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Biochemical and Genetic Analysis of Dominant-Negative Mutations Affecting a Yeast G-Protein γ Subunit

ANATOLY V. GRISHIN, JENNIFER L. WEINER,† AND KENDALL J. BLUMER*

Department of Cell Biology and Physiology, Washington University School of Medicine, St. Louis, Missouri 63110

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Heterotrimeric guanine nucleotide-binding proteins (G proteins) consisting of α, β, and γ subunits mediate signalling between cell surface receptors and intracellular effectors in eukaryotic cells. To define signalling functions of G_γ subunits (*STE18* gene product) involved in pheromone response and mating in the yeast *Saccharomyces cerevisiae*, we isolated and characterized dominant-negative *STE18* alleles. We obtained dominant-negative mutations that disrupt C-terminal sequences required for prenylation of G_γ precursors (CAAX box) and that affect residues in the N-terminal half of Ste18p. Overexpression of mutant G_γ subunits in wild-type cells blocked signal transduction; this effect was suppressed upon overexpression of G_β subunits. Mutant G_γ subunits may therefore sequester G_β subunits into nonproductive $G_{\beta\gamma}$ dimers. Because mutant G_γ subunits blocked the constitutive signal resulting from disruption of the G_α subunit gene (*GPA1*), they are defective in functions required for downstream signalling. Ste18p bearing a C107Y substitution in the CAAX box displayed reduced electrophoretic mobility, consistent with a prenylation defect. G_γ subunits carrying N-terminal substitutions had normal electrophoretic mobilities, suggesting that these proteins were prenylated. G_γ subunits bearing substitutions in their N-terminal region or C-terminal CAAX box (C107Y) supported receptor-G protein coupling in vitro, whereas C-terminal truncations caused partial defects in receptor coupling.

Heterotrimeric guanine nucleotide-binding proteins (G proteins), consisting of α, β, and γ subunits, transduce signals from cell surface receptors to various intracellular effectors in eukaryotic cells. In many organisms, there are multiple subtypes of G_α, G_β, and G_γ subunits that associate in various combinations to yield distinct types of heterotrimers. The properties of different heterotrimers indicate that various subtypes of G-protein subunits control signalling in several ways. For example, G_α subunit subtypes mediate selective coupling between receptors and G proteins and act selectively upon downstream effector molecules, including subtypes of adenylyl cyclase, phospholipase C, and ion channels (1, 15, 39, 44).

G_β and G_γ subunits also control signal transduction pathways at several steps. Specific G_β and G_γ subunit subtypes are required for coupling between certain receptors and effectors (11, 21, 22), presumably at the level of receptor-G protein interaction. $G_{\beta\gamma}$ subunits also control signalling pathways by regulating intracellular effector molecules, including phospholipase Cβ (2, 6, 7, 20) and type I and type II adenylyl cyclase (12, 17, 45, 46). Indeed, G_γ subunit subtypes determine the ability of $G_{\beta\gamma}$ dimers to regulate adenylyl cyclase in vitro (17). However, in general, the structural features of G_β and G_γ subunits responsible for specific signalling functions have not been defined. An exception is the CAAX box of G_γ subunits, the site of C-terminal prenylation (8, 13, 24, 27, 35), which is important for G_γ membrane localization (17, 30, 40) and efficient rhodopsin-transducin (14, 32, 54) and $G_{\beta\gamma}$-adenylyl cyclase (17) interactions.

The mating pheromone response pathway of the yeast *Saccharomyces cerevisiae* provides a useful model system for dissecting the functions of G_β and G_γ subunits in different signal transduction steps and for defining structural features of these subunits that influence their function. Pheromone binding to cell surface receptors elicits responses that prepare yeast cells for mating, including arrest in the G_1 phase of the cell cycle, induction of specific genes, and elaboration of morphological changes (23, 43). Genetic and biochemical studies indicate that pheromone receptors are coupled to a G protein whose α, β, and γ subunits are encoded, respectively, by the *GPA1*, *STE4*, and *STE18* genes (5, 10, 29, 52). Biochemical evidence indicates that G_α, G_β, and G_γ subunits form $G_{\alpha\beta\gamma}$ heterotrimers that can interact with pheromone receptors (5), and genetic analysis indicates that $G_{\beta\gamma}$ subunits rather than G_α subunits transduce signals to downstream components in the pathway (3, 9, 31, 53). Dominant-negative forms of yeast G_β and G_γ subunits that disrupt signalling, albeit by mechanisms yet to be defined biochemically, have been identified (26, 51).

To determine the functions and structural features of G_γ subunits required for different steps in the pheromone signal transduction pathway, we have isolated and characterized dominant-negative alleles of the *STE18* gene. Genetic and biochemical analyses have been used to address the properties of mutant G_γ subunits with regard to subunit association, prenylation, and downstream signalling and receptor-coupling activities.

MATERIALS AND METHODS

***S. cerevisiae* strains, media, and transformation.** *S. cerevisiae* strains used in this study are listed in Table 1. AG39-4C was derived as a meiotic segregant from a cross between a *MAT*α derivative of J57D and KMG49-1D. Standard growth media (YPD and SD) were used (36, 37). YPGal and SGal media were identical to YPD and SD except that they contained 2% galactose

* Corresponding author. Mailing address: Department of Cell Biology and Physiology, Washington University School of Medicine, Box 8228, 660 South Euclid Ave., Saint Louis, MO 63110. Phone: (314) 362-1668. Fax: (314) 362-7463.

† Present address: University of Vermont School of Medicine, Burlington, VT 05405.

TABLE 1. *S. cerevisiae* strains used in this study

Strain	Genotype	Reference or source
RK511-6B	*MAT***a** *ade2 his3-Δ1 his6 ura3-52 leu2-3,112 trp1 sst1-3*	4
RK511-6B-1	RK511-6B *ste18Δ::URA3*	This study
RK511-6B-12	RK511-6B *STE18*R33K,E36K::*URA3*	This study
RK511-6B-19	RK511-6B *STE18*Q94ter::*URA3*	This study
RK511-6B-28	RK511-6B *STE18*R47P::*URA3*	This study
RK511-6B-38	RK511-6B *STE18*R33K::*URA3*	This study
RK511-6B-54	RK511-6B *STE18*R47H,Q48K::*URA3*	This study
RK511-6B-74	RK511-6B *STE18*C107Y::*URA3*	This study
RK511-6B-75	RK511-6B *STE18*Q98ter::*URA3*	This study
RK511-6B-56A	RK511-6B *adh1Δ::ADH1pSTE18*R33K::*URA3*	This study
J57D	*MAT***a** *ade2 his3-11,13 ura3-52 leu2-3,112 trp1 can1*	15a
J57D-1	J57D *ste18Δ::ura3*	This study
J57D-12	J57D *STE18*R33K,E36K::*URA3*	This study
J57D-28	J57D *STE18*R47P::*URA3*	This study
J57D-54	J57D *STE18*R47H,Q48K::*URA3*	This study
J57D-56	J57D *STE18*R33K::*URA3*	This study
J57D-74	J57D *STE18*C107Y::*URA3*	This study
J57D-75	J57D *STE18*Q98ter::*URA3*	This study
W303-1B	*MATα ura3 leu2 trp1 his3 ade2 can1*	47
KMG49-1D	*MAT***a** *gpa1::HIS3 cln1::URA3 cln2::LEU2 ura3 leu2 his3 trp1 ade1*[pG1302]	18
YPH102	*MATα ade2 his3-Δ200 lys2 ura3-52 leu2-Δ1*	P. Hieter
AG39-4C	*MATα ade1 his3 ura3 leu2 trp1 gpa1::HIS3* [pG1302]	This study

and 0.1% raffinose instead of glucose. Yeast transformations were done by the alkali cation procedure (19).

Plasmids, mutagenesis, and genetic methods. pBH21 (provided by D. Jenness) is a 2μ-based plasmid carrying the *LEU2* gene and the *STE18* gene placed under control of the *ADH1* promoter. pL19 (provided by M. Whiteway) carries the *STE4* gene fused to the *GAL1* promoter (53). pAG3*STE4* is a pRS313 derivative that carries the *GAL1-STE4* expression cassette derived from pL19. Plasmid pVT-HA*STE18* (provided by F. Tamanoi) is a derivative of the pVT100-U expression vector (49) containing an influenza virus hemagglutinin (HA) epitope-tagged form of the *STE18* gene (13). Plasmid pRS315*STE18* was constructed by isolating a *Pst*I-*Sac*I fragment bearing the entire *STE18* promoter and coding region from plasmid M70p2 (50) and inserting it into *Pst*I-*Sac*I-cut pRS315. Yeast genomic DNA for Southern blots and PCR experiments was purified by published procedures (33). Mutagenesis of pBH21 was performed as described previously (38) except that hydroxylamine-treated DNA was purified by precipitation with isopropanol and transformed directly into yeast cells. Specific mutations in the *STE18* gene in pVT-HA*STE18* were constructed by oligonucleotide-directed mutagenesis (Amersham). Mutations in *STE18* were confirmed by sequencing reactions that used primers synthesized according to the published *STE18* sequence (52). For replacement of the chromosomal *STE18* gene with *STE18*^{dn} (dn for dominant-negative) alleles, derivatives of pBH21 carrying *STE18*^{dn} mutations were linearized with *Bcl*I, which cuts 185 bp downstream of the *STE18* coding sequence, and ligated with a 1.1-kb *Bgl*II fragment of pVT100-U containing the *URA3* gene. Plasmids carrying the *URA3* gene inserted between *STE18* coding and 3′ flanking sequences were isolated and cut with *Hin*dIII and *Sst*I to release a 1.75-kb *STE18-URA3* fragment. This fragment was purified and used for one-step gene replacement (34). The *STE18* coding sequence was disrupted as described above by using an *Sph*I-*Hin*dIII fragment from plasmid M59p7 (52). PCR was used to confirm the structure of chromosomal replacements. For integration of the *ADH1p-STE18*R34K expression cassette at the *ADH1* locus, a fragment bearing the *URA3* and *STE18* coding sequences flanked by noncoding regions of *ADH1* was purified and used to replace the *ADH1* chromosomal allele. The resulting replacement was confirmed by Southern blotting. Other genetic manipulations were performed by standard methods (36, 37).

Assays of pheromone response. Quantitative mating assays were performed essentially as described previously (42). Cells to be tested for mating ability (10^7 cells) and a fivefold excess of the mating-type tester YPH102 were mixed and incubated 8 h at 30°C. Mating was scored by complementation of auxotrophic markers. Mating efficiencies of cells expressing *STE18*^{dn} mutations were expressed relative to the mating efficiency of an isogenic wild-type control strain. Assays of pheromone-induced growth inhibition (halo assays) were performed as described previously (42). Assays of pheromone-induced gene expression used cells carrying pSL307, a plasmid that bears the pheromone-inducible reporter gene *FUS1-lacZ* (28). Cells in early logarithmic phase were treated with various concentrations of α-factor for 2 h, and β-galactosidase activity in permeabilized cells was determined as described previously (42).

Immunoblotting methods. Methods used to lyse cells with glass beads in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer have been described elsewhere (4, 5). Cells used for these experiments carried pVT-HA*STE18* or its derivatives expressing dominant-negative mutations. Protein samples from equal numbers of cells resolved on 20-cm 16% polyacrylamide gels were subjected to immunoblotting with the anti-HA monoclonal antibody 12CA5 (BAbCO). Immunoblots were developed by using enhanced chemiluminescence (Amersham).

Assays of receptor-G protein coupling. Derivatives of RK511-6B expressing various chromosomal *STE18* alleles were used as sources of membrane fractions. Methods for cell growth, extract preparation, and preparation of ^{35}S-labeled α-factor have been described elsewhere (4, 5). Assay mixtures contained membranes (500 μg of protein) and ^{35}S-labeled α-factor (10 nM, 50 Ci/mmol). For determinations of nonspecific binding, parallel reaction mixtures also contained excess unlabeled α-factor (1 μM). After a 1-h incubation, the mixture was diluted 1:100 into a solution containing 1 μM unlabeled α-factor and 10 μM GTPγS or into the same solution lacking GTPγS. Aliquots were withdrawn at intervals, filtered through polyethyleneimine-treated GF/C filters (Whatman), washed, and analyzed by liquid scintillation spectrometry. Data were expressed as the fraction of specific binding sites that remained bound as a function of time.

RESULTS

Isolation and sequence of *STE18* dominant-negative mutations. Because dominant-negative mutations disrupt certain functions of proteins while leaving other functions relatively unaffected (16), they may provide useful tools for identifying regions of G_γ subunits that influence their ability to associate with other G-protein subunits, interact functionally with pheromone receptors or signalling effectors, or undergo functionally important conformational changes. Accordingly, to gener-

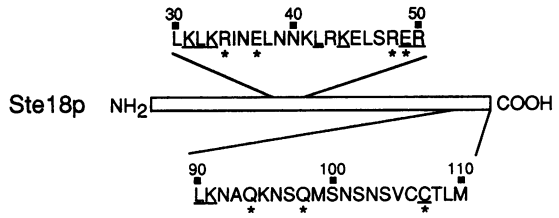

FIG. 1. Sequences altered by *STE18*^{dn} mutations. Amino acid sequences of the N- and C-terminal regions of Ste18p affected by *STE18*^{dn} mutations are displayed in the single-letter amino acid code. Residues affected by *STE18*^{dn} mutations are indicated by asterisks. The following *STE18*^{dn} mutations were identified: *STE18*R34K (three isolates); *STE18*E37K (one isolate); *STE18*R34K,E37K (double mutant, two isolates); *STE18*R48P (one isolate); *STE18*R48H,E49K (double mutant, two isolates); *STE18*Q94ter (one isolate); *STE18*Q98ter (one isolate); and *STE18*C107Y (three isolates). Residues conserved between Ste18p and bovine transducin γ (52) are underlined. Within the regions shown, the position of every 10th amino acid residue in Ste18p is indicated.

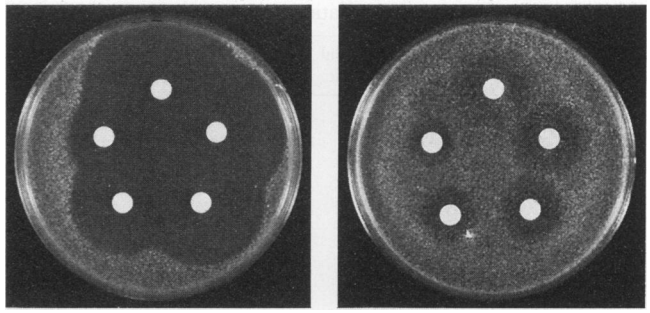

FIG. 2. Pheromone-induced growth arrest assays of wild-type cells overexpressing a *STE18*^{dn} allele. Control cells (RK511-6B, an *sst1* strain; left) and an isogenic derivative (RK511-6B-56A; right) carrying an *ADH1*-driven *STE18*^{dn} allele (*STE18*R34K) integrated at the *ADH1* locus were embedded in YPD top agar. Various amounts of α-factor (10, 2, 0.4, 0.08, and 0.016 μg, decreasing clockwise from the top) were applied on disks, and the plates were incubated for 2 days at 30°C. Cells overexpressing the *STE18*^{dn} allele gave rise to zones of growth inhibition (halos) that were smaller and turbid.

ate dominant-negative alleles of the gene encoding G_γ subunits (*STE18*) that are required for mating pheromone response in *S. cerevisiae*, we used hydroxylamine to treat a plasmid (pBH21) in which the *STE18* gene is expressed from a strong constitutive promoter (*ADH1*). Treated plasmid DNA was introduced by transformation into wild-type cells (J57D). Cells expressing putative dominant-negative *STE18* alleles that interfere with pheromone response were selected as colonies that grew in the presence of pheromone (α-factor, 1 μM). Thirteen transformants in which the pheromone-resistant phenotype was plasmid dependent were identified. Plasmids from these transformants were recovered in *Escherichia coli*, and the entire *STE18* coding region of each plasmid was sequenced to identify lesions responsible for the pheromone-resistant phenotype. Sequencing revealed that mutations were obtained resulting in the following substitutions in Ste18p: R34K (three isolates): R34K,E37K (double mutant, one isolate); E37K (one isolate); R48P (one isolate); R48H,E49K (double mutant, two isolates); Q94ter (one isolate); Q98ter (one isolate); and C107Y (three isolates). *STE18*^{dn} mutations therefore affected two regions of the G_γ subunit (Fig. 1): an N-terminal region containing residues 34 to 49, and the C-terminal region containing the CAAX box of Ste18p. Mutations affecting the CAAX box either substituted the essential cysteine residue for tyrosine (C107Y) or introduced nonsense codons upstream (Q94ter, Q98ter).

Overexpression of *STE18*^{dn} mutations in wild-type cells blocks signal transduction. Overexpression of *STE18*^{dn} alleles in wild-type cells could cause pheromone resistance because they promote recovery from pheromone-induced cell cycle arrest or because they encode signalling-defective G_γ subunits that block signalling, for example, by titrating an essential signalling component. To address these possibilities, we examined several signalling-related phenotypes of haploid cells that overexpressed various *STE18*^{dn} alleles from the *ADH1* promoter.

First, we determined if haploid cells overexpressing *STE18*^{dn} alleles could undergo pheromone-induced G_1 arrest. To eliminate possible ambiguities arising from plasmid loss, a *STE18*^{dn} allele (*STE18*R34K) was integrated and overexpressed at the *ADH1* locus. Halo assays were used in which control cells (RK511-6B, an *sst1* strain) or those overexpressing *STE18*^{dn} mutations (RK511-6B-56A) were plated in top agar and challenged with various doses of α-factor applied to disks. As shown in Fig. 2, control cells underwent growth arrest, as indicated by a zone of growth inhibition (halo) around the disks. Halos appearing in lawns of cells overexpressing the *STE18*^{dn} allele were smaller and turbid. Similar results were obtained with cells overexpressing any of the *STE18*^{dn} mutations from plasmids (data not shown). Formation of small turbid halos indicated that even at high levels of pheromone, growth arrest was transient or incomplete.

Second, we determined whether overexpression of *STE18*^{dn} alleles affected pheromone-induced expression of the *FUS1-lacZ* reporter gene (28, 48). Cells (J57D) carrying a *FUS1-lacZ* reporter plasmid (pSL307), and either a control plasmid or plasmids that overexpress *STE18*^{dn} alleles, were treated with various doses of α-factor. Pheromone-induced gene expression was monitored by measuring β-galactosidase activity in permeabilized cells. As indicated by the dose-response curves shown

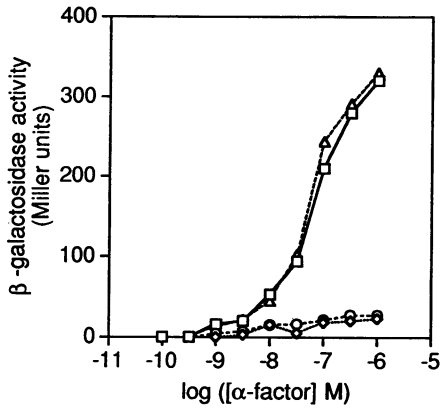

FIG. 3. Pheromone-induced transcription in wild-type cells and cells overexpressing *STE18*^{dn} alleles. Wild-type cells (J57D) carried a *FUS1-lacZ* reporter plasmid (pSL307) and either a control plasmid (YEp13; squares) or a plasmid that overexpresses the wild-type *STE18* gene (pBH21; triangles), *STE18*R34K (pBH21-R34K; circles), or *STE18*C107Y (pBH21-C107Y; diamonds). Plasmid-containing cells were treated for 2 h with the indicated concentrations of α-factor, and β-galactosidase activity was measured in permeabilized cells. The data presented are averages of three independent determinations for each plasmid combination.

TABLE 2. Mating phenotypes

STE18 allele overexpressed[a]	Chromosomal *STE18* allele	*STE4* overexpressed[b]	Relative mating efficiency[c]
STE18	*STE18*	−	1.0
		+	0.7
*STE18*R34K	*STE18*	−	1.4×10^{-3}
		+	0.35
*STE18*R34K,E37K	*STE18*	−	1.2×10^{-3}
		+	0.49
*STE18*R48P	*STE18*	−	1.1×10^{-3}
		+	0.36
*STE18*R48H,E49K	*STE18*	−	1.2×10^{-3}
		+	0.44
*STE18*Q94ter	*STE18*	−	1.0×10^{-3}
		+	0.38
*STE18*C107Y	*STE18*	−	2.0×10^{-3}
		+	0.41
*STE18*R34K	*ste18*Δ::*URA3*	−	$<10^{-5}$
		+	$<10^{-5}$
*STE18*Q94ter	*ste18*Δ::*URA3*	−	$<10^{-5}$
		+	$<10^{-5}$

[a] *STE18* alleles were overexpressed from the *ADH1* promoter on plasmid pBH21.
[b] The *STE4* gene was overexpressed from the *GAL1* promoter on plasmid pL19 or pAG3*STE4*.
[c] Mating efficiencies were determined relative to the mating frequency of wild-type cells (J57D) that carried the indicated plasmids. Values shown are averages of three determinations.

TABLE 3. Tetrad analysis[a]

STE18 genotype of parental diploid	No. of tetrads containing:		
	4 viable ascospores	3 viable ascospores	2 viable ascospores
STE18/*STE18*	0	0	18
*STE18*R34K/*STE18*	0	13	1
*STE18*R34K,E37K/*STE18*	3	11	0
*STE18*R48P/*STE18*	1	13	0
*STE18*R48H,E49K/*STE18*	1	8	2
*STE18*C107Y/*STE18*	2	7	1
*STE18*Q98ter/*STE18*	0	6	1

[a] Diploids heterozygous for *GPA1*, *gpa1*::*HIS3*, *STE18*, and *STE18*^{dn} were constructed by crossing AG39-4C (a *gpa1*::*HIS3 STE18* strain carrying the *GPA1* gene on plasmid pG1302) with J57D derivatives expressing various *STE18*^{dn} chromosomal replacements (the wild-type *STE18* gene was carried on plasmid pBH21 to permit mating). Control diploids homozygous for *STE18* and heterozygous for *GPA1* and *gpa1*::*HIS3* were constructed by crossing AG39-4C and J57D. After diploids were cured of plasmids pG1302 and pBH21, they were sporulated and asci were dissected. Viable ascospores produced colonies after a 2-day incubation at 30°C on YPD agar. No viable His$^+$ Ura$^-$ segregants were found in these tetrads, and all Ura$^+$ segregants were sterile.

in Fig. 3, overexpression of *STE18*R34K or *STE18*C107Y, but not wild-type *STE18*, interfered at least 10-fold with induction of *FUS1-lacZ* expression. *FUS1-lacZ* expression was blocked to a similar extent in cells overexpressing any of the other *STE18*^{dn} alleles (data not shown). The magnitude of this apparent signalling defect may be an underestimate because cells that spontaneously lose plasmids overexpressing *STE18*^{dn} alleles might induce *FUS1-lacZ* expression.

As a third means of assessing signalling efficiency, quantitative mating assays were performed. Compared with control cells (J57D) overexpressing the wild-type *STE18* gene, wild-type cells overexpressing *STE18*^{dn} alleles mated at efficiencies that were lower by 3 orders of magnitude (Table 2). Because signal transduction is essential for mating, low mating efficiencies indicated that overexpression of *STE18*^{dn} alleles in wild-type cells caused a strong signalling block.

To determine whether *STE18*^{dn} mutations were formally dominant, we introduced the wild-type *STE18* gene on a centromere-containing plasmid (pRS315*STE18*) into cells (RK511-6B derivatives) expressing chromosomal *STE18*^{dn} alleles from the *STE18* promoter. All of the *STE18*^{dn} mutations were tested in this way. In every case, plasmid-containing cells displayed wild-type pheromone sensitivity, as judged by halo assays (data not shown). Therefore, all of the *STE18*^{dn} mutations that we have identified were recessive when expressed in single copy. Nevertheless, we refer to these alleles as *STE18*^{dn} mutations because they are dominant when overexpressed in wild-type cells.

Mutant G_γ subunits could block signalling in wild-type cells by sequestering a limited pool of functional G_β subunits as inactive $G_{\beta\gamma}$ dimers. To test this hypothesis, we simultaneously overexpressed *STE18*^{dn} alleles and the G_β subunit gene, *STE4*, in wild-type cells (J57D). As predicted, co-overexpression of *STE4* with any of the *STE18*^{dn} alleles resulted in mating at nearly wild type efficiencies (Table 2), although we have not determined under these conditions whether the mating pathway is constitutively activated or dependent on mating pheromone. We did find that overexpression of *STE4* suppressed the mating defect caused by overexpression of *STE18*^{dn} mutations only in cells expressing a functional chromosomal *STE18* allele (Table 2). Overexpressed G_β subunits were therefore incapable of directly restoring activity to mutant G_γ subunits. These results therefore suggested that mutant forms of G_γ subunits may block signalling in wild-type cells by sequestering a limited pool of G_β subunits as signalling-defective $G_{\beta\gamma}$ dimers.

Replacement of the chromosomal *STE18* allele by *STE18*^{dn} mutations disrupts signalling. To determine directly whether *STE18*^{dn} alleles encode signalling-defective G_γ subunits, we replaced the wild-type *STE18* allele with various *STE18*^{dn} alleles in two different genetic backgrounds (Table 1) and evaluated the pheromone responsiveness of the resultant strains. Cells expressing *STE18*^{dn} alleles (except the C107Y allele) exhibited signalling defects similar to those of cells carrying a *ste18* null mutation: the inability to mate ($<10^{-5}$ of wild-type mating efficiency), undergo pheromone-induced cell cycle arrest (halo formation), or induce expression of a pheromone-responsive reporter construct (*FUS1-lacZ*) (data not shown). Cells expressing the C107Y allele did mate at detectable efficiencies (0.01 to 0.4% of the wild-type control level, depending on unknown factors in different strain backgrounds), although they did not respond significantly to pheromone, as judged by an inability to form halos and induce expression of *FUS1-lacZ* (data not shown).

As a further means of testing whether *STE18*^{dn} alleles encode signalling-defective G_γ subunits, we determined whether they can block the constitutive signal resulting from disruption of the G_α subunit gene, *GPA1* (3, 10, 29). Constitutive signalling causes a haploid-lethal phenotype because cells remain arrested in the G_1 phase of the cell cycle. For these experiments, we constructed diploids that were heterozygous for both a *gpa1* disruption (marked with *HIS3*) and a chromosomal *STE18*^{dn} replacement (marked with *URA3*). Upon sporulation of these strains, tetrad analysis was used to monitor the meiotic segregation of a *gpa1*-associated haploid-lethal phenotype (Table 3). In a control diploid heterozygous for the *gpa1*::*HIS3* disruption and homozygous for wild-type *STE18*, tetrads contained no more than two viable ascospores that were always His$^-$, consistent with a haploid-lethal phenotype of the *gpa1*::*HIS3* disruption. By contrast, tetrads derived from diploids heterozygous for the *gpa1*::*HIS3* disruption and

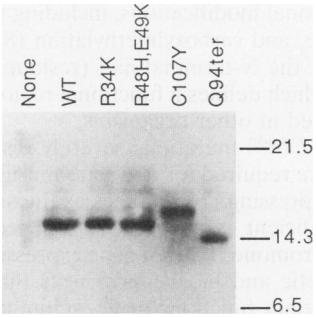

FIG. 4. Electrophoretic mobilities of wild-type and mutant G_γ subunits. HA epitope-tagged wild-type (WT) and mutant G_γ subunits were overexpressed from pVT-HA*STE18* derivatives in the haploid strain RK511-6B. Equivalent amounts of extracts prepared by lysis of cells in SDS-PAGE sample buffer were resolved by SDS-PAGE. Immunoblotting was done with anti-HA monoclonal antibody 12CA5 and an enhanced chemiluminescence detection system. Positions of molecular weight markers are indicated in kilodaltons. The overexpressed *STE18* alleles are indicated. Reduced mobility of Ste18p bearing the C107Y substitution indicated a prenylation defect.

a *STE18*^{dn} replacement frequently contained more than two viable ascospores. In these tetrads, the His^+ phenotype always segregated with a Ura^+ phenotype, indicating that segregants carrying the *gpa1* disruption were viable only if they also received a *URA3*-marked *STE18*^{dn} allele. This analysis indicated that *STE18*^{dn} mutations affecting either the N-terminal half or the CAAX box of Ste18p blocked the constitutive signal that otherwise occurs in the absence of G_α subunits. Thus, all of the mutant forms of G_γ subunits that we have identified were defective in functions required for signalling after the step in the pheromone response pathway controlled by the G_α subunit.

Prenylation of mutant G_γ subunits. Precursors of yeast G_γ subunits undergo posttranslational prenylation (13), probably at cysteine 107, the first residue of the Ste18p CAAX box. Because this modification is required for downstream signalling by $G_{\beta\gamma}$ subunits in yeast cells (13), prenylation defects probably account for the phenotypes of the C-terminal class of *STE18*^{dn} alleles. In contrast, signalling-defective forms G_γ subunits bearing N-terminal substitutions were likely to undergo prenylation because prenyltransferases primarily recognize sequences within or near the CAAX box of their protein or peptide substrates (8, 35).

To explore whether G_γ subunits bearing CAAX box or N-terminal substitutions were prenylated, we examined the electrophoretic mobilities of wild-type and mutant G_γ subunits that were tagged with an influenza virus HA epitope and expressed from the *ADH1* promoter in haploid cells (RK511-6B). Although this method does not determine directly whether G_γ subunits are prenylated, it has been used to demonstrate that Ste18p exhibits reduced electrophoretic mobility in *dpr1* (*ram1*) mutants (13), which are defective in the β subunit of a farnesyltransferase.

The results obtained from immunoblotting experiments are shown in Fig. 4. Wild-type Ste18p migrated as a single 15-kDa species. Consistent with prior studies (13), Ste18p bearing a C107Y substitution migrated more slowly than the wild-type protein, presumably because it was not prenylated. G_γ subunits truncated at position 94, proximal to the CAAX box, migrated as a 14.3-kDa species. Because these molecules are truncated, their mobility does not indicate whether prenylation occurred; however, because these molecules lack a CAAX box, we assume that they are not prenylated. In contrast, G_γ subunits bearing N-terminal substitutions (R34K or R48H,E49K) comigrated with wild-type G_γ subunits. Similar results were obtained with other N-terminal substitutions (data not shown). These results provided indirect evidence that substitutions in the N-terminal portion of Ste18p probably do not disrupt prenylation of the protein.

Effects of *STE18*^{dn} mutations on receptor-G protein coupling. Although G_γ subunits expressed from *STE18*^{dn} alleles were defective in functions required for downstream signalling, they could also cause defects in coupling between G-protein heterotrimers and pheromone receptors. Accordingly, we examined the ability of α-factor receptors to couple with G proteins in membrane fractions isolated from cells expressing various *STE18*^{dn} chromosomal replacements. Assays used for this purpose rely on the observation that at pH 8.0 and high ionic strength, the affinity of α-factor receptors for agonist decreases about 10-fold in the presence of guanine nucleotides (5). This effect presumably occurs because receptors and nucleotide-free G proteins form ternary complexes that bind α-factor with high affinity. Upon binding guanine nucleotides, G proteins are activated, thereby yielding receptors that bind α-factor with low affinity. These effects can be observed as guanine nucleotide-dependent increases in the dissociation rate of ^{35}S-labeled α-factor from receptors.

Figure 5 shows the results of receptor-G protein coupling assays using membranes prepared from wild-type cells, a *ste18* null mutant, and cells expressing various *STE18*^{dn} chromosomal replacements. In membranes prepared from wild-type cells, GTPγS-dependent increases in pheromone dissociation rates were observed. In membranes prepared from an isogenic *ste18* null mutant, α-factor dissociated rapidly from receptors in a GTPγS-independent manner, demonstrating that G_γ subunits are essential for supporting receptor-G protein coupling. In contrast, when membranes isolated from cells expressing *STE18*R34K or *STE18*C107Y were used, pheromone dissociation rates were similar to those observed in experiments using membranes from wild-type cells (Fig. 5). Similar results were obtained in experiments using membranes isolated from cells expressing the *STE18*R34K,E37K, *STE18* E37K, *STE18*R48P, and *STE18*R48H,E49K mutations (data not shown). These results indicated that missense mutations affecting either the CAAX box or the N-terminal half of Ste18p did not significantly perturb receptor-G protein coupling.

Quite different results were obtained in experiments using membranes from cells expressing nonsense mutations (*STE18*Q94ter and *STE18*Q98ter) that truncate Ste18p at points proximal to the CAAX box. Here there was evidence of reduced coupling between receptors and mutant G proteins. In the absence of GTPγS, α-factor dissociated from receptors at intermediate rates, and increases in ligand dissociation rates following the addition of GTPγS were less pronounced (Fig. 5).

One reason why C-terminally truncated G_γ subunits could cause receptor coupling defects in vitro is that they are expressed at significantly lower levels. To address this issue, we used densitometric scanning procedures to analyze immunoblots (Fig. 4) of extracts prepared from cells (RK511-6B) expressing HA-tagged forms of wild-type and mutant G_γ subunits. Mutant forms of G_γ subunits bearing the Q94ter truncation (coupling defective) and the C107Y substitution (coupling proficient) were expressed at similar levels (60 and 70%, respectively, of the levels of wild-type Ste18p). Furthermore, overexpression of C-terminally truncated G_γ subunits did not improve receptor-G protein coupling activity in vitro (data not shown).

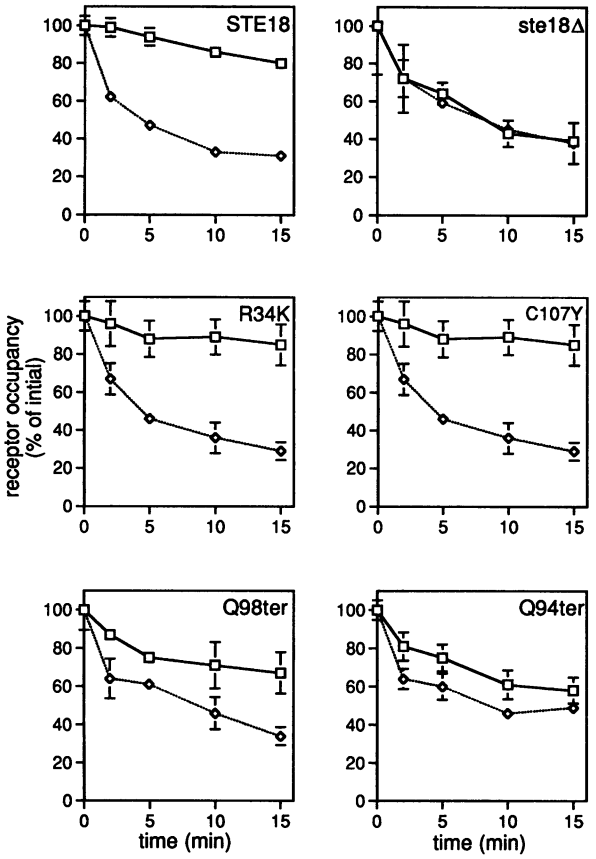

FIG. 5. Effects of *STE18*^{dn} mutations on receptor-G protein coupling activity in vitro. Membrane preparations isolated from wild-type cells (RK511-6B) or isogenic derivatives expressing the indicated *STE18*^{dn} chromosomal replacements were analyzed for receptor-G protein coupling activity in vitro as described in Materials and Methods. Dissociation of ^{35}S-labeled α-factor bound to receptors in the absence (squares) or presence (diamonds) of GTPγS (10 μM) was determined. Data were corrected for nonspecific binding (which was <30% of specific binding). Initial levels of receptor binding were as follows: *STE18*, 117 fmol/μg; *ste18*Δ, 15 fmol/μg; *STE18*R34K, 31 fmol/μg; *STE18*C107Y, 23 fmol/μg; *STE18*Q98ter, 19 fmol/μg; and *STE18*Q94ter, 30 fmol/μg. Data shown are averages of three independent determinations for each strain. Vertical bars indicate standard errors.

DISCUSSION

We have isolated eight dominant-negative alleles of the *STE18* gene, which encodes the G-protein γ subunit that is required for signal transduction in the mating pheromone response pathway of the yeast *S. cerevisiae*. Similar *STE18*^{dn} alleles have been described previously (51), but specific biochemical defects of the mutant G_γ subunits have not been established. The genetic and biochemical studies that we have performed suggest that dominant-negative mutations affecting yeast G_γ subunits influence coupling between G-protein heterotrimers and pheromone receptors and disrupt signalling between $G_{\beta\gamma}$ complexes and elements downstream in the pathway. Whether G_γ subunits physically interact with receptors or downstream signalling components remains to be established.

STE18^{dn} mutations that we have identified affect two regions of the G_γ subunit, in general agreement with a previous study (51). Three mutations affect the C-terminal CAAX box, a site for posttranslational modifications, including prenylation, proteolytic cleavage, and carboxylmethylation (8, 35). Five other mutations affect the N-terminal half (residues 34, 37, 48, and 49) of Ste18p, which defines a functional region of G_γ subunits not yet implicated in other organisms.

All of the *STE18*^{dn} mutations severely disrupt G_γ subunit functions that are required for downstream signal transduction because cells expressing these alleles as the sole source of G_γ subunits are deficient in pheromone-induced growth arrest, mating, and pheromone-induced gene expression. As discussed below, the genetic and biochemical tests that we have used indicate that certain functions of G_γ subunits are not significantly disrupted by these mutations, while other functions have been perturbed.

Several observations indicate that *STE18*^{dn} mutations preserve the ability of G_γ subunits to associate with G_α and G_β subunits. First, whereas mutations that completely disrupt association of G_β and G_γ subunits should be recessive, *STE18*^{dn} mutations are dominant when overexpressed. Second, most of the mutant G_γ subunits, including those bearing substitutions in the N-terminal region or a C107Y substitution in the CAAX box, support relatively efficient receptor-G protein coupling activity in vitro, which requires all three G protein subunits functioning presumably as heterotrimeric complexes (5). Third, because overexpression of *STE18*^{dn} mutations in wild-type haploid cells reduces mating efficiency in a manner that can be reversed by overexpression of G_β subunits, mutant G_γ subunits may exert their dominant-negative effects by sequestering a limited pool of G_β subunits into nonproductive $G_{\beta\gamma}$ dimers.

Whiteway et al. (51) suggest that *STE18*^{dn} alleles may exert their dominant-negative effects in wild-type cells by means other than sequestering G_β subunits. These investigators report that overexpression of *STE18*^{dn} alleles (except for *STE18*-A1) suppresses the constitutive growth arrest phenotype caused by overexpressing G_β subunits in wild-type cells, indicating that *STE18*^{dn} mutations interfere with signal transduction even in the presence of high levels of G_β subunits. We suggest that these findings are not necessarily inconsistent with our demonstration that co-overexpression of G_β subunits and *STE18*^{dn} mutations in wild-type cells allows signalling to occur, as indicated by nearly normal mating efficiencies. Although other models are possible, we suggest that wild-type cells co-overexpressing *STE18*^{dn} mutations and G_β subunits experience a partial signalling block that is sufficient to prevent constitutive growth arrest but not mating. A partial signalling block might occur if the levels of overexpressed dominant-negative G_γ subunits exceed the levels of overexpressed G_β subunits. Alternatively, even if overexpressed G_β subunits are present in excess, overexpressed dominant-negative G_γ subunits might still interfere with other components of the signalling pathway. Biochemical analysis will be required to determine the mechanisms responsible for the dominant-negative phenotypes caused by overexpression of *STE18*^{dn} mutations.

Our studies indicate that prenylation of G_γ subunits is not essential for coupling between G proteins and α-factor receptors in vitro. G_γ subunits bearing the C107Y substitution in the CAAX box exhibit reduced electrophoretic mobility, which based on prior studies (13) is consistent with a block in prenylation. Nevertheless, G_γ subunits bearing the C107Y substitution support relatively efficient coupling between receptors and G proteins in vitro. However, modest reductions in receptor-coupling efficiency cannot be excluded because the sensitivity of the assay that we use may be limiting. With this limitation in mind, we suggest that coupling between yeast G proteins and α-factor receptors is relatively insensitive to the

prenylation state of the G_γ subunit. Whether α-factor receptors and other G-protein-coupled receptors differ significantly in this respect is uncertain. However, it is known that rhodopsin-transducin interaction is promoted by prenylation of T_γ subunits (32, 54).

Interestingly, we find that truncation mutations (Q94ter and Q98ter) affecting the C-terminal region of yeast G_γ subunits cause partial defects in coupling between $G_{\alpha\beta\gamma}$ heterotrimers and α-factor receptors in vitro. Although receptor-coupling defects could be due to reduced expression of G_γ subunits, overexpression of truncated G_γ subunits does not improve receptor-coupling efficiency, and mutant forms of coupling-proficient (C107Y) and coupling-defective (Q94ter) G_γ subunits are expressed at similar levels.

Assuming that truncated G_γ subunits lacking a CAAX box are not prenylated, their receptor-coupling defects are probably not due to lack of this modification per se, because as noted above, G_γ subunits bearing a C107Y substitution in the CAAX box support relatively efficient coupling between receptors and G proteins. Accordingly, truncated G_γ subunits may cause partial defects in receptor coupling because peptide sequences that normally promote efficient receptor coupling, possibly by interacting directly with receptors, assuming a conformation that promotes interactions between receptors and G_α or G_β subunits, facilitating intracellular targeting of G-protein subunits, and/or serving as sites for further posttranslational modifications, are absent. These possibilities are being investigated.

Our studies also indicate that *STE18*^{dn} mutations profoundly disrupt signalling between $G_{\beta\gamma}$ subunits and components downstream in the pheromone response pathway. *STE18*^{dn} mutations affecting either the N- or C-terminal region of the protein can block the constitutive signal that is otherwise caused by disruption of the G_α subunit gene (*GPA1*). However, it remains to be determined if mutations affecting the N- and C-terminal regions of G_γ subunits disrupt signalling by similar or different mechanisms. Substitutions or truncations affecting the CAAX box may cause signalling defects because $G_{\beta\gamma}$ complexes are deficient in membrane association (13) or because prenylation of Ste18p is critical for $G_{\beta\gamma}$-effector interaction, as has been demonstrated for regulation of mammalian adenylyl cyclase by $G_{\beta\gamma}$ subunits in vitro (17).

G_γ subunits bearing N-terminal substitutions are apparently defective in essential signalling functions other than anchoring G_β subunits to the membrane, associating with G_α and G_β subunits, or coupling to pheromone receptors. Unlike G_γ subunits bearing the C107Y substitution in the CAAX box, those carrying N-terminal substitutions comigrate with wild-type G_γ subunits, indicating that they are probably prenylated. G_γ subunits bearing N-terminal substitutions do not block signalling solely by interfering with G-protein activation because they can block the constitutive signal that otherwise occurs in cell lacking the G_α subunit encoded by *GPA1*. Accordingly, we speculate that N-terminal residues affected by *STE18*^{dn} mutations (residues 34, 37, 48, and 49) may enable $G_{\beta\gamma}$ subunits to bind or regulate downstream signalling effectors or control a conformational change of $G_{\beta\gamma}$ subunits that is required for downstream signalling.

On the basis of alignments between Ste18p and bovine transducin gamma (52), the N-terminal region of Ste18p required for downstream signal transduction corresponds approximately to residues 10 to 30 in mammalian G_γ subunits. Because this region of mammalian G_γ subunit subtypes is somewhat more diverged than the C-terminal domain (39), we speculate that the N termini of mammalian G_γ subunits may also be required for signalling between $G_{\beta\gamma}$ subunits and intracellular effectors. Indeed, the N termini of mammalian G_γ and G_β subunits have been proposed to participate in three-stranded coiled-coil interactions with at least one signalling target, β-adrenergic receptor kinase (41). Whether signalling between yeast $G_{\beta\gamma}$ subunits and their downstream effectors, which may include the protein kinase homolog encoded by the *STE20* gene (25), occurs by similar or different mechanisms is currently under investigation.

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