA-3′, and 5′-CGTATTTCAAGCGCATAAAGTGGAA-CC-3′. These oligonucleotides were used for site-directed mutagenesis, and the DNA fragments were amplified with the 5′-GTTTTGTTGACGGAGAAGTTC-3′ primer and pGEX-3X. The 0.96-kb fragment of the STM1 gene was amplified from genomic DNA with the primers 5′-CCGGATCCGATTTTTAATGGA-3′ and 5′-CCGGATCCCAACAGCAAACTACTGTGTC-3′.
It grew normally when the ras1 gene was harvested on glass filters by rapid filtration. The cell samples were transferred into fresh thiamine-free EMM and incubated at 30 °C. pombe, of which the nucleotide sequences, confirmed by sequence analysis, caused changes in the expression of a gene under the thiamine-regulated nmt1 promoter; KSC3, a mutant derived from KSC1; KSC3+pWH031, KSC3 that contain a complementary genomic clone, pWH031. -Thi and +Thi represent cells grown in the absence or presence, respectively, of thiamine in EMM.

Assay of Intracellular cAMP Levels—The method described by Mochizuki and Yamamoto (47) was used to measure cAMP levels in the cells. Cells carrying pnt1-stm1 were grown first at 30 °C in EMM containing thiamine to the mid-log phase. Then the cells were transferred to thiamine-free EMM and grown for 12 h. 4 × 10^6 cells/ml were transferred into fresh thiamine-free EMM and incubated at 30 °C. Aliquots of these cells were taken every 3 or 6 h or after 18 h and harvested on glass filters by rapid filtration. The cell samples were immediately soaked in acidic ethanol (0.01 N HCl in ethanol) and extracted using a bead beater. The debris was removed by centrifugation at 12,000 × g for 3 min, and the amount of cAMP was determined using a cAMP assay kit according to the supplier’s protocol (Amersham Pharmacia Biotech TRK329). The amount of protein was measured using the dyes-binding method (48).

Nucleotide Sequence Deposition—The nucleotide sequence of the stm1 gene was submitted in 1995 and has been assigned GenBank™ accession number L49134.

RESULTS

Isolation of the stm1+ Gene, Which Suppressed a Synthetic Lethal Mutant of ras1—In an attempt to identify those genes that function in association with Ras1 in terms of delivering cell surface signals into the cell interior for the differentiation of S. pombe, we looked for novel genes that might function in linking poor nutritional status and pheromone signals with the cell surface signals into the cell interior for the differentiation that function in association with Ras1 in terms of delivering nutrient signals.

When we used this mutant to screen the genes that complement the lethal mutant phenotype of the ras1 gene under the thiamine-regulated nmt1 promoter, we isolated one such clone that suppresses a synthetic lethal mutant ofras1, which we designated as the STM1 gene.

Transcription of stm1 Is Induced by Nitrogen Starvation—Northern analysis of total RNAs probed with the coding region DNA of stm1 showed a 1.3-kb transcript. The transcript levels in the cells varied depending upon the medium in which the S. pombe cells were grown. As shown in Fig. 3A, the amount of stm1 transcripts in the cells grown in YEPD-rich medium was relatively low (lane 1). In the cells grown in EMM containing ammonium as a nitrogen source, transcription of stm1 increased (lane 2). This increment became higher in the cells grown in EMM containing proline, a poor nitrogen source (lane 3). Scanning and normalization of the stm1 transcript bands against a ribosomal protein gene (rp) transcript in each lane showed that transcription of stm1 in ammonium (EMM/NH₄Cl) or in proline (EMM/proline) was 5.7 or 13.3 times higher than that in EYPD, respectively. To test whether transcription of stm1 was regulated by depletion of the nitrogen source, the cells were grown first in EMM containing 0.5% ammonium chloride and then transferred to EMM free of ammonium chloride. Within 12 h, the transcript of stm1 increased markedly (Fig. 3B, lane 2), and levels remained high for at least 24 h (lane 3). In contrast to depletion of the nitrogen source, lowering of glucose concentration from 2% in EMM to 0.2% (starvation) did not elicit a change in the levels of the stm1 transcript (data not shown). These results indicate that transcription of the stm1 gene is induced during this nutritionally deficient physiological stage at a time when overall transcription declines dramatically (49, 50). Nitrogen-deficient signaling may be the major regulator for this induction.

Western analysis of the HA epitope-tagged Stam1, which was produced from the plasmid pnt1-stm1-HA3 in S. pombe cells, showed that Stam1 is present mainly in the membrane-enriched fraction rather than in the cytoplasmic fraction (Fig. 3C, lane 6). An in situ localization experiment in which Stm1 fused with jellyfish GFP was used revealed that Stam1 is likely to be associated with the plasma membrane (Fig. 3D), suggesting that Stam1 may function as a membrane protein.

Disruption of stm1 Facilitates G1 Arrest under Nitrogen-deficient Conditions—To explore Stam1 function, the stm1+ gene was disrupted by a one-step gene disruption method using the ura4+ gene as a marker gene (Fig. 4A). The entire coding region DNA of stm1, including a 100-base pair 5′-upstream and a 30-base pair 3′-downstream sequence was replaced with ura4+. A diploid h+/h+ strain that contained one wild type and
one disrupted copy of *stm1* was selected, converted into h+/k*″*, and sporulated. All of the resulting tetrads were viable on YE plates and showed 2:2 segregation on Ura− plates. All Uraspores were wild type. The *stm1*-disrupted haploid cells showed normal vegetative growth in nutritionally sufficient medium (YEPD or EMM+N, Fig. 4D). However, when the *stm1*-disrupted haploid cells were transferred to the nitrogen-depleted EMM (EMM−N, Fig. 4C), they exhibited G1 arrest much earlier than observed in the undispersed wild type 972 cells. Flow cytometric analysis showed that half of the wild type cells progressed to G2 after 4 h transfer to the nitrogen-deficient medium (Fig. 4B, lane 1), whereas most of the *stm1*-deleted cells shifted to G1 phase within 2 h and remained arrested in the G1 phase (Fig. 4B, lane 2). However the rapid G1 arrest shown by the *stm1*-deleted cells under nitrogen-deficient conditions was not observed in the cells deleted in both *stm1* and ras1 (*stm1* −, ras1−) genes (Fig. 4B, lane 4). Instead, the *stm1* and ras1− double-deleted cells showed continued cell cycle progression under nitrogen-deficient condition (Fig. 4B, lane 4, and C, filled circles). We consistently observed that *stm1* − ras1− cells continued to grow at least for 24 h after transfer to nitrogen-deficient EMM (EMM−N, Fig. 4C, filled circles). In EMM containing a sufficient nitrogen source (EMM+N), the *stm1* −, ras1− double-disrupted cells showed a significant decrease in vegetative cell growth (Fig. 4D, filled circles), whereas the cells disrupted only in the *stm1* gene grew like undisrupted wild-type cells (Fig. 4D, open squares). This finding suggests that even though the *stm1* gene itself is not essential for vegetative cell growth in nutritionally sufficient media, when ras1 is not functional, deletion of *stm1* causes defects not only in vegetative cell growth under nutritionally sufficient conditions but also in G1 arrest under nutritionally deficient conditions. It is possible that Ras1 can function cooperatively with Stm1 in regulating vegetative cell growth during the nitrogen deficient period.

**Overexpression of Stm1 Inhibits Vegetative Cell Growth and Lowers Intracellular cAMP Levels**—To investigate how *stm1* gene expression drives *S. pombe* cells to either G1 arrest or the G0 phase under nitrogen-deficient conditions, we examined the effects of *stm1* overexpression. The coding region DNA of *stm1* was fused with the thiamine-regulatable nmt1 promoter (pnt1-1stml). Expression of *stm1* was examined by growing *stm1* − haploid cells containing this plasmid in the absence of thiamine. The cells were first grown in EMM containing thiamine that repressed expression of *stm1*. Then the cells were transferred to thiamine-free EMM, as indicated in Fig. 5A, arrow a, and grown for a further 12 h to remove residual thiamine in the cells. For *stm1* induction, the cells were transferred again to fresh EMM containing no thiamine (Fig. 5A, arrow b), and the levels of the *stm1* transcript, cell growth, and morphology were examined at every 3–6 h thereafter. As shown in Fig. 5B, transcription of *stm1* increased markedly after 6 h of actual thiamine induction and continued to increase. DAPI and Calcofluor staining of wild type haploid cells (ED665) expressing *stm1* showed multisepta after 18 h of thiamine induction (Fig. 5C, panel 2). Vegetative cell growth in nitrogen sufficient EMM (EMM+N) was inhibited as *stm1* induction continued (Fig. 5D, open squares). Many of the cells were lethal after 24 h (Fig. 5E, lower panel). However, this severe growth inhibition and the multisepta-forming effects of Stm1 were not observed in the cells defective in the *gpa2* gene, a heterotrimeric G protein α2 gene known to be responsible for relaying nutritional signal at the initial stage of sexual development in *S. pombe* (19) (Fig. 5C, panel 4, and D, open triangles). This indicates that Gpa2 function is required for Stm1 to be effective in altering cell growth under nutritionally sufficient condition. In a homothallic haploid strain, h90, overexpression of Stm1 caused facilitated sporulation to occur even in a nitrogen-rich medium in which sporulation is not normally observed (Fig. 6A, panel 2). The addition of 2 μM cAMP and 5

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**References:**

1. **Fig. 2. Restriction map and deduced amino acid sequence of the *stm1* gene.** A, the cDNA region is represented by open boxes, and the open reading frame of *stm1* is indicated by a black box. B, comparison of the deduced amino acid sequence of *Stm1* with the functionally uncharacterized genes of *S. cerevisiae*. Identical amino acid sequences are boxed. Putative transmembrane domains I–VII are indicated.
mm caffeine, a treatment that regulates meiotic cell division in S. pombe and inhibits phosphodiesterase activity, respectively (19), suppressed the facilitated conjugation and sporulation phenotype caused by overexpression of Stm1 (Fig. 6A, panel 3).

To examine whether the hyper-sporulation effect of Stm1-overexpression in homothallic cells was due to changes in intracellular cAMP concentrations regulated mainly by the heterotrimeric Gα2 protein Gpa2 in S. pombe, the intracellular cAMP levels were monitored during stm1 induction. As shown in Table II (experiment 1), stm1 heterothallic haploid cells expressing Stm1 showed decreased cAMP levels as stm1 induction increased. Within 3 h after stm1 induction, intracellular cAMP levels did not change much. However after 6 h, when stm1 transcripts increased markedly (Fig. 5B), cAMP levels dropped to less than half of that observed in the uninduced state (2.5 versus 1.0). This value remained around 1.0 as stm1 transcripts continued to increase as thiamine induction progressed (Fig. 5B and Table II, experiment 1). The increase in stm1 transcripts correlates with the decrease in cAMP levels down to a certain level. No further decrease in cAMP was observed despite further increases in the levels of stm1 transcripts. In homothallic cells we observed similar results (Table II, experiment 3, KS2/JY4:1.2 versus 2.5). The values are similar to those shown for a deletion mutant of gpa2, responsible for modulating cAMP levels (Table II, experiment 2, HD3, 1.3).

Deletion of the stm1 gene itself, or the ras1 gene that is required for initiation of sexual differentiation in S. pombe, did not elicit any change in intracellular cAMP levels (Table II, experiment 2, HD1 and HD8, 2.8–2.9). Stm1 overexpression in the gpa2-deleted cells did not cause a further decrease in intracellular cAMP levels in gpa2 cells (1.3 in both HD3 and KS3). To test whether this decrease in cAMP was because of modulation of cAMP-phosphodiesterase (Pde1), which breaks down cAMP, we examined cAMP levels in the pde1 null mutant expressing stm1. As shown in Table II, the pde1 null mutant cells (HD9), which are defective in cAMP hydrolysis, showed cAMP levels four times higher than those observed in pde1+ cells (14 versus 3.3). This value decreased to 6.2 in the pde1 null
Fig. 5. Induction of the stm1 transcript and its effect on growth in nitrogen-rich medium. A, induction of the stm1 transcript. Heterothallic haploid cells containing pnmnt1-stm1 overexpression plasmid (KS1) were first grown in EMM in the presence of thiamine. To induce stm1 expression, the cells were transferred to thiamine-free EMM at a cell density of A_{540} = 0.2 (a). After being grown for 12 h, the cells were again transferred to a fresh EMM containing no thiamine (b). Cell samples were withdrawn every 3 or 6 h. B, Northern blotting of the total RNAs prepared from the cell samples (upper panel). The lower panel shows 18 S and 28 S RNAs in each lane of the same gel stained by ethidium bromide. C, morphology of the cells expressing Stm1. Heterothallic haploid wild type cells (panels 1 and 2) or the gpa2-deleted cells (panels 3 and 4) containing Stm1 overexpression plasmid pnmnt1-stm1 were grown in the presence (thi+) or absence (thi-) of thiamine in EMM. Cell morphology and septa were examined under a fluorescent microscope after 18 h of Stm1 induction. Arrowheads indicate the septa of the cells. D, growth curves of the Stm1-overexpressed cells. The stm1′ gpa2′ wild type cells (squares) or the stm1′ gpa2′ mutant cells (triangles) containing pnmnt1-stm1 were grown for 12 h in thiamine-free EMM and were transferred to either fresh EMM containing thiamine (thi+, filled squares or triangles) or no thiamine (thi–, open squares or triangles). Growth of the cells was monitored. E, rhodamine B (+Ph B) staining of the dead cells after 24 h of stm1 induction in the stm1′ gpa2′ cells. Upper panel, cells grown in the presence thiamine. Lower panel, cells grown in the absence of thiamine.

mutant cells overexpressing Stm1 (KS7). In the cells carrying deletions for both pde1 and gpa2 genes (HD11), cAMP levels were slightly higher than observed in undisrupted gpa2′ pde1′ cells. These results support the hypothesis that not cAMP hydrolysis but cAMP production itself is affected by Stm1 overexpression and also supports earlier findings by Isshiki et al. (19) who showed that a null mutation in gpa2 inhibits cAMP production in pde1-deleted strains. In the strain that has a mutation in the GTPase domain in Gpa2 (gpa2^{R176H}) we observed slightly higher levels of cAMP (HD13, 53). Overexpression of Stm1 in this strain did not alter this value very much (KS9, 49) as was observed in the gpa2 null mutant expressing Stm1 (HD13 and KS9, 13). Additional changes in the intracellular cAMP levels in gpa2 mutants overexpressing Stm1 were not observed. In the absence of functional Gpa2, expressed Stm1 could not affect intracellular cAMP levels (51). These results indicate that proper Gpa2 function is required for Stm1 to be effective in regulating intracellular cAMP and that gpa2^{R176H} is epistatic to stm1^{+} in cAMP regulation.

Stm1 Overexpression Leads to Increased Transcription of Meiosis-specific Genes—The finding that overexpression of Stm1 causes a reduction of intracellular cAMP levels and facilitates sexual development in homothallic cells (Table II, Fig. 6A) suggests that Stm1 might also affect the expression of
lane 7

grown in the presence of exogenous cAMP and caffeine (Fig. 6). These inductions were not observed in the cells of the homothallic haploid cells as control (Fig. 6B, lanes 3, 9).

Therefore, we examined whether transcription of ste11, mei2, or mam2 genes, required for initiation of meiosis (52–55), were induced by Stm1 overexpression. As shown in Fig. 6B, lanes 3 and 6, transcription of a meiosis-specific gene transcription factor, ste11, increased in both the heterothallic and homothallic haploid cells as stm1 transcript levels increased. The mei2 gene was also induced several-fold in the wild type cells expressing Stm1. These inductions were not observed in the cells grown in the presence of exogenous cAMP and caffeine (Fig. 6B, lane 7). Mating factor receptor mam2 expression also increased in a similar manner (Fig. 6B, lane 3). Thus the suppression of hyper-sporulation phenotype of Stm1 overexpression by the addition of cAMP and caffeine (Fig. 6A, panel 3) in growth media correlates with the inhibition of meiosis-specific gene transcription (Fig. 6B, lane 7). In the gpa2-disrupted cells, the increase in stm1 transcripts caused a marked induction of the ste11 transcript and a few-fold induction of the mei2 transcript (Fig. 6B, lanes 8 and 9), whereas intracellular CAMP levels remained less than half that of gpa2+ cells regardless of an increase of the stm1 transcript (Table II, HD3 and KS3). This showed that multiple copies of Stm1 affect transcription of ste11 responsible for the induction of pre-meiotic gene transcription even in the absence of Gpa2 function. It is already known that ste11 is regulated by stress-activated Sty1 protein kinase through Atf1 transcription factor in response to nitrogen limitation (64). The latter is a key signal that promotes sexual development in S. pombe, and we observed whether high expression levels of the ste11 transcript induced by Stm1 was due to a modulation of the sty1-associated pathway. The marked induction of ste11 caused by Stm1 overexpression in the gpa2-disrupted cells was abolished when the sty1 gene was deleted (Fig. 6, lanes 9 and 11). These results suggest that unless a stress-activated pathway gene such as sty1 is active, increased transcription of meiosis-specific genes caused by overexpressing Stm1 is not possible. Sty1 function is required for Stm1 to be effective in the induction of meiosis specific-gene transcription. Thus it is likely that the stress-activated sty1 gene is epistatic to stm1 in meiosis-specific gene transcription. This confirms an earlier finding by Shiozaki and Russell (64) who showed that the stress-activated Wis1-Sty1 protein kinases are upstream regulators of meiosis-specific gene transcription.

**Stm1 Is Likely to be Coupled with Gpa2, Which Is Involved in the Monitoring of Nutrition—Our observation that the stm1 transcript is induced under nitrogen-deprived conditions and that overproduction of Stm1 causes a reduction in internal cAMP levels led us to examine whether Stm1 can function through Gpa2, the mechanism by which intracellular cAMP production in S. pombe is known to be regulated in response to nutritional signals (19, 47). First, we examined whether Stm1 interacts directly with Gpa2 and affects the ability of Gpa2 to modulate intracellular cAMP levels. Using yeast two-hybrid experiments with Stm1 fused to the GAL4 binding domain (GAL4BD) and gpa2 fused to the GAL4 activation domain (GAL4AD), or vice versa, we observed β-galactosidase gene (lacZ) expression with both an X-gal filter assay and a quantitative liquid assay (Fig. 7A). In contrast, when the other Ga protein gene, gpa1, which is responsible for transmitting the pheromone signal for sexual development (18), was fused with GAL4 and co-transformed with the GAL4-stm1 fusion construct, we did not observe any β-galactosidase expression. This

### Table II

**Overexpression of Stm1 lowers the cAMP level**

| Strains | Relevant genotype | Plasmid | cAMP level (pmol/mg of protein) |
|---------|------------------|---------|--------------------------------|
| Exp. 1  |                  |         |                                |
| KS1     | h−               | prm1t-stm1 | 2.5 ± 0.3                     |
|         | 0 h−             | prm1t-stm1 | 2.2 ± 0.2                     |
|         | 3 h−             |prm1t-stm1 | 1.0 ± 0.2                     |
|         | 6 h−             |prm1t-stm1 | 1.0 ± 0.2                     |
|         | 12 h−            |prm1t-stm1 | 1.2 ± 0.2                     |
|         | 18 h−            |prm1t-stm1 | 0.80 ± 0.1                    |
|         | 24 h−            |prm1t-stm1 |                                |
| Exp. 2  |                  |         |                                |
| ED665   | h− sta1+ gpa2+ pde1− ras1+ | prm1t1  | 3.3 ± 0.2                     |
|         | h0 sta1+ gpa2+ pde1+ ras1+ | prm1t1  | 2.7 ± 0.2                     |
| HD1     | h− Δstm1         | prm1t1  | 2.8 ± 0.2                     |
| HD3     | h− Gpa2          | 1.3 ± 0.1 |
| HD8     | h− Δras1         | 2.5 ± 0.3 |
| HD9     | h− Pde1          | 14.0 ± 1.5 |
| HD11    | h− Gpa2 Δpde1    | 3.7 ± 0.2 |
| HD13    | h− gpa2R176H    | 5.3 ± 0.3 |
| Exp. 3  |                  |         |                                |
| ED665   | h− gpa2− pde1− ras1− | prm1t1  | 3.1 ± 0.2                     |
| JY4     | h0 gpa2− pde1+ ras1+ | prm1t1  | 2.5 ± 0.1                     |
| KS1     | h− gpa2− pde1− ras1− | prm1t1  | 1.4 ± 0.1                     |
| KS2     | h0 gpa2− pde1+ ras1+ | prm1t1  | 1.2 ± 0.2                     |
| KS3     | h− Gpa2          | 1.3 ± 0.1 |
| KS5     | h− Δras1         | 1.2 ± 0.2 |
| KS7     | h− Pde1          | 6.2 ± 0.4 |
| KS9     | h− gpa2R176H    | 4.9 ± 0.1 |
| Exp. 4  |                  |         |                                |
| ED665   | h− sta1+        | prm1t1  | 3.1 ± 0.2                     |
| HD1     | h− Δstm1         | prm1t1  | 2.8 ± 0.2                     |
| KS11    | h− Δstm1         | prm1t1  | 1.2 ± 0.2                     |
| KS12    | h− Δstm1         | prm1t1  | 1.4 ± 0.1                     |
| KS13    | h− Δstm1         | prm1t1  | 2.4 ± 0.1                     |
| KS14    | h− Δstm1         | prm1t1  | 2.5 ± 0.2                     |

*Induction time.

prm1t (2), inducible overexpression plasmid under the nmt1 or nmt2 promoter carrying LEU2 or ura4 selective marker, respectively.

Notes:
- Table II shows the effects of Stm1 overexpression on cAMP levels in different strains and plasmids.
- The cAMP levels were measured at short intervals after induction.
- Two samples were assayed, and the results were consistent in separate experiments.
FIG. 7. Interaction of Stm1 protein with the heterotrimeric G proteins. A, yeast two-hybrid interactions between Gα subunit proteins and Stm1. Stm1 fused to the GAL4 binding domain plasmid pGBT9 or the activation domain plasmid pGAD424 was co-transformed with either gpa1 or gpa2 fused to the GAL4 activation or binding domain plasmid. The degree of interaction between the two proteins was assessed by the amount of lacZ expression on the X-gal indicator filter and by a quantitative β-galactosidase liquid assay. The co-transformed cells were either patched on minimal plates or grown in 0.67% yeast nitrogen base, 2% glucose minimal medium to a cell density of A600 = 0.6–0.8. B, domains of Stm1 protein responsible for the interaction with Gpa2. Each fraction of stm1(D, C, and N) was amplified by PCR with the appropriate primers, cloned into the GAL4 binding domain plasmid pGBT9, and used to transform S. cerevisiae with full-length gpa2 fused at the GAL4 activation domain. Activation of β-galactosidase was examined using the X-gal filter assay. F indicates a full-length Stm1. C, in vitro binding of each fraction of Stm1 with Gpa2. Each fraction of stm1 shown in B was cloned into the GST fusion vector pGEX-3X, and the entire coding region DNA of gpa2 was fused to MBP. C-a, upper panel, E. coli crude cell extracts containing the expressed GST-Stm1 fusion proteins were analyzed by SDS-PAGE and stained with Coomassie Blue. Lower panel, the same gels as in the upper panel were blotted with anti-GST antibody to confirm whether each domain of Stm1 is expressed in E. coli. C-b, upper panel, the E. coli cell extracts shown in C-a were incubated with MBP (lane 2) or MBP-Gpa2 fusion protein (lanes 3–14) and purified on amylose-agarose beads in NETN buffer. The proteins on the beads were subjected to electrophoresis on a 12% SDS-polyacrylamide gel and then blotted onto a nitrocellulose membrane and analyzed with anti-GST (α-GST). Lane 1 shows the full-length GST-Stm1 itself expressed in the E. coli cell extract analyzed with anti-GST (α-GST). Lower panel, the same gels as in the upper panel were blotted with anti-MBP (α-MBP) antibodies. D, consensus sequences required for interaction with G proteins from several G protein-coupled receptors (58). STE2 and STE3 are the pheromone binding receptors of S. cerevisiae, and Mam2 and Map3 are the pheromone binding receptors of S. pombe. GPR1 is the recently cloned GPA2 associated protein found in S. cerevisiae (62). Stm1 possesses the consensus sequences between residues 197 and 202 in the D3 domain of the third cytoplasmic loop (indicated as bold letters in the text). E, predicted topology of Stm1 in the membrane. The putative Gpa2 binding domain including the third cytoplasmic domain is indicated. The arrows indicate the amino acid residues changed by site-directed mutagenesis.
showed that Stm1 could interact with Gpa2 but not with Gpa1. Meanwhile, when the mam2 gene encoding a receptor for pheromone M factor was fused with GAL4 and co-transformed with GAL4-gpa1, activation of β-galactosidase expression was observed. However when GAL4-mam2 was co-transformed with GAL4-gpa2, it did not activate β-galactosidase expression (Fig. 7A). Therefore, the Mam2 receptor, specific for the pheromone signal, interacts only with the pheromone-responsive Go subunit, Gpa1, and not with the nutrient responsive Go subunit, Gpa2. The Gβ subunit gene, gpb1, which we had identified previously (20), induced β-galactosidase expression both with stm1 and mam2 (data not shown). These results suggest that Stm1 has the specific ability to interact directly with Gpa2 in S. pombe cells and also with the Gβ subunit, Gpb1. For this reason we examined which domains of Stm1 were directly involved in these interactions. Different regions of stm1, encoding the amino acids of the corresponding domains (Fig. 7B, D1–D4) and 3′- or 5′-deleted stm1 (Fig. 7B, ΔC and ΔN) were cloned separately into the Gal4 binding domain. These fusion constructs were used for yeast two-hybrid experiments with the entire gpa2 coding sequences fused to the Gal4 activation domain. As shown in Fig. 7B, the fragments D3 and D4 of Stm1, which contained amino acids 175–228 and 229–271, respectively, showed activation of lacZ expression. Whereas deletion of the C-terminal 111 amino acid sequences, including the D3 and D4 regions, abolished lacZ expression (Fig. 7B, ΔC2), deletion of the N-terminal 142 amino acids did not affect the expression of the lacZ gene (Fig. 7B, ΔN3).

The C-terminal Domain of Stm1 Is Required for the Possible Interaction with Gpa2—To confirm the binding ability of Stm1 to Gpa2, in vitro binding between Stm1 and Gpa2 was tested using the GST and MBP fusion proteins produced by E. coli. The full-length stm1 or its different regions, as shown in Fig. 7B, were fused to the 3′-end of the glutathione S-transferase gene (GST-stm1) and were expressed in E. coli. The amount of each domain protein of Stm1 expressed was verified by SDS-PAGE (Fig. 7C-a, upper panel) and by Western blotting with anti-GST antibody (Fig. 7C-a, lower panel). The entire coding region DNA of the gpa2 gene fused to the maltose-binding protein gene (MBP-gpa2) was expressed in E. coli and purified on agarose beads. The purified MBP-Gpa2 (Fig. 7C-b, lower panel) was mixed with E. coli cell extracts expressing each domain protein as a GST-Stm1 fusion protein as shown in Fig. 7C-a. The proteins associated with MBP-Gpa2 were analyzed by SDS-PAGE and by Western blotting (Fig. 7C-b, upper panel). As found in the yeast two-hybrid experiments, the full-length Stm1 showed direct binding to Gpa2 (Fig. 7C-b, lanes 3 and 9). When E. coli cell extracts expressing different regions of Stm1 as GST fusion proteins were mixed with the purified MBP-Gpa2, the proteins that were deleted in the N-terminal half were found not to affect the ability of Stm1 to bind Gpa2 (Fig. 7C-b, lanes 12–14). However, deletion of a portion of the C-terminal region, domains 3 and 4 (ΔC2) abolished in vitro binding ability with Gpa2 (Fig. 7C-b, lane 11). GST fusion containing these D3 or D4 regional sequences alone was enough for binding to Gpa2 (Fig. 7C-b, lanes 6 and 7). The other regions did not show this binding ability with Gpa2 (lanes 4 and 5). The region between the amino acid sequences 175 and 271 (D3+D4) seems to be necessary for the interaction with Gpa2. This region contains the third cytoplasmic loop (Fig. 7E), known to be required for coupling with G proteins in other well characterized G protein-coupled receptors (56, 57). The amino acid sequences KHNKIQ conserved in several G protein-coupled receptors, e.g. STE2, STE3, and GPR1 of S. cerevisiae and Mam2 and Map5 of S. pombe (58), are present in the third cytoplasmic loop region between amino acids 193 and 207 (Fig. 7, D and E). To test whether these conserved sequences are actually involved in interaction with Gpa2 and might affect some of Gpa2 function, one or both of the conserved sequences at amino acid 197 (Ile) and 199 (Lys) were changed to alanine by site-directed mutagenesis as described under “Experimental Procedures.” The mutated stm1 was overexpressed in the absence of the chromosomal copy of stm1*, and the degree to which intracellular cAMP levels decreased with the overexpression of Stm1 was taken as a measure of the interaction between Stm1 and Gpa2. As shown in Table II, experiment 4, overexpression of the mutated Stm1 at the position 197 (HD1/pnmt1-stm1I197A) caused a reduction of cAMP levels to less than half of that observed in the uninduced state (1.4 versus 2.8–3.1). These levels were the same as those observed during overexpression of the un-mutated Stm1 (1.2, HD1/pnmt1-stm1). However overexpression of Stm1 mutated at the position 199 (HD1/pnmt1-stm1K199A) alone or at both positions 197 and 199 (HD1/pnmt1-stm1I197A.K199A) did not show a decrease in cAMP levels (2.4–2.5). This indicates that among the sequences at the putative Gpa2 binding site, at the third cytoplasmic loop, lysine at position 199 in Stm1 is critical for Gpa2 function in regulating cAMP production. In addition, as shown earlier with the gpa2 null mutant or the GTPase mutant gpa2R176H overexpressing Stm1 (Fig. 5C and Table II, KS9), the functional Gpa2 is also required if Stm1 is to be effective in modulating intracellular cAMP levels. Without functional Gpa2, Stm1 could not affect intracellular cAMP levels and/or inhibit cell growth.

**DISCUSSION**

Nutrient starvation in the fission yeast *S. pombe* leads to a number of physiological changes that accompany entry into the stationary phase or G1 (59–61). The cells in the stationary phase become differentiated in a way that allows the maintenance of viability for extended periods without added nutrients but enabling the yeast to retain the ability to resume growth promptly when appropriate nutrients become available (61). When mating partners are present the yeast undergoes sexual development. The decision whether to stay in the active growth cycle or to enter into the differentiation cycle is made by the coordinated interactions of complex molecules. For proper sexual differentiation, i.e., conjugation followed by meiosis and sporulation, the concerted action of a nutritional starvation signal, which brings *S. pombe* cells to arrest at G1, and a pheromone signal, which activates heterotrimeric G proteins and modulates MAP kinases via Ras1, are required. Integrated signals from the cell surface are required to arrest the ongoing cell cycle and facilitate entry into the differentiation cycle. Ras1 plays a critical role in this process by activating the MAP kinase pathway genes when the pheromone signal is accepted by a heterotrimeric G protein. Several elements are known to function together in association with Ras1, but before Ras1 can play its full part in sexual development, *S. pombe* cells must be in the physiological prerequisite state. This state can be initiated by nutritional starvation. The Ras1 function in activating the MAP kinase pathway genes such as byr2* and byr1* in response to pheromone is reasonably well characterized (12, 27, 30). In contrast, little is known about how the nutritional starvation signal is relayed to the downstream elements to execute the differentiation of *S. pombe*. In particular, the sensor capable of detecting nutritional deficiency and causing *S. pombe* cells to enter the mitotic cycle has yet to be identified. Moreover, the connection between nutritional deprivation and sexual development is poorly understood. The novel gene, *stm1**,* isolated in this study as a multicopy suppressor of a synthetic lethal mutant of ras1, encodes a molecule that may function as a sensor of changes in nutritional conditions and may signal the regulation of cell growth and/or differentiation.
The finding that the deletion of the *stm1* gene itself was not detrimental to cell growth under nutritionally rich conditions but caused facilitated entry into G1 arrest under nitrogen-starved conditions supports the notion that Stm1 is involved in regulating cell cycle progression under nitrogen-depleted conditions. Delayed entry into the G1 arrest under nitrogen-depleted conditions in the *stm1* and ras1 double-deleted mutant suggests that ras1 \(^\ast\) function is required for proper *stm1* function in regulating the ongoing cell cycle at the initial stages of nitrogen starvation.

The following results support the hypothesis that Stm1 functions through the heterotrimeric G protein-coupled signaling pathway: (i) the seven-transmembrane domain structure of Stm1; (ii) the requirement for the C-terminal third cytoplasmic loop that contains conserved sequences shown in several G protein-coupled receptors for interaction with Gpa2; and (iii) no change in intracellular cAMP levels by overexpression of Stm1 in a ligand-receptor-specific manner, changing its structure and activating some nitrogenous metabolites may bind to Stm1 in a ligand-specific pathway but also stress-activated pathways to synergistically activate the required gene functions for the initial stage of differentiation before the pheromone signal plays its part. The manner in which Stm1 senses intracellular nitrogen levels remains unknown, but there is a possibility that Stm1 senses this external nutritionally deficient status as a stress signal, modulates Gpa2, and at the same time activates a stress-activated pathway. The balanced function between the Gpa2-associated and Sty1-associated pathways may be critical in sensing the nutritional signals of cell and functioning accordingly.

A novel G protein-coupled receptor, Stm1, that we characterized in this study may function as a pivotal molecule, acting upstream of Ras1, which may allow cross-talk between nutritional starvation and pheromone signals. The degree of cross-talk between the two signals may determine the fate of *S. pombe* cells as to whether to remain in the mitotic cell cycle or to undergo the pheromone-dependent Ras1-mediated meiotic differentiation cycle under nutritionally unfavorable conditions.

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Isolation of a Novel Gene from *Schizosaccharomyces pombe*: stm1+ Encoding a Seven-transmembrane Loop Protein That May Couple with the Heterotrimeric Gα2 Protein, Gpa2

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Isolation of a novel gene from Schizosaccharomyces pombe: stm1+, encoding a seven-transmembrane loop protein that may couple with the heterotrimeric Ga2 protein, Gpa2.

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Page 40198: Fig. 7B was printed incorrectly. The correct figure is shown following.
**Fig. 7. Interaction of Stm1 protein with the heterotrimeric G proteins.** A, yeast two-hybrid interactions between Gα subunit proteins and Stm1. Stm1 fused to the GAL4 binding domain plasmid pGBT9 or the activation domain plasmid pGAD424 was co-transformed with either gpa1 or gpa2 fused to the GAL4 activation or binding domain plasmid. The degree of interaction between the two proteins was assessed by the amount of lacZ expression on the X-gal indicator filter and by a quantitative β-galactosidase liquid assay. The co-transformed cells were either patched on minimal plates or grown in YNB minimal medium to a cell density of A600 = 0.6–0.8. β-Galactosidase activity in the cells was measured with permeabilized cells as described by Miller (69).

B, domains of Stm1 protein responsible for the interaction with Gpa2. Each fraction of stm1(D, ΔC, and ΔN) was amplified by PCR with the appropriate primers, cloned into the GAL4 binding domain plasmid pGBT9, and used to transform S. cerevisiae with full-length gpa2 fused at the GAL4 activation domain. Activation of β-galactosidase was examined using the X-gal filter assay. F indicates a full-length Stm1. C, in vitro binding of each fraction of Stm1 with Gpa2. Each fraction of stm1 shown in B was cloned into the GST fusion vector pGEX-3X, and the entire coding region DNA of gpa2 was fused to MBP. Upper panel, E. coli crude cell extracts containing the expressed GST-Stm1 fusion proteins were analyzed by SDS-PAGE and stained with Coomassie Blue. Lower panel, the same gels as in the upper panel were analyzed by Western blotting with GST antibody to confirm whether each domain of Stm1 is expressed in E. coli. Lower panel, the same gels as in the upper panel were analyzed by Western blotting with MBP antibody to confirm whether each domain of Stm1 is expressed in E. coli. C-b, Western blotting analysis of the proteins binding to MBP-Gpa2. Upper panel, the E. coli cell extracts shown in C-a were incubated with MBP (lane 2) or MBP-Gpa2 fusion protein (lanes 3–14) and purified on amylose-agarose beads in NETN buffer. The proteins on the beads were subjected to electrophoresis on a 12% SDS-polyacrylamide gel and then blotted onto a nitrocellulose membrane and analyzed with anti-GST (α-GST). Lane 1 shows the full-length GST-Stm1 itself expressed in the E. coli cell extract analyzed with anti-GST (α-GST). Lower panel, the same gels as in the upper panel were blotted with anti-MBP (αMBP) antibodies. D, consensus sequences required for interaction with G proteins from several G protein-coupled receptors (58). STE2 and STE3 are the pheromone binding receptors of S. cerevisiae, and Mam2 and Map3 are the pheromone binding receptors of S. pombe. GPR1 is the recently cloned GPA2 associated protein found in S. cerevisiae (62). Stm1 possesses the consensus sequences between residues 197 and 202 in the D3 domain of the third cytoplasmic loop. E, predicted topology of Stm1 in the membrane. The putative Gpa2 binding domain including the third cytoplasmic domain is indicated. The arrows indicate the amino acid residues changed by site-directed mutagenesis.