CDC36 and CDC39 are negative elements in the signal transduction pathway of yeast

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Mutations in either the *CDC36* or *CDC39* gene cause yeast cells to arrest in G1 of the cell cycle at the same point as treatment with mating pheromone. We demonstrate here that strains harboring temperature-sensitive mutations in *CDC36* or *CDC39* activate expression of the pheromone-inducible gene *FUS1* when shifted to nonpermissive temperature. We show further that cell-cycle arrest and induction of *FUS1* are dependent on known components of the mating factor response pathway, the *STE* genes. Thus, the G1-arrest phenotype of *cdc36* and *cdc39* mutants results from activation of the mating factor response pathway. The *CDC36* and *CDC39* gene products behave formally as negative elements in the response pathway: they are required to block response in the absence of pheromone. Epistasis analysis of mutants defective in *CDC36* or *CDC39* and different *STE* genes demonstrates that activation requires the response pathway G protein and suggests that *CDC36* and *CDC39* products may control synthesis or function of the G_α subunit.

Introduction

Mutations in *CDC* genes of *Saccharomyces cerevisiae* lead to arrest of cells at specific positions in the cell cycle (Pringle and Hartwell, 1981). Mutations in four genes—*CDC28*, *CDC36*, *CDC37*, and *CDC39* (Reed, 1980)—cause cells to arrest in late G1 at a point termed START (Hartwell *et al.*, 1974). This is the same point in the cell cycle at which mating factors arrest cells, and cells arrested by mutations in any one of these four genes display morphological changes similar to those seen in cells treated with mating factors. The phenotypes of *CDC36* and *CDC39* mutants differ from mutants defective in *CDC28* and *CDC37* in at least one important way: the G1-arrest phenotype exhibited by *cdc36-ts* and *cdc39-ts* mutants is dis-

played only in **a** or α cells (Reed, 1980). **a**/ α *cdc36/cdc36* and **a**/ α *cdc39/cdc39* strains do not exhibit a uniform arrest point in the cell cycle, whereas **a**/ α *cdc28/cdc28* or *cdc37/cdc37* cells arrest at START. This observation suggests that the G1 arrest phenotype of *CDC36*- and *CDC39*-deficient strains is dependent on some gene product present in **a** or α cells but absent from **a**/ α cells. In addition, it has been shown that uncharacterized mutations that lead to α -factor resistance can suppress the cell-cycle arrest of *cdc36-ts* and *cdc39-ts* strains (Connolly *et al.*, 1983). These and other observations (Shuster, 1982) have led to the suggestion that *CDC36* and *CDC39* may be involved in the mating factor response pathway of *S. cerevisiae*.

Yeast cells have a signal transduction pathway that plays a key role in mating between the two yeast mating types **a** and α (reviewed by Cross *et al.*, 1988). Each of these haploid cell types secretes a specific peptide pheromone (**a**-factor and α -factor) to which the other cell type responds. In the presence of the appropriate pheromone, haploid cells arrest in G1 at START; induce transcription of particular genes such as *FUS1* that are required for mating (Trueheart *et al.*, 1987); and undergo a morphological change in which a projection is extended from one face of the cell, giving the cells an elongated shape (Levi, 1956). The signal transduction pathway that mediates this response has been well characterized genetically (Cross *et al.*, 1988). Mutations in genes coding for components of this pathway, the *STE* genes, were primarily identified by the isolation of mutants that are unable to mate (MacKay and Manney, 1974) or that are resistant to G1 arrest in the presence of α -factor (Hartwell, 1980). The pathway defined by these mutations consists of a specific receptor for each cell type, the *STE2* gene product in **a** cells (Burkholder and Hartwell, 1985; Nakayama *et al.*, 1985) and the *STE3* gene product in α cells (Nakayama *et al.*, 1985; Hagen *et al.*, 1986); a G-protein β subunit (G_β) homologue encoded by the *STE4* gene (Whiteway *et al.*, 1989); and a G_γ homologue encoded by the *STE18* gene (Whiteway *et al.*, 1989). The receptor and G protein appear to act

upstream of two presumptive protein kinases encoded by the *STE7* and *STE11* genes (Teague *et al.*, 1986; N. Rhodes and B. Errede, personal communication), a protein of unknown function encoded by *STE5*, and a transcriptional activator encoded by the *STE12* gene (Dolan *et al.*, 1989; Errede and Ammerer, 1989).

The *STE* genes are all required for response to pheromone. They therefore define a set of positive elements in the response pathway. Negative elements are required to block response in the absence of pheromone. Mutants defective in such genes have the phenotype of constitutive response. Only one negative element with known function has been identified in the transduction pathway, the G_α homologue encoded by the *SCG1*(*GPA1*) gene (Dietzel and Kurjan, 1987; Miyajima *et al.*, 1987; see also Jahng *et al.*, 1988). The *SCG1* gene product is thought to form a complex in vivo with *STE4* (G_β) and *STE18* (G_γ). Null mutations of the *SCG1* gene cause lethality in a cells or α cells but have no phenotype in a/α cells. The lethality of *SCG1* null mutations can be suppressed by null mutations in *STE4*, *STE5*, *STE7*, *STE11*, *STE12*, or *STE18* (Nakayama *et al.*, 1988; Whiteway *et al.*, 1989), which indicates that the lethality is caused by constitutive activation of the mating factor response and that these *STE* gene products act downstream of the *SCG1* protein in the pathway.

We have examined the possibility that mutations in *CDC36* and *CDC39* also cause arrest at START by inappropriate activation of the pheromone response pathway. We demonstrate here that this is the case. Strains harboring temperature-sensitive alleles of *CDC36* or *CDC39* activate expression of a pheromone-inducible gene when shifted to the nonpermissive temperature. For both *CDC* genes, this induction and cell-cycle arrest are dependent on a subset of the *STE* genes. These studies allow us to place *CDC36* and *CDC39* in a specific position in the signaling pathway.

Results

Inactivation of CDC36 or CDC39 results in expression of a pheromone-inducible gene

To determine whether the signal transduction pathway is activated in *CDC36* or *CDC39* mutants, we first asked whether expression of a pheromone-inducible gene, *FUS1*, is increased when cells carrying the temperature-sensitive mutations *cdc36-16* or *cdc39-1* are shifted to nonpermissive temperature. Strains were constructed that contain the temperature-sensitive mutations *cdc28-1*, *cdc36-16*, or *cdc39-1* and

have a *FUS1::lacZ* gene integrated into the chromosome, serving as a reporter for *FUS1* expression. The *FUS1::lacZ* gene joins the *FUS1* regulatory region and amino terminus to the structural gene for β -galactosidase and exhibits pheromone-inducible β -galactosidase synthesis (McCaffrey *et al.*, 1987). The *FUS1* gene cannot be induced in strains that carry a *ste* mutation (McCaffrey *et al.*, 1987). In contrast, strains that are defective in *SCG1* express *FUS1* constitutively (Nakayama *et al.*, 1988). For our experiments, cells were grown in liquid culture to log phase at 23°C and then shifted to 37°C. Aliquots were removed at intervals after temperature shift and assayed for β -galactosidase activity and examined for cell-cycle arrest.

Shift to the nonpermissive temperature of the *cdc36-16* and *cdc39-1* strains induced β -galactosidase expression 20- and 200-fold, respectively, by 5 h (Figure 1). Treatment of the wild-type a strain with α -factor caused rapid induction of β -galactosidase synthesis to the same level seen in the *cdc39-1* mutant. Half-maximal induction occurred at ~45 min after exposure of the wild-type strain to α -factor, and at 2–3 h after shifting the *cdc39-1* mutant to high temperature. Although induction observed for the *cdc36-16* mutant is evident, the maximal level of activity is only ~20% of the level resulting from α -factor induction of the wild-type strain. In contrast, the wild-type strain untreated with α -factor and the *cdc28-1* mutant did not exhibit induction of *FUS1*. The *cdc36-16* mutant exhibited a higher basal level of β -galactosidase activity than the other strains, ~10-fold more than wild type. This may reflect reduced activity of the *CDC36* product in these strains even under permissive conditions.

These cultures were also examined to determine the time course of arrest in G1 (Figure 2). In *S. cerevisiae*, only cells in G1 are unbudded; those in other phases of the cell cycle have buds of characteristic sizes. Thus G1 arrest can be scored as an increase in the percentage of unbudded cells in the population. All of the *cdc* mutants arrested in G1 by 5 h after shift to nonpermissive temperature. A significant fraction of the unbudded cells in the *cdc* cultures displayed an elongated morphology similar to that seen for wild-type cells treated with α -factor (data not shown). The time course of arrest of α -factor-treated wild-type cells and the *cdc36-16* and *cdc39-1* mutants roughly paralleled the kinetics of β -galactosidase synthesis in these cultures: α -factor treatment induced arrest sooner than did shifting the *cdc39-1* mutant; the population of *cdc36-16* mutant cells was slower to arrest than the *cdc39-1* mutants; and the

Figure 1. Time course of induction of *FUS::lacZ*. Cultures were grown to early log phase at 23°C and then shifted to 37°C. For wt + α -factor, synthetic α -factor (Sigma) was added to a final concentration of 25 μ g/ml at the same time as these cells were shifted to 37°C. At intervals, an aliquot of each culture was removed and assayed for β -galactosidase activity as described in Methods. Strains used were BH1001 (wt), AN15-4C (*cdc28-1*), AN9-2D (*cdc36-16*), and AN5-8B (*cdc39-1*).

cdc36-16 population did not attain the same level of arrest as the α -factor-treated cells. The lower level of cell-cycle arrest and induction of *FUS1* exhibited by the *cdc36-16* strain may reflect a less severe defect in CDC36 function rather than a fundamental difference between CDC36 and CDC39 (although differences between the *cdc36-16* and *cdc39-1* mutants were noted, as discussed below). Induction of β -galactosidase activity indicates that the pheromone response pathway has been activated in *cdc36-16* and *cdc39-1* cells. The observation that the *cdc28-1* strain arrested in G1 but did not exhibit induction of *FUS1* indicates that activation of this gene is not a general phenomenon associated with arrest of cells in G1.

Position of CDC36 and CDC39 in the signaling pathway determined by double-mutant analysis

The above observations demonstrate that *cdc36-16* and *cdc39-1* mutants display several phenotypes of pheromone-treated cells: cell-cycle arrest at START, changes in cell morphology, and induction of novel gene products.

We next asked if these phenotypes were dependent on the known components of the signal transduction pathway encoded by the *STE* genes. For this purpose, we constructed strains carrying either *cdc36-16* or *cdc39-1*, *FUS1::lacZ*, and a *ste* mutation. First, a set of strains was constructed that contained insertion or deletion alleles of *STE2*, *STE4*, *STE5*, *STE11*, *STE12*, and *STE18* as well as *FUS1::lacZ*, as described in Methods. These strains were then crossed by spheroplast fusion to *cdc36-16* and *cdc39-1* strains (KK2A and KK5B, respectively; see Table 7). Diploids produced in this fashion were sporulated and tetrads dissected to identify tetratype asci which contained the desired *cdc ste* double mutant as well as *CDC ste*, *cdc STE*, and *CDC STE* sister segregants that served as controls. The *ste* and *ts* phenotypes of segregants from these crosses could be scored independently of each other and thus did not prejudice the phenotype of the *cdc ste* double mutant (see Methods).

Epistasis analysis of *cdc39*

Both cell-cycle arrest and induction of *FUS1::lacZ* after shift to the nonpermissive tempera-

Figure 2. Time course of cell cycle arrest. The cultures assayed for β -galactosidase activity (Fig. 1) were observed microscopically for arrest of cells in G1, which is indicated by an accumulation of unbudded cells (see text).

ture were examined in the double-mutant strains. In the first assay, all four segregants from a tetatype ascus were grown to early log phase at 23°C and then shifted to 37°C. At intervals, aliquots of cells were removed and scored as budded or unbudded cells by microscopic observation. Furthermore, the morphology of the unbudded cells was examined to determine whether they exhibited the elongated morphology associated with cells arrested by α -factor.

A *ste2 cdc39-1* strain exhibited G1 arrest and displayed altered morphology after shift to the nonpermissive temperature in a manner identical to a *cdc39-1 STE* strain. However, double mutants carrying *cdc39-1* and a *ste4*, *ste5*, *ste11*, *ste12*, or *ste18* mutation did not accumulate unbudded cells, and, importantly, the unbudded cells present in the culture did not exhibit any morphological changes after temperature shift (Table 1). These double-mutant strains were also analyzed for induction of *FUS1::lacZ* after temperature shift. A *cdc39-1* strain that was also *ste4*, *ste5*, *ste11*, *ste12*, or *ste18* did not exhibit induction, whereas a *ste2 cdc39-1* strain exhibited induction similar to a *cdc39-1 STE* strain (Table 2). These observa-

tions correlate precisely with the results of the cell-cycle arrest assay.

We interpret these data to indicate that the cell-division cycle phenotype of *cdc39-1* is caused by activation of the pheromone response pathway. The requirement for *STE* gene products demonstrates that the pathway is activated. Further, because arrest is independent of the α -factor receptor (*STE2*), we conclude that, in a cells at least, cell-cycle arrest is not caused by inappropriate synthesis of α -factor. This epistasis analysis indicates that *CDC39* functions in the response pathway after the receptor (the *STE2* product) and before or in conjunction with the G-protein subunits coded by *STE4* and *STE18*. In a strictly formal sense, *CDC39* can be construed as a negative regulator of the pheromone response pathway, acting at or before the step mediated by *STE4* and *STE18*.

Epistasis analysis of *cdc36*

An analysis identical to that just described for *cdc39-1* was carried out for *cdc36-16*. Results are shown in Tables 3 and 4. Double mutants carrying *cdc36-16* and *ste2* mutations arrested

Table 1. Epistasis analysis of *cdc39-1*

Genotype of segregant	% Unbudded cells ^a	Morphology of unbudded cells ^b	Number of tetrads examined
<i>wt</i>	63	+	3
<i>ste2</i>	56	+	
<i>cdc39</i>	84	—	
<i>ste2 cdc39</i>	87	—	
<i>wt</i>	46	+	5
<i>ste4</i>	40	+	
<i>cdc39</i>	75	—	
<i>ste4 cdc39</i>	51	+	
<i>wt</i>	42	+	3
<i>ste18</i>	36	+	
<i>cdc39</i>	70	—	
<i>ste18 cdc39</i>	45	+	
<i>wt</i>	46	+	2
<i>ste5</i>	42	+	
<i>cdc39</i>	70	—	
<i>ste5 cdc39</i>	58	+	
<i>wt</i>	45	+	2
<i>ste11</i>	46	+	
<i>cdc39</i>	78	—	
<i>ste11 cdc39</i>	49	+	
<i>wt</i>	51	+	2
<i>ste12</i>	47	+	
<i>cdc39</i>	77	—	
<i>ste12 cdc39</i>	56	+	

^a Percentages are the means for all segregants of a given genotype. Thus, 87% is the mean obtained from three separate *ste2 cdc39* double mutants. Cultures were assayed 5–6 h after temperature shift as described in Methods. At least 200 cells were scored in each culture.

^b +, wild-type morphology; —, elongated morphology indicative of arrest by α -factor. Cultures were considered to exhibit elongated morphology if >20% of the unbudded cells in the culture were abnormal. In cultures that are designated wild type, <1% of the unbudded cells were abnormal.

in G1 and exhibited altered morphology after temperature shift. These double mutants induced expression from *FUS1::lacZ* after temperature shift. In contrast, *ste4*, *ste5*, *ste11*, *ste12*, or *ste18* null mutations suppressed all of these phenotypes in a *cdc36-16* background. Once again there is a precise correlation between cell-cycle arrest, morphology, and induction of *FUS1::lacZ*.

The conclusions that we draw from these data are identical to those for *CDC39*. The cell-division cycle phenotype of *cdc36-16* mutants is caused by inappropriate activation of the pheromone response pathway. This activation is clearly not an autocrine response in that it does not require the pheromone receptor (at least in *a* cells). The epistasis analysis suggests that *CDC36*, like *CDC39*, may function in the pheromone response pathway in conjunction with or prior to *STE4* and *STE18*.

Relationship of *CDC39* and *CDC36* to *SCG1*

The results presented above demonstrate that loss of *CDC36* or *CDC39* function can activate the pheromone response pathway and that this activation requires the G-protein complex. In yeast, the components of the G protein are believed to be the *SCG1*, *STE4*, and *STE18* gene products, which correspond to G_{α} , G_{β} , and G_{γ} subunits, respectively. The yeast G protein is assumed to function similarly in many respects to G proteins of higher eukaryotes (see Stryer and Bourne, 1986; Gilman, 1987; Neer and Clapham, 1988), although direct biochemical analysis of the yeast components has not yet been reported. The G_{α} subunit can bind to either GDP or GTP. When in the GDP-bound state, G_{α} associates with $G_{\beta\gamma}$; in the GTP-bound state, G_{α} dissociates from $G_{\beta\gamma}$. Activated receptors stimulate GDP-GTP exchange, which causes release of G_{α} from $G_{\beta\gamma}$. The current view for yeast is that free $G_{\beta\gamma}$ is the active signal in the pathway: G_{α} is a negative component of the pathway which keeps $G_{\beta\gamma}$ inactive when bound to it (Dietzel and Kurjan, 1987; Blinder *et al.*, 1989; Whiteway *et al.*, 1989). It should be noted that these hypothetical relationships between *STE4*, *STE18*, and *SCG1* have not yet been demonstrated biochemically.

The observation that *STE4* and *STE18* are required for activation of the pathway in *cdc36-ts* and *cdc39-ts* mutants suggests that loss of *CDC36* or *CDC39* leads to the production of free $G_{\beta\gamma}$. There are two general ways in which this might occur. One possibility is that the *CDC* products interact with the G-protein subunits to govern the function of the G-protein complex, for example, by keeping G_{α} bound to $G_{\beta\gamma}$ in the absence of pheromone. Another possibility is that the *CDC* products control synthesis of G_{α} .

Table 2. Induction of *FUS1::lacZ* in *cdc39-1 ste* double mutants

Relevant genotype	β -Galactosidase activity ^a
<i>wt</i>	<0.1
<i>cdc39-1</i>	94
<i>cdc39-1 ste2</i>	84
<i>cdc39-1 ste4</i>	<0.1
<i>cdc39-1 ste5</i>	<0.1
<i>cdc39-1 ste11</i>	<0.1
<i>cdc39-1 ste12</i>	<0.1
<i>cdc39-1 ste18</i>	<0.1

^a Liquid cultures assayed as described in Methods 6 h after temperature shift. Numbers reported are means from at least two different double-mutant segregants of each genotype.

Table 3. Epistasis analysis of *cdc36-16*

Genotype of segregant	% Unbudded cells ^a	Morphology of unbudded cells ^b	Number of tetrads examined
<i>wt</i>	31	+	3
<i>ste2</i>	41	+	
<i>cdc36</i>	80	—	
<i>ste2 cdc36</i>	95	—	3
<i>wt</i>	38	+	
<i>ste4</i>	44	+	
<i>cdc36</i>	72	—	
<i>ste4 cdc36</i>	45	+	3
<i>wt</i>	41	+	
<i>ste18</i>	37	+	
<i>cdc36</i>	80	—	
<i>ste18 cdc36</i>	51	+	4
<i>wt</i>	42	+	
<i>ste5</i>	38	+	
<i>cdc36</i>	65	—	
<i>ste5 cdc36</i>	46	+	2
<i>wt</i>	36	+	
<i>ste11</i>	47	+	
<i>cdc36</i>	75	—	
<i>ste11 cdc36</i>	58	+	2
<i>wt</i>	51	+	
<i>ste12</i>	47	+	
<i>cdc36</i>	59	—	
<i>ste12 cdc36</i>	49	+	

^a Percentages are the means for all segregants of a given genotype. Thus, 95% is the mean obtained from three separate *ste2 cdc36* double mutants. Cultures were assayed 5–6 h after temperature shift as described in Methods. At least 200 cells were scored in each culture.

^b +, wild-type morphology; —, elongated morphology indicative of arrest by α -factor. Cultures were considered to exhibit elongated morphology if >20% of the unbudded cells in the culture were abnormal. In cultures that are designated wild type, <1% of the unbudded cells were abnormal.

or $G_{\beta\gamma}$ subunits, for example, by regulating their transcription or posttranslational modification. In this case, *cdc36* or *cdc39* mutants might lack functional G_{α} subunits or overproduce $G_{\beta\gamma}$ subunits.

These two general models can be distinguished by examining the behavior of *cdc36* or *cdc39* mutant strains that also carry a mutation affecting function of the G_{α} subunit. The *scg1-ctt1* allele of *SCG1* encodes a truncation that removes the five carboxy-terminal amino acid residues of G_{α} (Kurjan and Dietzel, 1988; J. Kurjan, personal communication). This mutation causes a nonmating phenotype because of a defect in response to mating factors. The *scg1-ctt1* mutation leads to production of a G_{α} subunit that is thought to be unable to dissociate from $G_{\beta\gamma}$, perhaps because it cannot interact with the activated receptor (Stryer and Bourne, 1986; J. Kurjan, personal communication), although the

precise molecular basis for this inability is not yet known. If *cdc36* and *cdc39* mutants are defective in synthesis of G_{α} subunits or overproduce $G_{\beta\gamma}$ subunits, then the *scg1-ctt1* mutation should not affect cell-cycle arrest or induction of *FUS1::lacZ* in *cdc36* or *cdc39* mutants. On the other hand, if *CDC36* and *CDC39* products control function of the G-protein complex, then some alterations of G_{α} might block cell-cycle arrest and *FUS1::lacZ* induction in *cdc36* and *cdc39* mutants and thus behave like mutations in *STE4* and *STE18*.

Diploids heterozygous for *scg1-ctt1* and *cdc36-16* or *cdc39-1* were constructed by spheroplast fusion, and the resulting tetratype asci were analyzed as described above. The results (Table 5) show that *cdc36-16 scg1-ctt1* and *cdc39-1 scg1-ctt1* strains differ from each other. The *scg1-ctt1* mutation did not affect the phenotype of the *cdc36-16* mutant: segregants carrying both mutations exhibited cell-cycle arrest and morphological alterations identical to *cdc36-16* segregants carrying a wild-type *SCG1* gene. Induction of *FUS1::lacZ* activity in this strain background is low but is similar in *SCG1* and *scg1-ctt1* strains. In contrast, *cdc39-1 scg1-ctt1* strains do not exhibit cell-cycle arrest or induction of *FUS1::lacZ*. Of seven double mutants analyzed, four exhibited normal morphologies at nonpermissive temperature, and three contained a small fraction of unbudded cells that exhibited an elongated morphology. The reason for this heterogeneous behavior is not known. These observations distinguish the *cdc36-16* phenotype from that of *cdc39-1*, and they are consistent with the view that the *CDC36* gene product controls synthesis of the G_{α} or $G_{\beta\gamma}$ subunits, whereas *CDC39* protein regulates the function of these subunits.

Table 4. Induction of *FUS1::lacZ* in *cdc36-16 ste* double mutants

Relevant genotype	β -Galactosidase activity ^a
<i>wt</i>	<0.1
<i>cdc36-16</i>	30
<i>cdc36-16 ste2</i>	29
<i>cdc36-16 ste4</i>	<0.1
<i>cdc36-16 ste5</i>	<0.1
<i>cdc36-16 ste11</i>	<0.1
<i>cdc36-16 ste12</i>	<0.1
<i>cdc36-16 ste18</i>	<0.1

^a Liquid cultures assayed as described in Methods 6 h after temperature shift. Numbers reported are means from at least two double-mutant segregants of each genotype.

Table 5. Epistasis relationship of *cdc36-16* and *cdc39-1* to *scg1-ctt1*

Genotype of segregant	% Unbudded cells ^a	Morphology of unbudded cells ^b	β -Galactosidase activity ^c
<i>wt</i>	43	+	n.t.
<i>scg1-ctt1</i>	42	+	n.t.
<i>cdc39-1</i>	60	—	6.0
<i>scg1-ctt1 cdc39-1</i>	45	+ ^d	<0.1
<i>wt</i>	38	+	<0.1
<i>scg1-ctt1</i>	38	+	n.t.
<i>cdc36-16</i>	73	—	1.8
<i>scg1-ctt1 cdc36-16</i>	70	—	0.6

^a See legend to Table 1 for description. At least 400 cells were scored in each culture. For *cdc39-1*, seven tetrad asci were examined. For *cdc36-16*, four tetrad asci were scored.

^b See legend to Table 1.

^c See legend to Table 2. At least four segregants of each genotype were tested. n.t., not tested.

^d For three of the seven *cdc39-1 scg1-ctt1* segregants examined, a low but significant fraction (10%) of the unbudded cells were abnormal. This observation may indicate that the suppression of *cdc39-1* by *scg1-ctt1* is not as complete as with the *ste* null mutations. In no case was β -galactosidase induction observed in these cultures.

Effect of mutations in CDC36 and CDC39 on mating by *ste* mutants

An earlier indication that CDC36 and CDC39 might act in the pheromone response pathway was the observation that *cdc36-ts* and *cdc39-ts* mutations can partially alleviate the mating defects in *ste4* and *ste5* mutants (Shuster, 1982). Jenness *et al.* (1987) report a similar suppression of *ste4* by *cdc39-ts* but only in a *bar1* or *sst2* background. These studies employed temperature-sensitive alleles of both CDC and STE genes and showed that suppression was observed only for specific *ste* mutations. This suppression is at odds with our conclusions on the positions of CDC36 and CDC39 relative to STE4 and STE5 in the signal transduction pathway. Because *ste4* and *ste5* null mutations block cell-cycle arrest and *FUS1::lacZ* induction, we deduce that CDC36 and CDC39 act before STE4 and STE5 in the pathway. We therefore expect that *cdc36* and *cdc39* mutations would not alleviate the mating defect of *ste4* and *ste5* mutants. To address this apparent contradiction, we examined the effect of *cdc36-16* and *cdc39-1* mutations on mating by strains that carry null mutations in STE2, STE4, and STE5 (Table 6). Whereas *cdc36-16* and *cdc39-1* partially suppress the mating defect of a *ste2* mutant, they do not improve the mating ability of a *ste4* or *ste5* mutant. These results conform to our expectations. Previous reports have suggested that the suppression of *ste4-ts* and *ste5-ts* by *cdc36* and *cdc39* may be indicative of direct interaction between these gene products. Although the inability of these *cdc* mutants to suppress the mating defect of null

mutations in *ste4* and *ste5* does not rule out this possibility, we prefer to interpret earlier observations on suppression to have resulted perhaps from leakiness of some *ste4-ts* and *ste5-ts* mutations.

Discussion

We demonstrate here that loss of CDC36 or CDC39 in temperature-sensitive mutants

Table 6. Quantitative mating analysis of double mutants

Relevant genotype	Mating frequency ^a
<i>wt</i>	3.1×10^{-1}
<i>ste2</i>	$<4 \times 10^{-7}$
<i>ste4</i>	6×10^{-7}
<i>ste5</i>	$<1.8 \times 10^{-7}$
<i>cdc39-1 ste2</i> ^b	8.4×10^{-4}
<i>cdc39-1 ste4</i> ^c	2×10^{-7}
<i>cdc39-1 ste5</i> ^c	4×10^{-7}
<i>cdc36-16</i>	1.2×10^{-1}
<i>cdc36-16 ste2</i> ^b	1.1×10^{-3}
<i>cdc36-16 ste4</i> ^c	$<5 \times 10^{-7}$
<i>cdc36-16 ste5</i> ^c	$<2 \times 10^{-7}$

^a Frequency calculated as the number of diploid cells/total cells.

^b Frequencies reported are means for three different MAT α isolates.

^c Frequencies reported for double mutants are the means for three different isolates, two MAT α and one MAT α .

Strains used are BH1001 (*wt*), 16Az4 (*ste2*), AN1001 (*ste4*), and AN1002 (*ste5*), which are listed in Table 7. Other strains are from Tables 1 and 3.

causes activation of the mating factor response pathway and that this is the reason that cells carrying mutations in these genes arrest at START. Our evidence is twofold: first, expression of a pheromone-inducible gene, *FUS1*, is activated by temperature shift; second, induction of *FUS1*, formation of morphologically altered cells, and cell-cycle arrest are all dependent on known components of the response pathway encoded by the *STE* genes. These findings substantiate and extend prior suggestions that *CDC36* and *CDC39* are involved in the response pathway (Shuster, 1982; Connolly *et al.*, 1983). Epistasis analysis using *cdc36-ts* or *cdc39-ts* mutations in combination with null mutations of the *STE* genes demonstrates that activation of the response pathway by *cdc36* or *cdc39* mutations requires the G-protein complex of the response pathway. Because induction of *FUS1* is not seen in strains arrested at START by temperature-sensitive mutations in *CDC28* (or *CDC37*; unpublished observations), induction of *FUS1* is not a general response to arrest at START.

Two types of START-arrest mutants

These results demonstrate that there are two types of START-arrest mutants in *S. cerevisiae*. One type is defined by mutations in *CDC28* and *CDC37*, the products of which are required for cell-cycle progression through START. *CDC28* codes for a protein kinase whose activation appears to be essential to pass START (Mendenhall *et al.*, 1987). The function of the *CDC37* gene product is not known. We suggest that it might be required for activation of the *CDC28* kinase or a substrate of this kinase.

The second type of START-arrest mutant is defined by mutations in *CDC36* and *CDC39*. These genes are not required for progression of the cell cycle through START. Rather, loss of their function activates the pathway of cell-cycle arrest ordinarily evoked by mating factors. There are several other genes that exhibit similar behavior: *SCG1* (also called *GPA1* and *CDC70*) (Dietzel and Kurjan, 1987; Miyajima *et al.*, 1987; Jahng *et al.*, 1988), *SRM1* (Clark and Sprague, 1989), and *CDC72* (Reed *et al.*, 1988). These genes share the feature that inactivation of any of them partially alleviates the mating defect of strains lacking mating factor receptors (*STE2* and *STE3*). Furthermore, null mutations in *STE4* can suppress the START-arrest phenotypes of mutations in any of these genes (Nakayama *et al.*, 1988; Clark and Sprague, 1989; S. Reed, personal communication).

There are thus five genes reported that behave as negative elements in the pheromone

response pathway and appear to act before or in conjunction with *STE4* (G_{β}). A simple model that incorporates these observations is that these genes control the production of functional G_{α} or the activity of G_{α} . In either case, loss of these products would result in the loss of functional G_{α} and would cause activation of the mating response pathway. The *SRM1* protein appears to be localized to the nucleus and thus might be required for transcription of *SCG1* or one of the *CDC* genes (K. Clark, personal communication). The observation that *CDC36* and *CDC39* are essential in a/α cells (Reed, 1980), even though the mating factor response pathway is inactive in these cells, indicates that these gene products are required for more than regulation of this pathway. One possibility is that *CDC36* and *CDC39* also affect the function of other GTP-binding proteins that are essential for vegetative growth of yeast.

A distinction between *CDC36* and *CDC39*

The observation that a mutation of *SCG1* (*scg1-ctt1*) can affect the behavior of a *cdc39-ts* mutation suggests that *CDC39* may modulate the activity of G_{α} . The *scg1-ctt1* mutation apparently leads to the production of a G complex in which G_{α} and $G_{\beta\gamma}$ remain associated with each other even when the receptor is activated by mating factors. We find that inactivation of *CDC39* in a *cdc39-ts* mutant does not provoke dissociation of this mutant G_{α} . Therefore, deletion of the five carboxy-terminal amino acid residues of G_{α} both renders it insensitive to the activated receptor and relieves the requirement for *CDC39* function to maintain association of G_{α} with $G_{\beta\gamma}$. One possible explanation for these observations is that this region of G_{α} is involved in triggering the release of GDP: the activated receptor might interact with this region to stimulate release of GDP from G_{α} -GDP and thereby allow formation of G_{α} -GTP. In contrast, *CDC39* might interact with this region of the protein to prevent GDP release in unstimulated cells. Thus, the role of *CDC39* in wild-type cells might be to stabilize G_{α} -GDP (Figure 3) and thereby maintain its association with $G_{\beta\gamma}$.

In contrast, *cdc36-16* is not suppressed by *scg1-ctt1*. This observation suggests that *CDC36* may be involved in some step in the production of G_{α} , in which case behavior of a *cdc36* mutant will not be affected by alteration of G_{α} (Figure 3). Another possibility is that *CDC36* is a negative regulator of $G_{\beta\gamma}$ production; hence, *cdc36* mutants will have more $G_{\beta\gamma}$ than can be bound by G_{α} . Yet a third possibility is that *CDC36* modulates an activity of G_{α} unaf-

Figure 3. Schematic model for the action of CDC36 and CDC39 in the mating factor response pathway. The CDC36 and CDC39 products are both required to keep the pathway in the OFF state in the absence of mating factor (A). CDC36 may do this by maintaining the level of SCG1 protein (G_{α}). Another possible role for CDC36 (see text) not shown in the figure is that it is a negative regulator of $G_{\beta\gamma}$. In unstimulated cells (A), the CDC39 product may prevent conversion of SCG1-GDP to SCG1-GTP (for example, by blocking dissociation of GDP from G_{α} -GDP) and thereby maintain G_{α} association with $G_{\beta\gamma}$. In the presence of mating factor (B), the activated STE2 gene product (indicated by asterisk) stimulates the conversion of SCG1-GDP to SCG1-GTP, leading to liberation of $G_{\beta\gamma}$ and cellular response. The activated STE2 product may stimulate GDP-GTP exchange by directly inhibiting CDC39, or it may act independently of CDC39.

ected by the *ctt1* mutation. In any event, this observation distinguishes the *cdc36-16* phenotype from that of *cdc39-1*.

It should be possible to gain a clearer idea of the roles of CDC36 and CDC39 by direct examination of the G-protein subunits in strains carrying *cdc36* or *cdc39* mutations. An analysis similar to the one used here, studying the behavior of *cdc36-ts* or *cdc39-ts* mutants that carry mutations in *SCG1* known to affect different

properties of the protein—such as GDP-GTP exchange, receptor interaction, or the affinity of G_{α} for $G_{\beta\gamma}$ —should also be helpful in defining the exact roles that CDC36 and CDC39 play in G-protein function.

Methods

Strains and media

Strains are listed in Table 7. Standard media and genetic methods were used (Mortimer and Hawthorne, 1969; Hicks and Herskowitz, 1976) unless otherwise noted. AN1001, AN1002, and AN1003 are derivatives of strain BH1001 that were constructed by one-step gene replacement (Rothstein, 1983) of the *STE4*, *STE5*, and *STE18* genes with null mutations of these genes (resulting from an insertion carrying the *LEU2* gene). A plasmid containing the *ste4::LEU2* allele was provided by V. MacKay (ZymoGenetics), a plasmid containing the *ste5::LEU2* allele was the gift of G. Sprague, and the *ste18::LEU2* plasmid was provided by M. Whiteway. Although several *ste* mutations suppress the cell-cycle arrest of *cdc36-16* and *cdc39-1*, the *ste* mutations do not suppress the temperature-sensitive lethality of these *cdc* mutations. Therefore, the *ste* and *ts* phenotypes of segregants could be scored independently by scoring mating at 23°C and growth at 37°C. The *FUS1::lacZ* gene is on a Ylp5 vector,

Table 7. Strains

Strain	Genotype	Source or reference
16A24	<i>MATα ura3-52 leu2 met1 lys5 bar1 ste2::TRP1</i>	L. Marsh
BH1001	<i>MATα ura3-52 his4-519 leu2 trp1 can1-101 FUS1::lacZ(URA3)</i>	B. Hoopes
BH1005	derivative of BH1001, <i>ste12::TRP1</i>	B. Hoopes
BH1008	derivative of BH1001, <i>ste11Δ</i>	B. Hoopes
AN1001	derivative of BH1001, <i>ste4::LEU2</i>	this work
AN1002	derivative of BH1001, <i>ste5::LEU2</i>	this work
AN1003	derivative of BH1001, <i>ste18::LEU2</i>	this work
KK2A	<i>MATα ura3 lys5 cdc36-16</i>	this work
KK5B	<i>MATα ura3 lys5 cdc39-1</i>	this work
JH1-CTT1	<i>MATα leu2-3,112 trp1-1 can1-100 ura3-1 ade2-1 his3-11,15 scg1-ctt1</i>	J. Kurjan
AN5-4B	<i>MATα ura3 leu2 his4 FUS1::lacZ(URA3) cdc39-1</i>	this work
AN9-4B	<i>MATα ura3 leu2 trp1 his4 FUS1::lacZ(URA3) cdc36-16</i>	this work
AN5-8B	<i>MATα ura3 his4 trp1 leu2 FUS1::lacZ(URA3) cdc39-1</i>	this work
AN9-2D	<i>MATα ura3 leu2 his4 FUS1::lacZ(URA3) cdc36-16</i>	this work
AN15-4C	<i>MATα ura3 FUS1::lacZ(URA3) cdc28-1</i>	this work
IH1792	<i>MATα cry1 lys1</i>	lab collection
IH1793	<i>MATα lys1</i>	lab collection

carrying a *URA3* marker, which was integrated into the chromosome at *FUS1*. Segregation of *FUS1::lacZ* was scored by segregation of *Ura*⁺.

Spheroplast fusion

Construction of diploids was performed in a manner similar to that of Rose *et al.* (1986). The α and the α strain to be mated were grown overnight at 23°C, then diluted into 50 ml YEPD broth and grown at 23°C to OD₆₀₀ of 1.0. Cells were pelleted in 50-ml conical tubes and resuspended in 5 ml 1 M sorbitol. Undiluted glucanase (100 μ l, DuPont) and 50 μ l of a 10 mg/ml solution of zymolyase (ICN Biochemicals) were added, and the cells were incubated for 75 min at 30°C. Spheroplasts were then pelleted for 10 min at 1200 rpm, washed once with 5 ml STC buffer [1M Sorbitol, 10mM tris(hydroxymethyl)aminomethane (Tris) pH7.5, 10mM CaCl₂], and gently resuspended in 0.4 ml of STC buffer. 0.2 ml of each cell type were mixed in a 15-ml conical tube; 2 ml PEG solution (40% PEG-4000, 10mM Tris pH7.5, 10mM CaCl₂) was added; and the mixture was left for 20 min at room temperature. The mixture was then pelleted for 10 min at 1200 rpm, resuspended in 1 ml STC, and plated onto selective plates in 10 ml restoration agar (Hinnen *et al.*, 1978).

Cell-cycle arrest assays

Fresh overnight cultures of strains to be tested were diluted 1:50 in YEPD and grown at 23°C to OD₆₀₀ of 0.1. An aliquot of cells was removed and fixed in 3.7% formaldehyde, and cells were scored in a phase contrast microscope as budded or unbudded to ensure that the cells in the culture were distributed throughout the cell cycle before temperature shift. The culture was shifted to 37°C and grown for 6 h. At this time, a second aliquot was removed, fixed, and scored for the percentage of unbudded cells in the culture, and the morphology of the unbudded cells was examined. For strains that carried a *cdc* mutation, at least 400 individual cells were scored in each culture.

β -galactosidase assays

Fresh overnight cultures of strains to be tested were diluted 1:100 and grown at 23°C to OD₆₀₀ of 0.1. Aliquots were removed at this point and assayed for β -galactosidase activity. The remaining culture was shifted to 37°C for 6 h and then assayed for β -galactosidase activity. Assays were performed as described by Stern *et al.* (1984). Units of β -galactosidase activity were calculated as $[1000 \times OD_{420}]/[OD_{600} \times \text{time (min)} \times \text{vol cells (ml)}]$.

Quantitative mating assays

Assays were performed largely as in Shuster (1982), except that the restrictive temperature was 37°C instead of 34°C. Cells (2×10^6) from an exponentially growing culture of each strain to be assayed were mixed with an equal number of cells of the appropriate mating tester strain (IH1792 and IH793) and then filtered onto a 0.45- μ m nitrocellulose filter (Millipore). The filter was rinsed with 5 ml of YEPD and then placed on a YEPD plate and incubated for 7 h at 37°C. Cells were resuspended in 5 ml of SD by 10 s vigorous vortexing followed by sonication for 5 s. Dilutions were plated on YEPD to determine total colony-forming units and on SD minimal plates to select for diploids. Mating frequency was calculated as diploid cells/total cells.

Acknowledgments

We thank Vivian MacKay, George Sprague, and Malcolm Whiteway for plasmids; Steve Reed, Leland Hartwell, Janet Kurjan and Barbara Hoopes for strains; and Karen Clark, Beverly Errede, Steve Reed and Janet Kurjan for communication of results before publication. We are grateful to the members of the Herskowitz laboratory for helpful discussion, to Kerrie Andow for help with the figures, and to Nancy Hollingsworth for comments on the manuscript. We give special thanks to Henry Bourne for discussion, comments on the manuscript, and, in particular, for his suggestions concerning the role of *CDC39*. This work was supported by a Program Project Grant (GM31286) to I.H. A.M.N. is a Howard Hughes Medical Institute Predoctoral Scholar, F.C. is a Merck Scholar, and K.K. is a National Science Foundation Predoctoral Scholar.

Received: February 13, 1990.

Revised and accepted: March 19, 1990.

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