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Three α-Subunits of Heterotrimeric G Proteins and an Adenylyl Cyclase Have Distinct Roles in Fruiting Body Development in the Homothallic Fungus *Sordaria macrospora*

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ABSTRACT

Sordaria macrospora, a self-fertile filamentous ascomycete, carries genes encoding three different α-subunits of heterotrimeric G proteins (*gsa*, G protein *Sordaria* alpha subunit). We generated knockout strains for all three *gsa* genes (Δgsa1, Δgsa2, and Δgsa3) as well as all combinations of double mutants. Phenotypic analysis of single and double mutants showed that the genes for Gα-subunits have distinct roles in the sexual life cycle. While single mutants show some reduction of fertility, double mutants Δgsa1Δgsa2 and Δgsa1Δgsa3 are completely sterile. To test whether the pheromone receptors PRE1 and PRE2 mediate signaling via distinct Gα-subunits, two recently generated Δpre strains were crossed with all Δgsa strains. Analyses of the corresponding double mutants revealed that compared to GSA2, GSA1 is a more predominant regulator of a signal transduction cascade downstream of the pheromone receptors and that GSA3 is involved in another signaling pathway that also contributes to fruiting body development and fertility. We further isolated the gene encoding adenylyl cyclase (AC) (*sac1*) for construction of a knockout strain. Analyses of the three ΔgsaΔsac1 double mutants and one Δgsa2Δgsa3Δsac1 triple mutant indicate that SAC1 acts downstream of GSA3, parallel to a GSA1–GSA2-mediated signaling pathway. In addition, the function of STE12 and PRO41, two presumptive signaling components, was investigated in diverse double mutants lacking those developmental genes in combination with the *gsa* genes. This analysis was further completed by expression studies of the *ste12* and *pro41* transcripts in wild-type and mutant strains. From the sum of all our data, we propose a model for how different Gα-subunits interact with pheromone receptors, adenylyl cyclase, and STE12 and thus cooperatively regulate sexual development in *S. macrospora*.

In eukaryotes, heterotrimeric GTP-binding proteins consisting of α-, β-, and γ-subunits interact with activated heptahelical transmembrane receptors (G protein-coupled receptors, GPCRs) and transduce various environmental signals to stimulate morphogenesis and cellular response. Upon activation by an extracellular signal, the receptor promotes the exchange of GDP for GTP on the Gα-subunit of the heterotrimeric G protein. This in turn leads to the dissociation of Gα from the βγ-complex and each complex can bind and regulate effectors that then can propagate signals into the cell (Hamm 1998; Lengeler *et al.* 2000). During evolution, G protein subunit genes have expanded enormously in number and diversity. The most complex situation is found in the genome of humans where 27 different genes encoding for Gα-subunits are found (Albert and Robillard 2002). On the basis of sequence similarity, the mammalian Gα-subunits have been divided into four families: (1) G_s activates adenylyl cyclase (AC), (2) G_i inhibits adenylyl cyclase, (3) G_q activates phospholipase C (PLC), and (4) Gα-subunits currently having an unknown function (Hamm 1998). In the genome of the yeast *Saccharomyces cerevisiae*, only two genes for Gα-subunits (*GPA1* and *GPA2*) have been detected and these are known to play significant roles in mating and filamentous growth (Kübler *et al.* 1997; Schrick *et al.* 1997). During sexual development of *S. cerevisiae,* two haploid mating types, **a** and α, communicate via pheromones. While **a**-cells express genes for a lipopeptide pheromone (**a**-factor) and the GPCR Ste2p sensing the extracellular α-pheromone, α-cells express genes for a peptide pheromone (α-factor) and the GPCR Ste3p sensing the **a**-factor. In both cell types, Ste2p and Ste3p are coupled to Gpa1p, one of the two Gα-subunits that forms a conventional heterotrimeric G protein with βγ-subunits Ste4p/18p (Dohlman and Thorner 2001). Recent studies by Slessareva *et al.* (2006) revealed a new function for Gpa1p, when they discovered that this Gα-subunit not only is located at the plasma membrane, but

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also is present at the endosomes where it stimulates phosphoinositide 3-kinase (PI3K) to produce PI 3-phosphate. Thus, this Gα-subunit contributes activity to the mating response pathway by signaling external signals to internal cellular compartments. The second Gα-subunit of *S. cerevisiae*, Gpa2p, senses nutrients and controls filamentous growth. This subunit acts upstream of the adenylyl cyclase that generates the second messenger cAMP in response to glucose (Versele *et al.* 2001).

To study the function of different Gα-subunits within a multicellular eukaryote, filamentous fungi are ideal model systems for such experimental investigations. Genomic sequencing has revealed that most filamentous ascomycetes have three Gα-subunits (Liu and Dean 1997; Chang *et al.* 2004; Kays and Borkovich 2004). Among them, Gα-subunits of the fungal group I share specific sequence similarities with the mammalian G_i subunits, while the subunits of group III have been assigned as G_s subunits on the basis of their functionality in stimulating adenylyl cyclases like their mammalian counterparts. The third fungal Gα-subunit placed in group II has no known mammalian counterpart (Bölker 1998). Currently, the best studied example among filamentous fungi is *Neurospora crassa*, where the three Gα-subunits are known to contribute significantly to sexual and vegetative development (Ivey *et al.* 1996; Baasiri *et al.* 1997; Kays *et al.* 2000; Kays and Borkovich 2004). In this heterothallic fungus, disruption of the genes encoding the Gα-subunits has an effect not only on fruiting body development, but also on the fertilization process. In *N. crassa,* fertilization is accomplished by growth of the trichogyne from a protoperithecium toward the male for which either a conidiospore or a somatic cell can act as a male cell. In both cases, the male cell must be derived from a strain of the opposite mating type. In *N. crassa*, this fertilization process is a prerequisite for fruiting body formation and ascospore development (Springer 1993; Davis 2000). Mutations of different components of the signaling pathway affect different steps of the complex fertilization process. For example, if the fusion of the male cell with the trichogyne is impaired as in the Gα-mutant Δgna-1 (Kim and Borkovich 2004), later steps of the sexual developmental pathway are blocked and therefore cannot be analyzed.

We previously established the homothallic ascomycete *Sordaria macrospora* as a model system to investigate sexual development and to determine key players controlling fruiting body differentiation. *S. macrospora* lacks any structures for asexual propagation like conidia, thus no overlapping developmental processes occur. Therefore, *S. macrospora* is an ideal model organism to study sexual differentiation. Moreover, developmental defects are immediately apparent in this self-fertile fungus without the necessity of fertilization (Pöggeler *et al.* 2006a). To date, several *S. macrospora* pro mutants that develop protoperithecia but no perithecia have been generated and characterized, thereby revealing essential components of fruiting body development (Masloff *et al.* 1999; Nowrousian *et al.* 1999, 2007a; Pöggeler and Kück 2004; Kück 2005; Engh *et al.* 2007). In addition, despite the fact that *S. macrospora* completes the sexual cycle without a mating partner, two pheromone-precursor (*ppg1* and *ppg2*) and two pheromone-receptor genes (*pre1* and *pre2*) were shown to be involved in sexual development (Pöggeler and Kück 2000; Mayrhofer and Pöggeler 2005; Mayrhofer *et al.* 2006; Pöggeler *et al.* 2006b). The two receptors show significant amino acid similarities to the pheromone receptors in *S. cerevisiae* (Pöggeler and Kück 2001).

Here, we present a genetic analysis of knockout strains Δgsa1, Δgsa2, and Δgsa3 that correspond to the three genes encoding Gα-subunits that act in different ways on sexual development and growth. We further address the question whether the Gα-subunits interact genetically with adenylyl cyclase, STE12 and PRO41. For this purpose, we generated 18 double mutants and a single triple mutant from the above-described mutant strains. With a total of 27 mutants, we genetically dissected the signaling pathway upstream and downstream of the Gα-subunits. To the best of our knowledge, double mutants carrying a deleted *gsa* gene together with a disrupted adenylyl cyclase, pheromone receptor, and ste12 transcription factor gene are described for the first time for a filamentous fungus. In addition, we analyzed the impact of the G protein signaling network on previously known developmentally regulated genes using expression analysis. The sum of our data led us to propose a model on how Gα-subunits interact differently with upstream or downstream signaling components and how they act on developmental processes.

MATERIALS AND METHODS

Strains, media, and growth conditions: Cloning and propagation of recombinant plasmids were performed in *Escherichia coli* strain XL1Blue MRF′ (Stratagene, La Jolla, CA) under standard culture conditions (Sambrook and Russel 2001). All *S. macrospora* strains were cultivated on cornmeal or CM medium (Esser 1982; Nowrousian *et al.* 1999). For supplementation of the Δsac1 strain with cAMP, 3.4 mm of dibutyryl-cAMP (db-cAMP) (Biolog Life Science Institute, Bremen, Germany) was added to solid cornmeal medium. For RNA extraction, strains were grown for 5 days in synthetic crossing medium as described previously (Nowrousian *et al.* 2005). Growth rates were measured in race tubes as described by Nowrousian and Cebula (2005). Transformation of *S. macrospora* was performed according to Nowrousian *et al.* (1999) with 0.4 g Glucanex 200 G (Novozymes Switzerland AG, Neumatt, Dittingen, Switzerland) for cell wall degradation. Details for all *S. macrospora* strains are given in Table 1.

Identification and DNA sequencing of three genes for G protein α-subunits and of a gene encoding adenylyl cyclase from *S.macrospora*: To isolate the *gsa* genes encoding Gα-

subunits, two different strategies were used. While *gsa1* and *gsa3* were isolated by direct amplification of *S. macrospora* genomic DNA with primers that were designed according to the sequence of the homologous *N. crassa* genes, *gsa2* was identified by screening a *S. macrospora* cosmid library (Pöggeler *et al.* 1997). The heterologous oligonucleotides used for all three genes encoding Gα-subunits were based on the *N. crassa* sequence as previously described (accession nos. U56090.1, AF004846, and AF281862, Nowrousian *et al.* 2004; Pöggeler and Kück 2006). All DNA sequencing was performed by GATC Biotech AG (Konstanz, Germany). Primers were synthesized at MWG Biotech AG (Eversberg, Germany). PCR amplicons of the *gsa1* and *gsa3* open reading frames were obtained with primer pairs gna1-3 and gna1-4 and gna3-1 and gna3-6, respectively (Table 2). Sequences adjacent to the *gsa1* and *gsa3* genes were obtained by inverse PCR. Prior to amplification, genomic DNA from the *S. macrospora* wild type was digested with *Pvu*I (5′ region) or *Nco*I (3′ region) (for *gsa1*) and *Ava*I (for *gsa3* 5′ region). Ligation and further amplification of the flanking regions were performed according to Nowrousian *et al.* (2007b) with the following primer pairs: gsa1-7 and gsa1-8 for *gsa1* 5′, gsa1-9 and gsa1-10 for *gsa1* 3′, and gsa3-7 and gsa3-8 for *gsa3* 5′ (Table 2). Using oligonucleotide primers gna2-1 and gna2-2, a *S. macrospora* indexed cosmid library was screened for *gsa2* (Pöggeler *et al.* 1997). This led to the isolation of cosmid D10 containing the *gsa2* gene of *S. macrospora* and its flanking sequences.

For the isolation of the *sac1* gene encoding the adenylyl cyclase, the *S. macrospora* cosmid library was screened with a *sac1*-specific fragment that was generated by PCR amplification using *S. macrospora* genomic DNA as template and the *N. crassa cr-1* specific oligonucleotides 1091 and 1092 (accession no. D00909.1, Table 2). This resulted in the isolation of cosmid H2, carrying the 3′ end of the coding region of *sac1* that was further sequenced by primer walking and random insertion of pGPS2.1-hph plasmid (Dreyer *et al.* 2007) using the Tn7L and Tn7R sequences of the integrated transposable element. The 5′ end of the *sac1* gene and its 5′ flanking region was amplified from genomic *S. macrospora* DNA with an oligonucleotide (u-cr-1) homologous to the corresponding *N. crassa* sequence and a *S. macrospora*-specific oligonucleotide (sac10s). The resulting 4.2 kb amplicon was cloned in pDrive (Qiagen GmbH, Hilden, Germany) and the recombinant plasmid pDAde was sequenced by means of primer walking. Details for the plasmids used in this study are given in Table 3.

Sequence analysis: DNA and protein sequence data were obtained from the public databases at NCBI (http://www.ncbi.nlm.nih.gov/sites/entrez) or for *N. crassa* sequences, at the Broad Institute (http://www.broad.mit.edu/annotation/genome/neurospora/Home.html). BLAST analysis (Altschul *et al.* 1990) was performed at the NCBI (http://blast.ncbi.nlm.nih.gov/Blast.cgi) or the Broad Institute (http://www.broad.mit.edu/annotation/genome/neurospora/Blast.html). Sequence alignments were carried out with ClustalW (http://align.genome.jp, Thompson *et al.* 1994), and the corresponding graphical editing was performed with GeneDoc (http://www.psc.edu/biomed/genedoc, Nicholas *et al.* 1997).

Preparation of nucleic acids, hybridization protocols, and PCR: Isolation of *S. macrospora* genomic DNA was carried out as described by Pöggeler *et al.* (1997). Southern blotting and hybridization was done according to standard techniques (Sambrook and Russel 2001) using ^{32}P-labeled DNA probes. For the construction of probes, PCR fragments carrying the *gsa1*, *-2*, *-3*, *sac1*, or *hph* gene were amplified from *S. macrospora* genomic DNA or pZHK2 (Kück and Pöggeler 2004) as template. PCR was performed with HotStarTaq DNA polymerase (Qiagen GmbH, Hilden, Germany), HotMaster Taq (Eppendorf AG, Hamburg, Germany), GoTaq (Promega, Madison, WI) or the Taq and Pwo DNA polymerase blend from the Expand Long Template PCR system (Roche AG, Basel, Switzerland) according to manufacturer's protocols. Extraction of total RNA and quantitative real-time PCR were performed as described previously (Nowrousian *et al.* 2005). Oligonucleotides that were used as primers for quantitative real-time PCR are given in Table 2.

Generation of Δgsa1, Δgsa2, Δgsa3, and Δsac1 knockout strains: Knockout constructs for homologous recombination in *S. macrospora* were generated using PCR-based fusions (Shevchuk *et al.* 2004). The flanking regions of the three *gsa* and the *sac1* genes were amplified with *S. macrospora* genomic DNA as template using primer pairs located upstream or downstream of the corresponding gene (Table 2; supplemental Figure S1A, oligonucleotides given in italics). The gene for the *hph* resistance marker was amplified with primer pair hph-f and hph-r (Table 2; supplemental Figure S1A, given in italics) from plasmid pZHK2 (Kück and Pöggeler 2004) and fused to the amplified flanking regions. The PCR products obtained served as template for the next step to generate the complete knockout constructs with nested primers (gsa1-2 and gsa1-5, gsa2-2 and gsa2-5, gsa3-2 and gsa3-5, and sac2 and sac5) as specified in Table 2 and supplemental Figure S1A (given in italics). To generate mutant strains Δgsa1, Δgsa2, Δgsa3, and Δsac1, the resulting PCR amplicons were used to transform the wild-type or Δku70 strain impaired in heterologous recombination (Pöggeler and Kück 2006). In each of the four strains, the corresponding gene was substituted by the *hph* gene through homologous recombination. Recombinant strains carrying the *hph* cassette instead of *gsa1*, *-2*, *-3*, or *sac1* were identified by PCR with primer pairs that are specific for sequences external and internal of the knockout cassette (see Table 2 and supplemental Figure S1A, given in gray). The number of integrated *hph* copies in the genome of the mutant strains was determined by Southern analysis with an *hph*-specific probe amplified with the primer pair hph-f and hph-r (Table 2) from the plasmid pZHK2 (Kück and Pöggeler 2004). Fungal transformants are often heterokaryotic and thus mycelia carry transformed and nontransformed nuclei. Therefore, single spore isolates were generated by crossing the putative knockout mutants with strain r2 (S67813) or fus1 (S23442), having red-colored ascospores (Table 1). To rescue the phenotype of single mutants Δgsa1 (J223) and Δgsa3/r2 (S72902), plasmid pD202 and pD194.1, respectively, each carrying a corresponding wild-type copy of the disrupted genes, were cotransformed with plasmid pD-NAT1, carrying the nourseothricin resistance gene *nat1* (Table 3, Kück and Hoff 2006). To complement the phenotype of the Δgsa1Δgsa2 (J283) and Δgsa1Δsac1 (S73097) double mutants, plasmid pD202 carrying the *gsa1* gene, was cotransformed with plasmid pD-NAT1. Cosmid H122 F6 (FGSC, Table 3), containing the entire *N. crassa cr-1* gene, was cotransformed with pD-NAT1 to rescue the Δsac1 mutant.

Double- and triple-knockout strains: Double- and triple-knockout strains (Table 1) were generated by crossing the single knockout strains using conventional genetic methods as described by Esser and Straub (1958). Asci from recombinant perithecia were isolated and spore isolates of selected asci were analyzed by PCR and by backcrossing.

Microscopic investigations: *S. macrospora* strains were grown on solid cornmeal medium on slides in glass petri dishes as described by Engh *et al.* (2007). Ascospore germination rates were determined after incubation for 5 hr on solid cornmeal medium containing 0.5% (w/v) sodium acetate. Light microscopy was carried out with an Axiophot microscope (Carl Zeiss AG, Oberkochen, Germany), and pictures were captured by an AxioCam using the Axiovision digital imaging system. Adobe Photoshop CS2 was used to edit images.

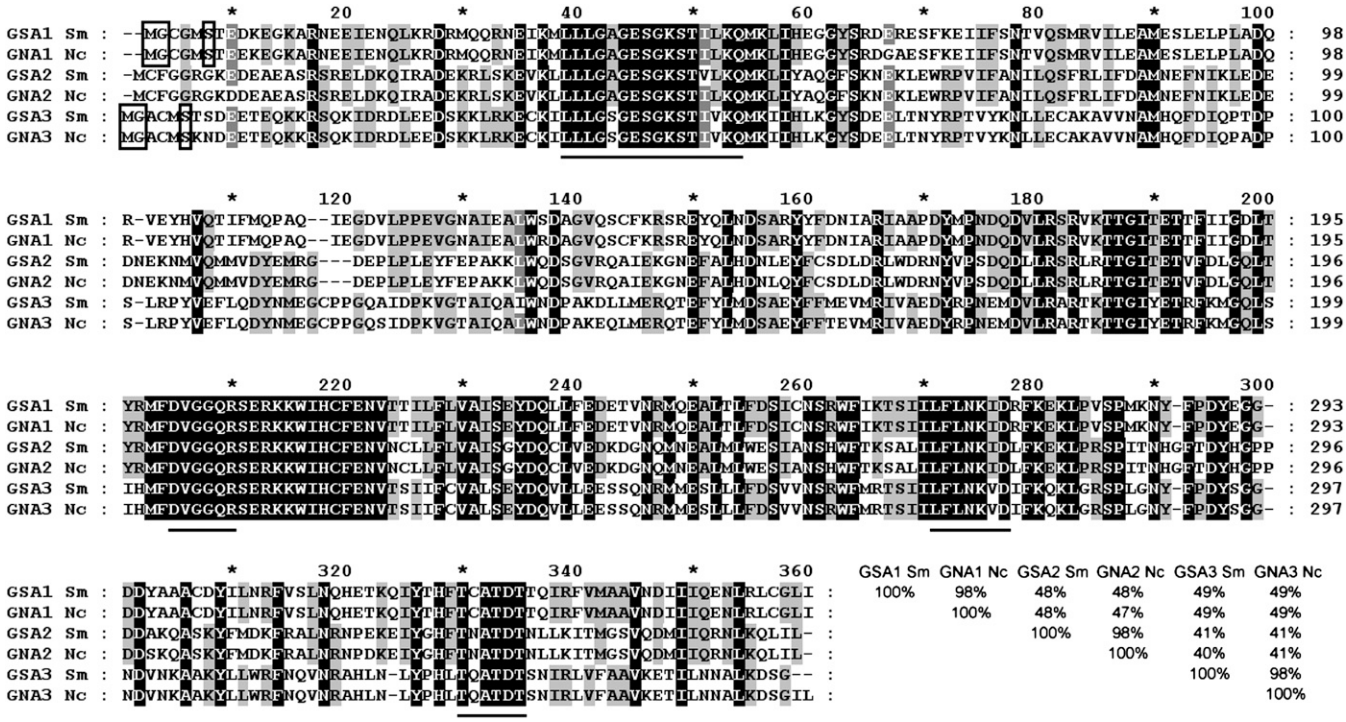

FIGURE 1.—Comparison of amino acid sequences from *S. macrospora* (Sm) GSA1, GSA2, and GSA3 proteins (accession nos. CAP09209, CAP09210, and CAP09211) with their *N. crassa* (Nc) orthologs (accession nos. AAB37244.1, Q05424, and XP_962205.1). The amino acids with the solid background are identical in all three subunits of both species. Sequence identity between four or five sequences is indicated by shading. Conserved regions that are predicted to play a role in the interaction with GTP are underlined. Putative myristoylation sites are marked with boxes. Amino acid identity of Gα-subunits is given at the end in percentages.

RESULTS

Isolation and characterization of three genes for G protein α-subunits and a gene for adenylyl cyclase from *S. macrospora*: Previously, we showed that *N. crassa* and *S. macrospora* have a high degree of nucleic acid identity with an average of 89.5% within exonic sequences (NOWROUSIAN *et al.* 2004). Therefore, primers based on the *N. crassa* sequence were designed and used for the PCR-mediated isolation of three genes encoding Gα-subunits from *S. macrospora*. The resulting amplicons were used for DNA sequencing and their identities were confirmed by comparison with the homologous sequences from *N. crassa*. Similar to the corresponding gene designations in *N. crassa*, the *S. macrospora* genes were named *gsa1*, *gsa2*, and *gsa3* (G protein *Sordaria* alpha subunit). Using an inverse PCR-based strategy, the flanking sequences of the *gsa1* and *gsa3* genes were isolated. In the case of *gsa2*, a PCR-based strategy was used to isolate a cosmid clone encoding the GSA2 protein (PÖGGELER *et al.* 1997). DNA sequencing of the three *gsa* genes revealed that the predicted amino acid sequences from all genes are closely related to the corresponding *N. crassa* homologs. The amino acid sequences of GSA1, -2, and -3 display 98% sequence identity with their counterparts from *N. crassa* (Figure 1). Further conservation is seen when the positions and numbers of introns are compared. All introns are located at similar positions when compared with the homologous genes from *N. crassa* (supplemental Figure S1A).

The predicted polypeptides encoded by the three *gsa* genes exhibit conserved domains (Figure 1) that are considered to be directly involved in guanine-nucleotide interaction (SIMON *et al.* 1991; SKIBA *et al.* 1996; BOHM *et al.* 1997). Both GSA1 and GSA3 have a putative myristoylation site (MGXXXS) at the N terminus (BUSS *et al.* 1987; GORDON *et al.* 1991), but this motif is absent from GSA2 (Figure 1). GSA1 is further characterized by the consensus sequence CXXX at the carboxy terminus that is susceptible to modification by the pertussis toxin (SIMON *et al.* 1991; BÖLKER 1998). These two features indicate that GSA1, just like its *N. crassa* counterpart, is evolutionarily related to the $G\alpha_i$-subfamily of mammals that inhibits adenylyl cyclase (IVEY *et al.* 1996). As already shown by others, the amino acid sequences for Gα-subunits are highly homologous within filamentous fungi (BÖLKER 1998; KAYS *et al.* 2000; PARSLEY *et al.* 2003). The proteins encoded by *gsa1*, *gsa2*, and *gsa3* from *S. macrospora* display significant identities to corresponding proteins from other fungi with the highest amino acid identity to the Gα-subunits from *N. crassa* (data not shown).

To isolate the gene encoding the adenylyl cyclase, a probe was amplified with *S. macrospora* genomic DNA as

TABLE 1

***Sordaria macrospora* strains used in this study**

Strain	Relevant genotype and phenotype	Reference or source
S48977	Wild type	Our culture collection
S23442	*fus1*, spore color mutant	Our culture collection
S67813	*r*2, spore color mutant	Our culture collection
S66001	Δ*ku70::nat*	Pöggeler and Kück (2006)
S2-2-1	Δ*pre1::hph*	Mayrhofer *et al.* (2006)
S60441	Δ*pre2::hph*	Mayrhofer *et al.* (2006)
J223	Δ*gsa1::hph*	This study
10-49-1	Δ*gsa2::hph*	This study
S72902	Δ*gsa3/r2::hph*	This study
K23	Δ*sac1::hph*	This study
S68567	Δ*ste12/fus::hph*	Nolting and Pöggeler (2006)
S46357	*pro41*	Nowrousian *et al.* (2007a)
J283	Δ*gsa1::hph*/Δ*gsa2::hph*	This study
J125	Δ*gsa1::hph*/Δ*pre1::hph*	This study
S71427	Δ*gsa1::hph*/Δ*pre2::hph*	This study
S73097	Δ*gsa1::hph*/Δ*sac1::hph*	This study
S80762	Δ*gsa1::hph*/Δ*ste12/fus::hph*	This study
S81063	Δ*gsa1::hph/pro41*	This study
S75575	Δ*gsa2::hph*/Δ*gsa3/r2::hph*	This study
S68487	Δ*gsa2::hph*/Δ*pre1::hph*	This study
S68093	Δ*gsa2::hph*/Δ*pre2::hph*	This study
S72594	Δ*gsa2::hph*/Δ*sac1::hph*	This study
S83053	Δ*gsa2::hph*/Δ*ste12/fus::hph*	This study
S81167	Δ*gsa2::hph/pro41*	This study
S73402	Δ*gsa3/r2::hph*/Δ*gsa1::hph*	This study
K101	Δ*gsa3/r2::hph*/Δ*pre1::hph*	This study
S75162	Δ*gsa3/r2::hph*/Δ*pre2::hph*	This study
J423	Δ*gsa3/r2::hph*/Δ*sac1::hph*	This study
S83821	Δ*gsa3*/r2*::hph*/Δ*ste12/fus::hph*	This study
S81284	Δ*gsa3/r2::hph/pro41*	This study
S77487	Δ*gsa2::hph*/Δ*gsa3/r2::hph*/Δ*sac1::hph*	This study

template and heterologous oligonucleotides specific for the *N. crassa* adenylyl cyclase gene (oligonucleotides 1091 and 1092, Table 2). The resulting fragment was used for screening of an indexed *S. macrospora* cosmid library (Pöggeler *et al.* 1997). This led to the identification of a cosmid clone that was subjected to DNA sequencing, resulting in the identification of an open reading frame for a predicted adenylyl cyclase. The corresponding gene was named *sac1* (*Sordaria* adenylyl cyclase1) and the comparison of the *S. macrospora* sequence with the corresponding *N. crassa cr-1* gene displayed a similar exon-intron structure with respect to position of the introns (supplemental Figure S1A). The deduced amino acid sequence (accession no. CAP09208) exhibits an identity of 92.5% to the *N. crassa* homolog CR-1 (accession no. BAA00755.1). SAC1 displays a domain distribution typical for fungal adenylyl cyclases. The conserved amino acid motif DXNLN is located close to the N terminus, representing a putative Gα-binding site (Ivey and Hoffman 2005), followed by a RA (*R*as *a*ssociation) domain. The central core of the enzyme consists of a leucin-rich repeat domain. At the C terminus, a serine/threonine protein phosphatase-like catalytic domain (type 2C, PP2Cc) is located immediately upstream of the single catalytic domain of the adenylyl cyclase (Baker and Kelly 2004).

Construction and phenotypic description of three G protein α-subunit deletion strains: To functionally and genetically analyze the Gα-subunit genes, we constructed three knockout strains. As shown in supplemental Figure S1A, linear fragments were generated that contain the *hph* gene, flanked by genomic sequences of the different *gsa* genes. For *gsa1* and *gsa2*, the marker gene replaces the complete open reading frame for the corresponding Gα-subunit, while for *gsa3*, two-thirds of the N-terminal coding region are substituted by the *hph* resistance gene. *S. macrospora* transformants often contain wild-type as well as mutant nuclei and are thus heterokaryotic. To obtain homokaryotic strains, single ascospore isolates were generated from all three above-described knockout strains. Since we crossed these strains with the spore color mutants r2 or fus1 (Table 1), disruption strains often carry an additional spore color mutation. Apart from the red spore color, r2 and fus1 strains resemble the wild-type phenotype of *S. macrospora*. For Δgsa3, only single-spore isolates with an r2 background were isolated. In all knockout strains, the substitution of the *gsa* genes by the hygromycin B resistance gene (*hph*) was further verified by PCR and Southern analysis as outlined in the materials and methods and shown in supplemental Figure S1.

The characterization of the mutant strains revealed several phenotypic changes compared to the wild type. For example, we found a reduction of the growth rate ranging from 22% (Δgsa3) to 29% (Δgsa2) and 37% (Δgsa1). With respect to sexual development, we observed that Δgsa2 has wild-type-like fertility, while the two other mutant strains exhibit major differences. In Δgsa1, the number of fruiting bodies is reduced by ~50% (Figure 2A). Moreover, in wild type grown on solid media, mature fruiting bodies appear after 7 days, whereas in Δgsa1, the first mature fruiting bodies did not appear until after 11 days of growth. The wild-type phenotype was restored when Δgsa1 was transformed with a full-length copy of the *gsa1* gene (pD202, Table 3, data not shown). Δgsa3 has a wild-type-like phenotype as regards fruiting body development, and also similar to the wild type, fruiting bodies develop within 7 days (Figure 2A). However, Δgsa3 ascospores have a lower germination rate with a 95% reduction compared to

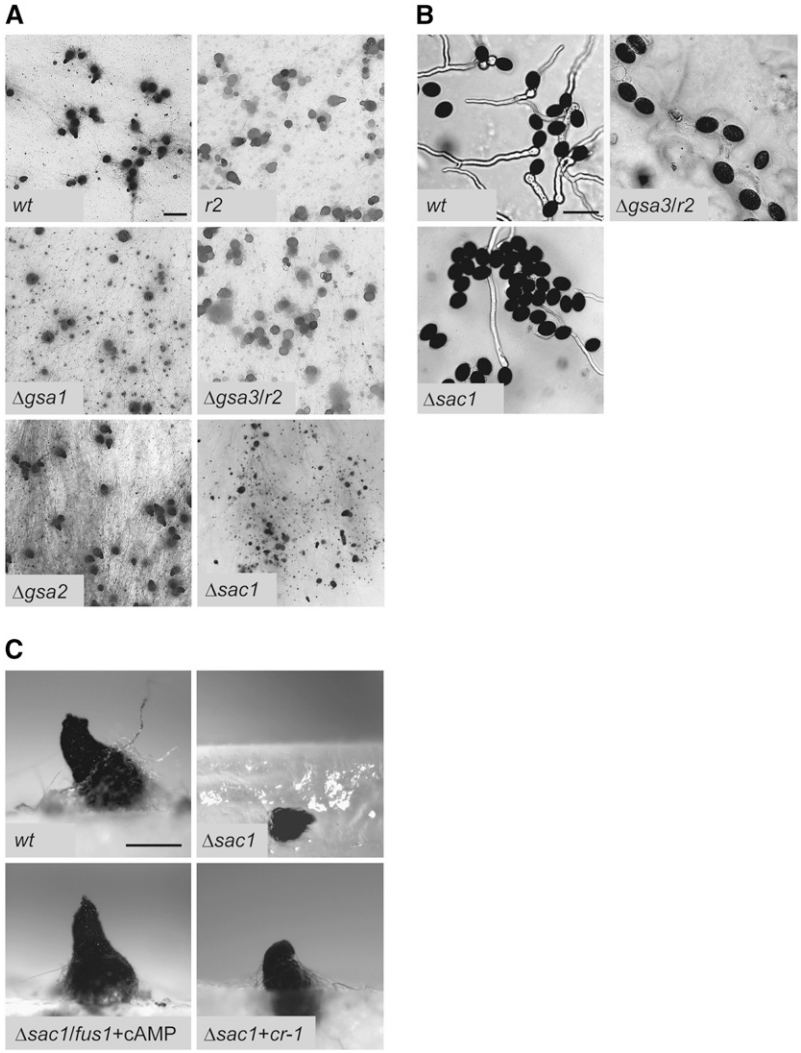

FIGURE 2.—Fruiting body development and ascospore germination of wild type, Δgsa, and Δsac1 mutant strains. r2 and fus1 are spore color mutants that show wild-type fertility. (A) Perithecia on solid cornmeal medium after 11 days of growth. The scale bar represents 1 mm. (B) Ascospore germination of wild-type and mutant strains Δsac1 and Δgsa3/r2. Spores were incubated for 5 hr on solid cornmeal medium with 0.5% (w/v) sodium acetate. The scale bar represents 50 μm. (C) Lateral view on perithecia of wild type, Δsac1, Δsac1/fus1 supplemented with cyclic AMP (+cAMP), and retransformant Δsac1 + *cr-1* on solid cornmeal medium after 9 days of growth. The scale bar represents 200 μm.

wild type (Figure 2B). This phenotype can be rescued through complementation with a full-length *gsa3* sequence (pD194.1, Table 3, data not shown).

Construction and phenotypic characterization of a Δsac1 disruption strain: Similar to the disruption strategy described above for the *gsa* genes, a *sac1* deletion strain was generated for further functional analysis. A set of primers was used to generate a DNA fragment containing the *hph* gene flanked by genomic sequences from the *sac1* gene (see supplemental Figure S1A). This linear DNA fragment was used to transform a Δku70 recipient strain and the resulting transformants were analyzed with different sets of primers (Table 2, supplemental Figure S1A, data not shown). Six fungal transformants were generated in this way and all of them contained a disrupted *sac1* gene, but only three of them were homokaryotic. By crossing of a primary, homokaryotic Δsac1 mutant with a fus1 strain, we obtained a Δsac1 strain lacking the *ku70* deletion but carrying a single copy of the *hph* gene (see corresponding Southern analysis in supplemental Figure S1B). This strain served for further functional analysis. Although this strain is fertile, the fruiting bodies have a size that is reduced by 30% compared with wild-type perithecia (Figure 2A). In addition, a significant number of fruiting bodies are embedded in the agar and the germination rate of the ascospores is only ~30% (Figure 2B). When the Δsac1 strain was transformed with cosmid H122 F6 (Table 3) carrying the entire *N. crassa cr-1* gene the ascospore germination rate was restored to the wild-type level, thus demonstrating that deletion of *sac1* is causally related to the observed phenotype (data not shown). Additionally, perithecia of the rescued strain developed at the air-to-surface interface of solid medium, as shown in Figure 2C (Δ*sac1* + *cr-1*). Perithecial formation at the air-to-surface interface was also restored by adding cAMP-derivative dibutyryl-cAMP to the culture medium, indicating that the embedded perithecia of the Δsac1 mutant are due to a lack of cAMP (Figure 2C, Δ*sac1*/*fus1* + cAMP). Besides, the growth rate of the mutant was increased from 59 to 74% compared to the wild type when supplemented with cAMP.

gsa double mutants carrying the Δ*gsa1* mutation are unable to develop fruiting bodies: To study the genetic

TABLE 2

Oligonucleotides used in this study

Oligonucleotide	Sequence (5′–3′)	Specificity
gna1-1	GAAGCAGATGAAGCTTATCCA	*gsa1*
gna1-2	TCGAACATACGGTACGTAAGA	*gsa1*
gna1-3	ATGGGTTGCGGAATGAGTACAGAG	*gna-1 N. crassa*
gna1-4	AAACCGCAGAGACGCAGGTTCTC	*gna-1 N. crassa*
gsa1-1	CGATCGCATCGGTCTTCGTTTC	5′ flank *gsa1*
gsa1-2	AGCGCTGCCATGCCCGACAAT	5′ flank *gsa1*
gsa1-3	GAATTCTCAGTCCTGCTCCTTTTGGCGA CTTGTTGTAACTCTTGG	5′ flank *gsa1* with *hph* overhang
gsa1-4	TCCTCTAGAGTCGACCTGCAGCATTGAA CCCAGTCTAATTTTTCAC	3′ flank *gsa1* with *hph* overhang
gsa1-5	TTCCCCCACACTGCCCATGAAAG	3′ flank *gsa1*
gsa1-6	CCATGGGCTTGAGTCCCACTAC	3′ flank *gsa1*
gsa1-7	CTGTACTCATTCCGCAACCCAT	*gsa1* (inverse PCR)
gsa1-8	GTCGACCATCTTGAAGCAGATG	*gsa1* (inverse PCR)
gsa1-9	CCAGTGGCAACTCTAGGGACT	*gsa1* (inverse PCR)
gsa1-10	GAGAACCTGCGTCTCTGCGG	*gsa1* (inverse PCR)
5′gsa1	TCAATGAGCGCTGCCATGCCCG	5′ flank *gsa1*
3′gsa1	ACTTTCCCCCACACTGCCCATGA	3′ flank *gsa1*
gna2-1	AAGTTGATCTATGCACAAGG	*gna-2 N. crassa*
gna2-2	AAATATTCTAGTGGTAGAGG	*gna-2 N. crassa*
gna2-3	ATGTGTTTCGGGGGTCGTGG	*gsa2*
gna2-4	ACAGGATAAGTTGTTTGAGGTTC	*gsa2*
gsa2-1	GTGGGCCTAGCATGCAGAAT	5′ flank *gsa2*
gsa2-2	TGATCGTCCCTGTCTCCATTG	5′ flank *gsa2*
gsa2-3	GAATTCTCAGTCCTGCTCCTTATGTGTT GGATCCGTGTTGCAGAG	5′ flank *gsa2* with *hph* overhang
gsa2-4	TCCTCTAGAGTCGACCTGCACACATCCGA TACATCTGCTTCCGCA	3′ flank *gsa2* with *hph* overhang
gsa2-5	CCAACGTGAGGGAGAGGTGA	3′ flank *gsa2*
gsa2-6	TGCAAGCTAACTTGAAATCTCC	3′ flank *gsa2*
5′gsa2	GGCTCCCACCACACTCGCTGTCTGTC	5′ flank *gsa2*
3′gsa2	ATCATCGAGAATAGCTCCTCTGTAC	3′ flank *gsa2*
gna3-1	ATGGGCGCATGCATGAGC	*gsa3 S. macrospora; gna-3 N. crassa*
gna3-2	ACTTGGCGGCCTTGTTGAC	*gsa3*
gna3-6	TCATAGAATACCGGAGTCTTTAAG	*gna-3 N. crassa*
gsa3-1	CCCATCTCCCTCCCGCAAGAT	5′ flank *gsa3*
gsa3-2	TTGCCTGCCTGCCTTTCACCTTTACC	5′ flank *gsa3*
gsa3-3	GAATTCTCAGTCCTGCTCCTATGAGGCAGTC GACGATGGTCCG	5′ *gsa3* with *hph* overhang
gsa3-4	TCCTCTAGAGTCGACCTGCACGTAGCGAAC GCAAGAAGTGGATT	3′ *gsa3* with *hph* overhang
gsa3-5	CCGGAGTCTTTAAGAGCGTTGTTG	3′ flank *gsa3*
gsa3-6	TCATAGAATACCGGAGTCTTTAAG	3′ flank *gsa3*
gsa3-7	TTCTTCTGCTCCGTCTCCTC	*gsa3* (inverse PCR)
gsa3-8	AAGAAGTGGATTCACTGCTTCG	*gsa3* (inverse PCR)
5′gsa3	GCATTGCCTGCCTGCCTTTCACCTTT	5′ flank *gsa3*
sac1	ACTGCCGACGGGAAGCTCAATG	5′ flank *sac1*
sac2	ATGGCAGATGGGAGTGGTGGTAC	5′ flank *sac1*
sac3	GAATTCTCAGTCCTGCTCCTACTACTAGGAGA CCACAGCCATTCCACG	5′ *sac1* with *hph* overhang
sac4	TCCTCTAGAGTCGACCTGCATCAAGCTGCATC ATCATCACCATCTGCTC	3′ *sac1* with *hph* overhang
sac5	ATCCAGGACCCAAAGACTAACC	3′ flank *sac1*
sac6	TATATGTAACTGTCGCAGGTGG	3′ flank *sac1*
sac-f	AGGCTGGTGCTTACCTACCG	*sac1*
sac-r	ATTGAACAGATGAGAACGACC	3′ flank *sac1*
sac10s	ATGATAGAATTGAAGCGTGG	*sac1*
5′sac1	ATAATGCTAGTTGTACCTCTAAGAGAGTCG	5′ flank *sac1*

(*continued*)

TABLE 2

(Continued)

Oligonucleotide	Sequence (5′–3′)	Specificity
3′sac1	ACATACACAGATTCGATCACCATCACCGATG	3′ flank *sac1*
1091	AAGACGGAAGGAGACGCATTTATG	*cr-1 N. crassa*
1092	CTCAAGGCCTTTGAGCTTCTTCTC	*cr-1 N. crassa*
u-cr-1	TGCGGCTGATTATGGAGGA	*cr-1 N. crassa*
cr-1for	AGCGCAGCAATTCAAGGGACAGC	*cr-1 N. crassa*
cr-1rev	GTTGCAAGAGCGCCATAGGGC	*cr-1 N. crassa*
D5	CACCACCACACAGAGGAAAC	5′ flank *ste12*
hph-f	AGGAGCAGGACTGAGAATTC	*hph* cassette
hph-r	TGCAGGTCGACTCTAGAGGA	*hph* cassette
hph-if	TCCAGTCAATGACCGCTGTTATG	*hph* cassette internal
hph-ir	TCCAACAATGTCCTGACGGACA	*hph* cassette internal
h3	GGGCCCGAAACGAACTAGAGTTCTAG	*hph* cassette internal
SSU1	ATCCAAGGAAGGCAGCAGGC	*SSU rRNA* (real-time PCR)
SSU2	TGGAGCTGGAATTACCGCG	*SSU rRNA* (real-time PCR)
ste12for	CTTCGCAGCATGCCAATATG	*ste12* (real-time PCR)
ste12rev	GCGCGGAAATGAGGAAATAC	*ste12* (real-time PCR)
SMU2767for	GTGGCCGCTCGGTTTTATTG	*pro41* (real-time PCR)
SMU2767rev	TCACCTGGTAAATCGCAGCGT	*pro41* (real-time PCR)

interactions between *S. macrospora gsa* genes, all three possible double mutants Δgsa1Δgsa2, Δgsa1Δgsa3, and Δgsa2Δgsa3 were generated by conventional crossing of single mutants. The genotype of the double mutants was confirmed by PCR analysis (data not shown). The two Δgsa1Δgsa2 and Δgsa1Δgsa3 double mutants are completely blocked in their sexual development. As can been seen in Figure 3A, development to mature fruiting bodies is prevented in these two double mutants even after prolonged growth. Moreover, both double mutants only produce protoperithecia with a frequency that is below that seen for each single mutant or the wild type (Figure 3B). To exclude the possibility that a further mutation is responsible for the sterile phenotype, fertility was restored by introducing a full-length copy of *gsa1* (pD202, Table 3) into the Δgsa1Δgsa2 and Δgsa1Δgsa3 double mutants (Figure 3A, Δ*gsa1*Δ*gsa2* + *gsa1;* Δ*gsa1*Δ*gsa3/r2* + *gsa1*). Characterization of the strains was done by PCR amplification with primer pairs specific for the two *gsa* deletions or the wild-type *gsa* genes (data not shown). The phenotype of the rescued strains resembled the Δgsa2 phenotype (Δ*gsa1*Δ*gsa2* + *gsa1*) and the Δgsa3 phenotype with reduced ascospore germination (Δ*gsa1*Δ*gsa3/r2* + *gsa1*). In contrast to the double mutants carrying the Δ*gsa1* disruption, Δgsa2Δgsa3 displays a phenotype that resembles that of the Δgsa3 single mutant (Figure 3A) including reduced ascospore germination and growth rates.

ΔgsaΔpre double mutants: Previously, we identified two transcriptionally expressed pheromone receptor genes, *pre1* and *pre2*, in the genome of *S. macrospora* (Pöggeler and Kück 2001). Functional characterization of the *pre* genes has shown that single knockout strains have a wild-type-like fruiting body development. However, deletion of both receptor genes completely eliminates fruiting body formation (Mayrhofer *et al.* 2006).

To determine which of the Gα-subunits transmits the pheromone signal from the G protein-coupled receptors, six double mutants were generated by crossing all

TABLE 3

Plasmids and cosmids used in this study

Plasmid/cosmid	Feature	Reference
pDrive	UA-based PCR cloning	Qiagen
pGPS2.1-hph	pGPS2.1 carrying *hph*, Transprimer-2 element	Dreyer *et al.* (2007)
pD-NAT1	pDrive with *nat1*	Kück and Hoff (2006)
pD194.1	pDrive with *gsa3* and 5′ flanking region	This study
pD202	pDrive with *gsa1* and flanking regions	This study
pDAde	pDrive with *sac1* 5′ flanking region	This study
D10	Cosmid from pool 2012-2116A containing *gsa2*	Pöggeler *et al.* (1997)
H2	Cosmid from pool 2213-2308VIB containing *sac1*	Pöggeler *et al.* (1997)
H122 F6	*N. crassa cr-1* in pLorist6Xh	Fungal Genetics Stock Center (FGSC)

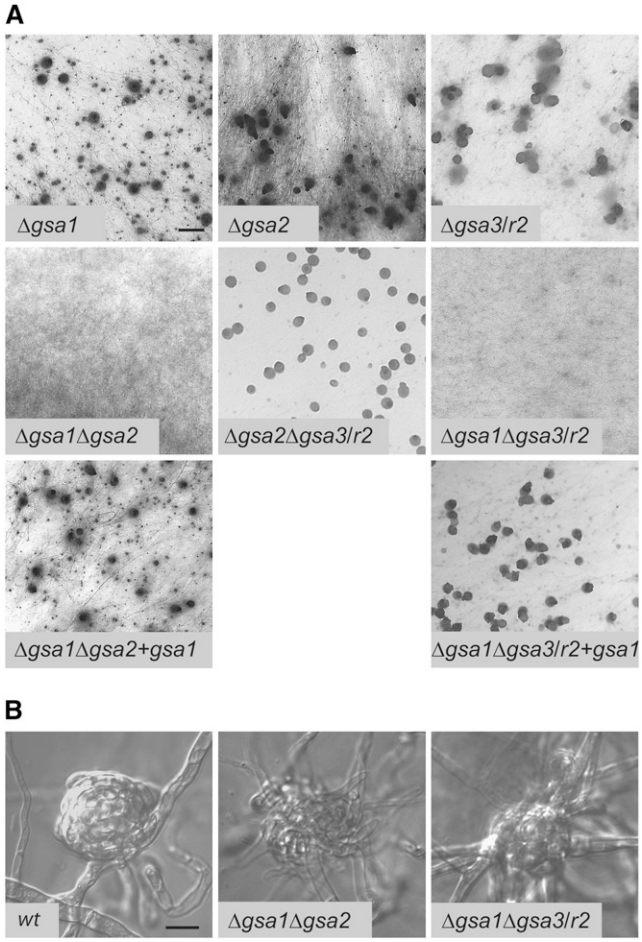

FIGURE 3.—Phenotypic characterization of fruiting body development of Gα-single and -double mutants. (A) Perithecial development on solid cornmeal medium after 11 days. Bar, 1 mm. (B) Microscopic images of protoperithecia from wild-type and double mutants Δgsa1Δgsa2 and Δgsa1Δgsa3/r2. Bar, 10 μm.

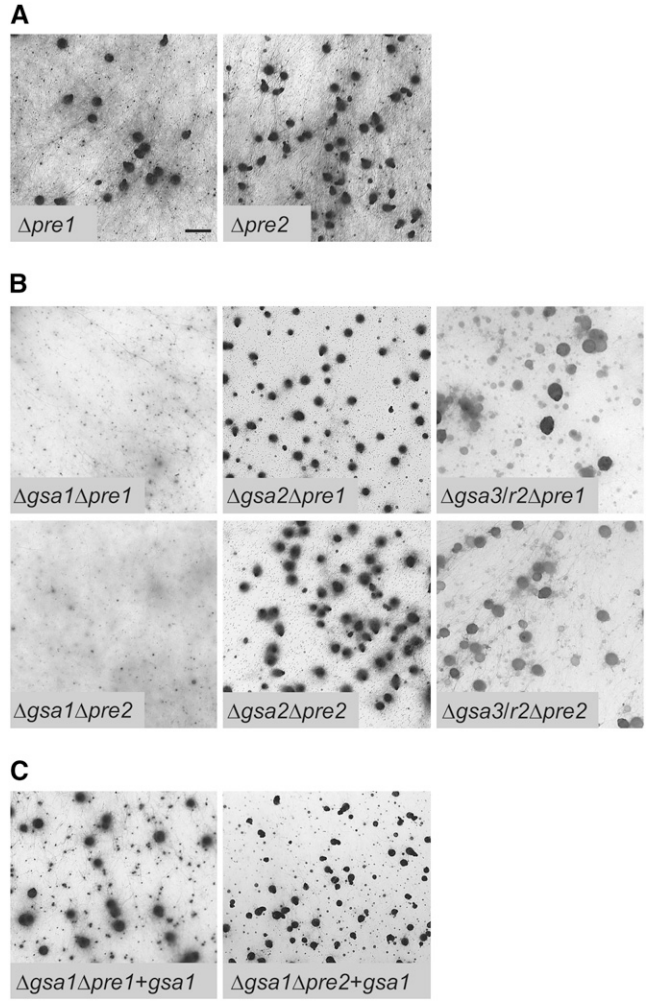

FIGURE 4.—Phenotypic characterization of fruiting body development in Gα-subunits/pheromone receptor mutants. Δpre receptor mutants (A) and ΔgsaΔpre double mutants (B) are shown after growth for 11 days on solid cornmeal medium. (C) Transformation with *gsa1* (+*gsa1*) restores fertility in the Δgsa1Δpre mutants. Bar, 1 mm.

Δgsa strains with either Δpre1 or Δpre2 (Figure 4A). Similar to the procedures described above, the genotypes of double mutants were confirmed by PCR analysis (data not shown). Phenotypic characterization of the six double mutants showed clear differences in their capacity to complete the sexual cycle. While Δgsa2Δpre1 and Δgsa2Δpre2 have a wild-type-like phenotype, both Δgsa1Δpre1 and Δgsa1Δpre2 display a severe impairment of fruiting body development (Figure 4B). For example, the number of perithecia is drastically reduced to a level of 0.5% when compared to the Δpre1 and Δpre2 single mutants. To confirm that this reduction is not due to the acquisition of another mutation, both double mutants (Δgsa1Δpre1; Δgsa1Δpre2) were complemented with a full-length copy of the *gsa1* gene (pD202, Table 3). As shown in Figure 4C, Δgsa1Δpre1 + *gsa1* and Δgsa1Δpre2 + *gsa1* strains are phenotypically identical to the Δpre1 and Δpre2 single mutants.

In addition to the above-described double mutants, Δgsa3Δpre1 and Δgsa3Δpre2 mutants were generated and both double mutants exhibit a Δgsa3-like phenotype (reduced germination rate of ascospores).

ΔgsaΔsac1 double and triple mutants: To study the genetic interactions between the adenylyl cyclase and the Gα-subunits, the three possible double mutants (Δgsa1Δsac1, Δgsa2Δsac1, and Δgsa3Δsac1) and a Δgsa2 Δgsa3Δsac1 triple mutant were generated by conventional crossings. The genotype of the mutants was confirmed by PCR analysis (data not shown). The morphological characterization of the mutants showed the following phenotypes: Δgsa2Δsac1 and Δgsa3Δsac1 resemble Δsac1 with small perithecia embedded in the agar and a highly reduced germination rate of ascospores (Figure 5A). A dramatic change in phenotype was observed in the Δgsa1Δsac1 double mutant. As shown in Figure 5A, this strain is completely sterile and unable to form any perithecia, and only protoperithecia are detectable (Figure 5B). When Δgsa1Δsac1 was trans-

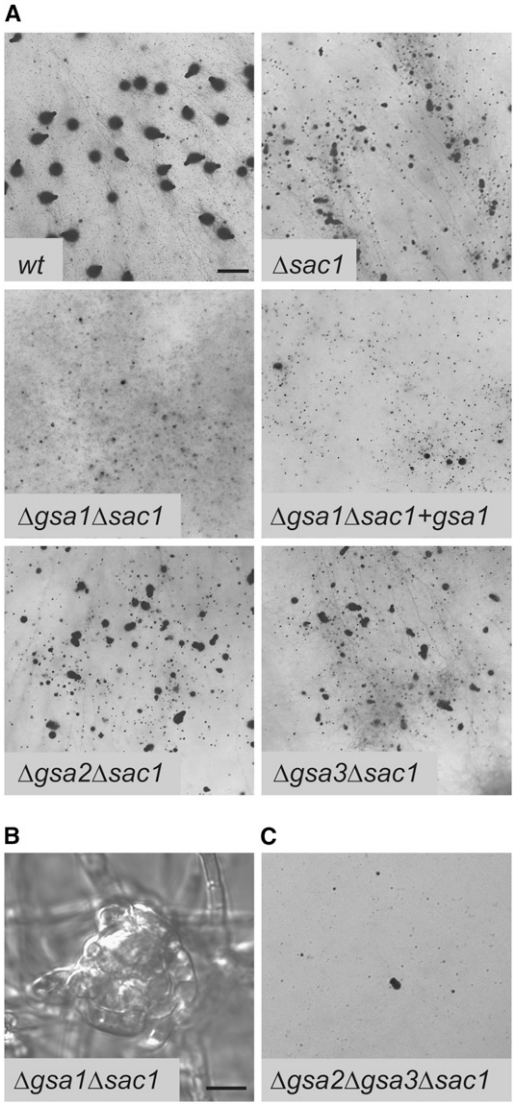

FIGURE 5.—Phenotypic characterization of Δgsa/Δsac1 double mutants and Δgsa2Δgsa3Δsac1 triple mutant. (A) Perithecial development of wild type, Δsac1, and Δgsa/Δsac1 double mutants after 11 days of growth on solid cornmeal medium. Bar, 1 mm. (B) Protoperithecial development in the sterile double mutant Δgsa1/Δsac1. Bar, 10 μm. (C) Δgsa2Δgsa3Δsac1 triple mutant after 11 days of growth on solid cornmeal medium.

formed with a full-length copy of *gsa1* (pD202, Table 3), development of a few perithecia compared to the Δsac1 single mutant was restored (Figure 5A, Δ*gsa1*Δ*sac1* + *gsa1*).

Finally, the triple-mutant strain Δgsa2Δgsa3Δsac1 exhibits a phenotype similar to the above-described Δgsa1Δpre strains showing a drastically reduced number of perithecia compared to the wild type (Figure 5C). To the best of our knowledge, this is the first description in filamentous fungi of all possible double mutants carrying a disrupted adenylyl cyclase gene together with one of the deleted *gsa* genes.

ΔgsaΔste12 double mutants: In *S. cerevisiae,* the Ste12p transcription factor acts downstream of a signaling cascade that links pheromone receptors to a MAP kinase cascade via heterotrimeric G protein signaling (DOHLMAN and THORNER 2001). Loss of the Ste12p function results in the inability of haploid *S. cerevisiae* cells to mate (HARTWELL 1980). However, the orthologous transcription factor STE12 of *S. macrospora* is required only for the correct morphogenesis of asci and ascospores. The cell walls of asci and ascospores of the Δste12 mutant strain are fragile and few of the spores are able to germinate (NOLTING and PÖGGELER 2006). To study the functional connections between the GSA subunits and the STE12 transcription factor in *S. macrospora*, three ΔgsaΔste12 double mutants were generated by conventional crossings. The genotype of the mutants was verified by PCR analysis (data not shown). While Δste12 single and the Δgsa2Δste12 double mutants show a wild-type-like formation of perithecia containing fragile asci and ascospores, the Δgsa1Δste12 mutant develops few fruiting bodies (Figure 6, A and B). Furthermore, perithecia of the Δgsa1Δste12 mutant contain only few asci compared with the Δste12 single or the Δgsa2Δste12 double mutants (Figure 6B). The Δgsa3Δste12 mutant exhibits a more severe phenotype lacking any perithecia. Instead only protoperithecia without asci initials are produced (Figure 6A).

Expression analysis of developmentally regulated genes in Δgsa and Δsac1 mutants: Microarray studies in the chestnut blight fungus *Cryphonectria parasitica* have revealed a downregulation of the *ste12* homolog *mst12* in a Δcpg-1 mutant strain that carries a deletion of the Gα-gene that is orthologous to *gsa1* (DAWE *et al.* 2004). We therefore studied the expression of the *ste12* transcript of *S. macrospora* in all Gα-single and -double mutants, in the Δsac1 mutant, and in the two previously characterized mutants pro1 and per5 (MASLOFF *et al.* 1999; NOWROUSIAN *et al.* 1999) by quantitative real-time PCR. To connect the Gα-genes to other factors involved in fruiting body formation, we included the gene *pro41* in the analysis, which is developmentally regulated during fruiting body development (NOWROUSIAN *et al.* 2007a). The *pro41* gene encodes a membrane protein of the endoplasmic reticulum and is essential for fruiting body development; the corresponding mutant forms protoperithecia but no mature perithecia (NOWROUSIAN *et al.* 2007a). As shown in Figure 7A, *ste12* transcript levels are significantly downregulated in the Δsac1 mutant in *S. macrospora*, but not in any of the other strains that were analyzed including Δgsa1. This indicates a high degree of diversification in the functions of G protein subunits in different fungal species. As was shown previously, *pro41* is significantly downregulated in mutant pro1 (NOWROUSIAN *et al.* 2007a). The only other mutant that displays a significant transcriptional downregulation of *pro41* is the Δgsa1Δgsa3 double mutant. This might indicate that G protein signaling has some

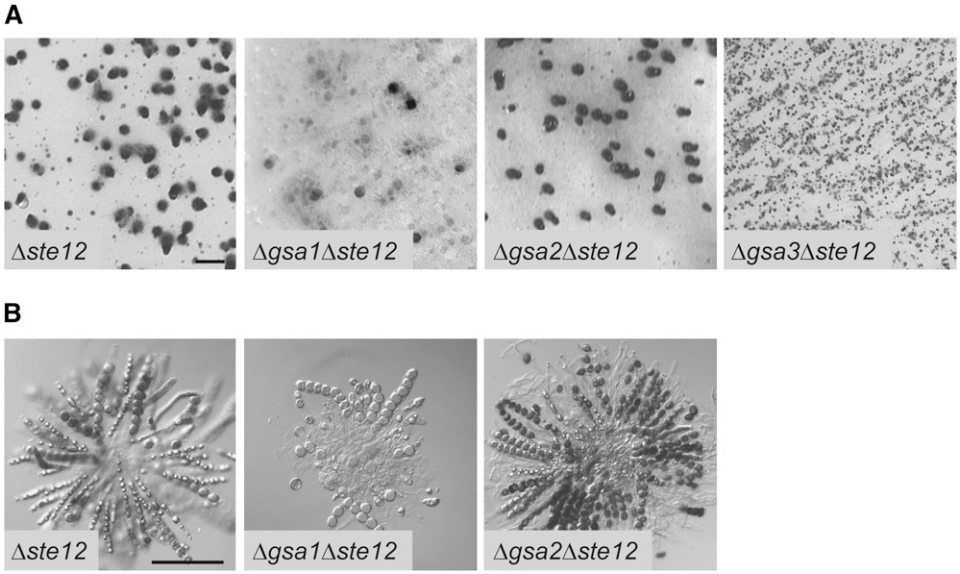

FIGURE 6.—Phenotypic characterization of Δste12 single and ΔgsaΔste12 double mutants. (A) Perithecial development of Δste12 single and Δgsa/Δste12 double mutants after 11 days of growth on solid cornmeal medium. Bar, 1 mm. (B) Microscopic images of asci from Δste12 single and Δgsa1Δste12 and Δgsa2Δste12 double mutants after 11 days of growth on solid cornmeal medium. The Δgsa3Δste12 mutant does not produce perithecia and therefore lacks any asci. Bar, 100 μm.

involvement in the regulation of *pro41* expression; however, only deletion of major parts of the pathway leads to a significant reduction in *pro41* transcript amounts. Taken together with the phenotypes of Gα-protein single and double mutants, this is another indication that the Gα-proteins are part of a signaling network that is at least partly buffered against loss of one subunit. We also generated double mutants of pro41 with all three Gα-genes, and as expected the mutants display the same phenotype as pro41 in that they are sterile and form only protoperithecia (Figure 7B).

DISCUSSION

Three Gα-subunits have different functions during fungal development: All three predicted GSA polypep-

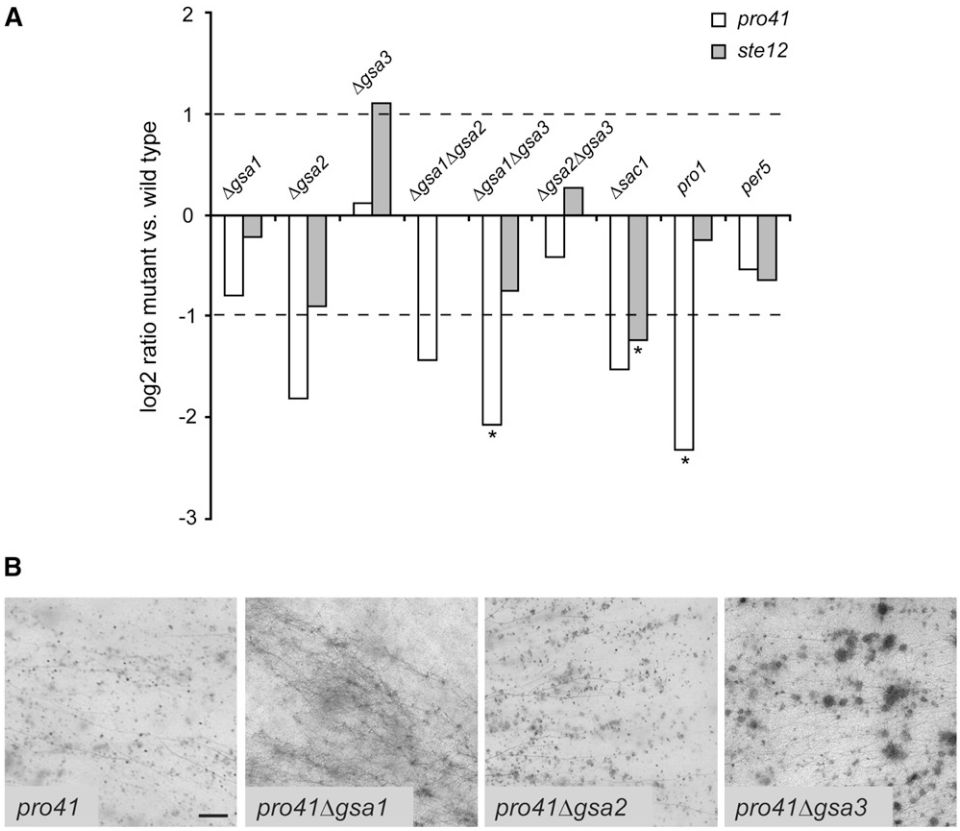

FIGURE 7.—Comparison of transcript levels of *pro41* and *ste12* between different mutants and phenotypes of pro41Δgsa double mutants. (A) Quantitative real-time PCR data are given as logarithmic values of the mutant/wild-type ratios (logarithm to the base 2 for the mean of at least two independent experiments). Real-time PCR results were tested for the significance of differential expression at $P = 0.001$ using REST (PFAFFL *et al.* 2002); genes that are expressed significantly differently in the mutant compared to the wild type are indicated by an asterisk. (B) Perithecial development of pro41 single and pro41Δgsa double mutants after 11 days of growth on solid cornmeal medium. Bar, 1 mm.

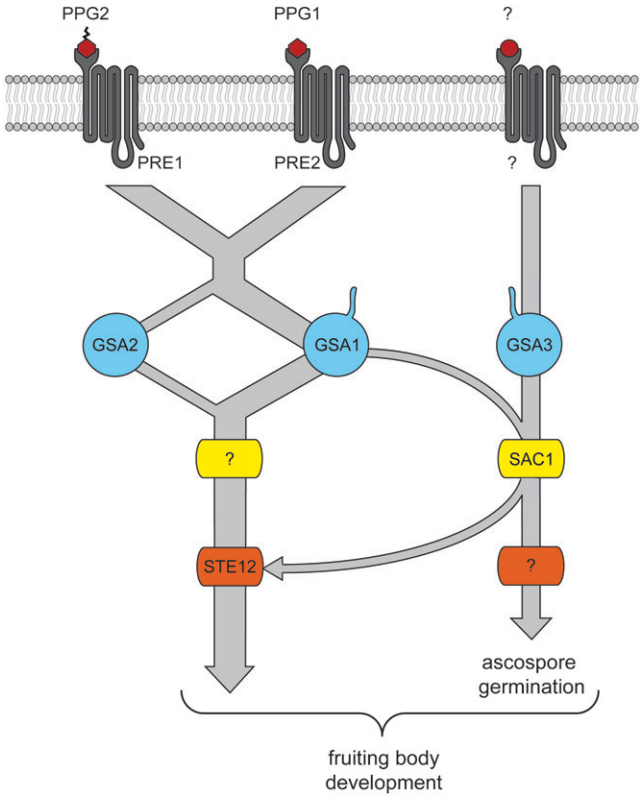

FIGURE 8.—A model for the predicted G protein α-subunit signaling in *S. macrospora*. GSA1 and GSA2 propagate signals within the pheromone signaling pathway, in which GSA1 is the predominant regulator of fruiting body development upstream of the STE12 transcription factor. GSA3 and SAC1 act on sexual development in a less characterized, parallel signaling pathway. Putative myristoylation of Gα-subunits GSA1 and GSA3 is indicated by tails. Putative farnesylation of PPG2 is shown by a serrated tail.

tides are structurally similar to those described for other ascomycetes and can be classified into fungal groups I–III as previously described (BÖLKER 1998). While GSA1 corresponds to Gpa1p from *S. cerevisiae* (group I), GSA2 is more similar to Gpa1 from *Schizosaccharomyces pombe* (group II). The GSA3 subunit shows the highest homology to Gpa2 from both *S. cerevisiae* and *S. pombe* (group III, BÖLKER 1998). Similar to the Δgna mutants from *N. crassa*, the *S. macrospora* Δgsa deletion strains exhibit a reduced vegetative growth rate. Like its *N. crassa* counterpart, Δgsa1 shows defects in sexual development and reduced perithecium number. However, unlike the *N. crassa* mutant that produces aberrant perithecia without any ascospores (IVEY *et al.* 1996), Δgsa1 forms wild-type-like fruiting bodies with fertile ascospores. In *Aspergillus nidulans*, the corresponding ΔfadA mutant strain displays a different phenotype that has reduced vegetative growth and a complete block in cleistothecia formation (ROSÉN *et al.* 1999); therefore, these mutant strains illustrate the high degree of diversity in the function of this Gα-subunit.

The phenotype of the Δgsa2 strain is very close to the wild type, indicating a minor role of this Gα-subunit in sexual development. Single and double mutants suggest that the function of GSA2 can be substituted by GSA1 in the Δgsa2 mutant. However, as Δgsa1 has reduced fertility, GSA2 cannot fully substitute the GSA1 function in a Δgsa1 mutant, indicating that the activity of GSA1 dominates over that of GSA2 in *S. macrospora* with respect to sexual development. These findings correlate well with those obtained with the corresponding *N. crassa* Δgna mutants (KAYS and BORKOVICH 2004).

As mentioned before, GSA3 from *S. macrospora* belongs to the same group (III) of fungal Gα-subunits as Gpa2 from the yeasts *S. cerevisiae* and *S. pombe*. The Gpa2 subunits from these yeasts are responsible for nutrient sensing and control of filamentous growth (LENGELER *et al.* 2000; VERSELE *et al.* 2001; IVEY and HOFFMAN 2005). The group III fungal Gα-subunits have also been proven to stimulate the adenylyl cyclase pathway in many different fungal species (BÖLKER 1998; LENGELER *et al.* 2000; D'SOUZA and HEITMAN 2001; VERSELE *et al.* 2001). The *S. macrospora* Δgsa3 mutant shows a late developmental block, resulting in a drastically reduced germination rate of the ascospores. This finding is similar to the Δ*GPA2* mutant of *S. pombe* and to the defect observed in the corresponding Δgna-3 mutant from *N. crassa*, indicating that activation of this pathway might be an essential step in the germination process (KAYS *et al.* 2000; HATANAKA and SHIMODA 2001). Interestingly, the corresponding Δgna-3 mutant from *N. crassa* develops smaller perithecia that are submersed in the agar (KAYS *et al.* 2000), a phenotype that was not observed in the corresponding *S. macrospora* mutant, but in the Δsac1 disruption strain. The differences between Δgsa1, Δgsa2, and Δgsa3 mutants suggest that they are involved in different steps of sexual development, such as fruiting body development and germination of the ascospores. Expression analysis of several developmentally regulated genes in the Gα-mutants also indicates distinct roles for each of the subunits.

In Figure 8, a summary of our data is displayed in a model. According to this model different Gα-subunits interact with pheromone receptors or adenylyl cyclase and thus cooperatively regulate sexual development in *S. macrospora*. The *S. macrospora* double mutants Δgsa1Δgsa2 and Δgsa1Δgsa3 show a developmental block at the stage of protoperithecium formation. As depicted in Figure 8, in the case of the Δgsa1Δgsa2 mutant this would be explained by inactivation of the pathway downstream of the pheromone receptors. In the case of the Δgsa1Δgsa3 mutant, GSA2 is not able to compensate for the lack of GSA1. The corresponding *N. crassa* Δgna-1Δgna-2 and Δgna-1Δgna-3 mutants are also sterile, but progress somewhat further in development in that they develop aberrant perithecia lacking any ascospores (KAYS and BORKOVICH 2004). Moreover, comparison of the Δgsa2Δgsa3 double mutant with its *N. crassa* counterpart revealed that both mutants produce perithecia, but in *N. crassa* perithecia are sub-

mersed in the agar whereas they are present in their normal position in *S. macrospora*. In summary, our genetic data lead us to conclude that *gsa2* partially substitutes for *gsa1*, while *gsa3* is part of a parallel pathway also necessary for wild-type-like fertility. The sterile phenotype of both Δgsa1 double mutants and the fertile phenotype of the Δgsa2Δgsa3 strain indicate that GSA1 is the major component for fruiting body development, while GSA2 and GSA3 play a supplementary role. In addition, GSA3 is involved in ascospore germination (Figure 8).

Double mutants of Gα-subunits and pheromone receptors indicate a predominant role of *gsa1* in fruiting body development: Here we report for the first time phenotypes resulting from deletion of all Gα-subunit genes in combination with pheromone receptor genes in filamentous fungi. All receptor mutants in a Δ*gsa1* genetic background can be clearly distinguished from those in a Δ*gsa2* background. Whereas the Δgsa2Δpre1 and Δgsa2Δpre2 double mutants are fertile, the Δgsa1Δpre1 and Δgsa1Δpre2 mutants are almost totally blocked in sexual development, producing a strongly reduced number of perithecia compared with the single mutants and the wild-type strain. This phenotype resembles that of the recently described Δpre1 Δppg1 and Δpre2Δppg2 double mutants (Mayrhofer *et al.* 2006). The drastically reduced fertility of the Δgsa1Δpre mutants and the wild-type-like fruiting body formation of Δgsa2Δpre mutants point to the crucial role of the GSA1 subunit in transducing the signals from the PRE pheromone receptors in *S. macrospora*. The data are consistent with our model in which GSA1 is the main player in transducing the signal from the pheromone receptors whereas GSA2 plays a minor role (Figure 8). As indicated by the severe phenotype of the Δgsa1Δpre double mutants compared to the Δgsa1 single mutant, GSA2 alone can not properly transmit the signal, especially with only half of the receptors present. Thus, the data obtained with double mutants carrying a disrupted *gsa* gene together with a disrupted receptor gene indicate that the pheromone receptors interact differently with GSA1 or GSA2. *S. macrospora* is homothallic and both receptor genes are coexpressed within a single cell (Pöggeler and Kück 2001). The knockout of a single receptor in *S. macrospora* has no obvious effect on fruiting body development and indicates that one receptor can compensate for the loss of the other receptor (Mayrhofer *et al.* 2006). This is supported by a growing body of evidence suggesting that G protein-coupled receptors exist as homo- or heterooligomers (Bulenger *et al.* 2005; Overton *et al.* 2005). Overton *et al.* (2005) propose in a model that oligomerized receptors can activate a single G protein heterotrimer or alternatively each receptor monomer activates individual G proteins.

Deletion of adenylyl cyclase affects vegetative growth and sexual development in *S.macrospora*: In *S. macrospora*, the deletion of the adenylyl cyclase-encoding gene *sac1* leads to reduced mycelial growth. Similar phenotypes were described also for other adenylyl cyclase mutant strains from filamentous fungi including *Magnaporthe grisea, Sclerotinia sclerotiorum, Aspergillus fumigatus, Trichoderma virens* and *N. crassa* (Terenzi *et al.* 1974; Choi and Dean 1997; Liebmann *et al.* 2003; Jurick and Rollins 2007; Mukherjee *et al.* 2007). The addition of dibutyryl-cAMP to the Δsac1 strain of *S. macrospora* partially restores the growth defect as was previously shown for the close relative *N. crassa* (Terenzi *et al.* 1974; Rosenberg and Pall 1979). Besides the vegetative phenotype, the deletion of the adenylyl cyclase gene leads to an impairment in fruiting body development. In *M. grisea*, disruption of the adenylyl cyclase gene causes female sterility as no perithecia are produced (Choi and Dean 1997) and similarly, apothecium formation is eliminated in *S. sclerotiorum* (Jurick and Rollins 2007). In *N. crassa,* the *cr-1* mutant exhibits a delay in fruiting body and ascospore formation (Ivey *et al.* 2002). However, the *S. macrospora* Δsac1 mutant has reduced fertility with a significant number of the fruiting bodies being embedded in the solid media, thereby leading to fewer ascospores being discharged by the perithecia. Similar findings were also observed in the *N. crassa* Δgna-3 mutant (Kays *et al.* 2000). The Δsac1 ascospores have a highly reduced germination rate and this phenotype also resembles that of Gα-subunit 3 disruption mutants from *N. crassa* (Kays *et al.* 2000) and *S. macrospora*. A severe block in the initial step of ascospore germination has also been reported for an adenylyl cylase disruption strain of *S. pombe* (Hatanaka and Shimoda 2001). While we were able to restore perithecial formation of the Δsac1 mutant at the air-to-surface interface by adding db-cAMP to the culture medium, the ascospore germination rate was not elevated by this cAMP analog (data not shown). This might be due to the highly impermeable ascospore cell wall that may prevent the uptake of cAMP in the cell. While a vegetative fungal cell is surrounded by a two-layer cell wall, an ascospore cell wall consists of four layers, of which the two outer layers have an ascospore-specific composition (Neiman 2005). The most outward layer, composed predominantly of dityrosine molecules, is highly impermeable (Briza *et al.* 1990).

Genetic interaction of Gα-subunits and the adenylyl cyclase of *S.macrospora*: G protein-mediated signaling in fungi is transmitted via three major signal transduction pathways. Besides the MAP kinase and the phospholipase C (PLC)/PKC pathway, the activity of the AC can be regulated by heterotrimeric G proteins (McCudden *et al.* 2005). The latter pathway leads to the generation of the second messenger cAMP which in turn modulates the activity of protein kinase A (PKA) and thereby the activity of downstream effectors. In some fungi, the activity of the adenylyl cyclase is additionally regulated by Ras-GTPases. However, Gα-subunits of heterotrimeric G proteins are well known to regulate the activity of fungal adenylyl cyclases in different ascomy-

cetes and basidiomycetes (Bölker 1998; Lengeler *et al.* 2000; D'Souza and Heitman 2001; Versele *et al.* 2001).

To analyze the genetic interactions between the Gα-subunits and adenylyl cyclase in *S. macrospora*, we generated double mutants lacking each of the Gα-subunits in combination with the adenylyl cyclase. As one would expect from crossing the wild-type-like Δgsa2 mutant, the Δgsa2Δsac1 has the same phenotype as the Δsac1 mutant, similar to the Δgsa2Δgsa3 mutant that displays the Δgsa3 phenotype. The Δgsa3Δsac1 double mutant resembles the Δsac1 phenotype as it develops small perithecia that are embedded in the agar, indicating that *sac1* is epistatic to *gsa3* (Figure 8). This is further verified by the ascospore germination deficiency found in both mutants. The genetic interaction between *gsa3* and *sac1* is in agreement with the observations made for *N. crassa*, where GNA-3, the corresponding Gα-subunit, regulates the protein level of adenylyl cyclase (Kays *et al.* 2000). In *S. pombe*, a direct interaction of the GSA3 ortholog Gpa2 with the adenylyl cyclase Git2 was demonstrated (Ivey and Hoffman 2005). Disruption of *gpa2* or *git2* results in retarded spore germination (Hatanaka and Shimoda 2001), and thus resembles the reduced rate of ascospore germination in Δgsa3 or Δsac1 from *S. macrospora* and Δgna-3 from *N. crassa* (Kays *et al.* 2000). We therefore propose that the *S. macrospora* GSA3-SAC1 pathway is a prerequisite for efficient spore germination supposedly by sensing nutrients through a yet unidentified receptor (Figure 8). This signal transduction resembles glucose sensing in *S. cerevisiae* through the Gpr1p-Gpa2p-Cyr1p pathway (Colombo *et al.* 1998; Kraakman *et al.* 1999).

The most significant phenotype concerning fruiting body development was observed in the Δgsa1Δsac1 double mutant that produces protoperithecia, but is unable to develop any perithecia. This implies that cAMP is required to develop mature perithecia in a Δ*gsa1* background. To further analyze the function of SAC1 in fruiting body development, we generated a Δgsa2Δgsa3Δsac1 triple mutant. While the Δgsa2Δgsa3 mutant is fully fertile concerning fruiting body development, the triple mutant is almost sterile showing a drastically reduced number of perithecia, thus confirming a functional interaction of the Gα-subunit GSA1 and the adenylyl cyclase SAC1 in fruiting body development. Interestingly, a direct regulation of adenylyl cyclase activity through the corresponding Gα-subunit GNA-1 from *N. crassa* has already been suggested (Ivey *et al.* 1999; Kays and Borkovich 2004). The involvement of both parallel pathways in fruiting body formation is verified by the sterile phenotype of the Δgsa3Δste12 double mutant, which only develops protoperithecia.

Taken together, we propose that both gene products, GSA3 and SAC1, act in a common signaling pathway. Additionally, the sterility of the Δgsa1Δsac1 double mutant indicates that the GSA3/SAC1 pathway functions in parallel to the GSA1/GSA2 pathway in fruiting body development. Both pathways are linked by the functional interaction of GSA1 with SAC1, as indicated by the Δgsa2Δgsa3Δsac1 triple mutant. The sterility of the Δgsa3Δste12 mutant is strong evidence for STE12 being one of several key regulators downstream of the pheromone receptors. This is reminiscent of signaling networks in the basidiomycete *Ustilago maydis*, where the pheromone-response factor Prf1, a transcription factor which recognizes pheromone response elements, is a key signaling node that mediates crosstalk between cAMP and MAP kinase pathways (Krüger *et al.* 1998; Feldbrügge *et al.* 2006).

In summary, the data presented here represent a comprehensive analysis of the contribution of all Gα-subunits combined with both pheromone receptor genes as well as downstream effector adenylyl cyclase and the transcription factor STE12 in fungal fruiting body development. Use of this powerful genetic approach will allow further dissection and better understanding of the key components of signaling pathways in filamentous fungi.

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