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Mutational analysis of the yeast a-factor transporter STE6, a member of the ATP binding cassette (ABC) protein superfamily

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STE6, the yeast a-factor transporter, is a member of the ATP binding cassette protein superfamily, which also includes the mammalian multidrug resistance protein and the cystic fibrosis gene product. These proteins contain two homologous halves, each with six membrane spanning segments and a predicted ATP nucleotide binding domain. To assess the importance of the two halves of STE6, and to examine the functional significance of residues conserved among members of the ATP binding cassette superfamily, we introduced mutations into the nucleotide binding domains of STE6. Our analysis demonstrates that both halves of STE6 are critical for function and that some, but not all, mutations analogous to those known to result in cystic fibrosis impair STE6 activity. To examine further the functional contribution of each half of the STE6 protein, we severed the *STE6* coding sequence and expressed the two halves of the transporter as separate polypeptides. Whereas 'half-molecules' are unable to provide transport function individually, co-expression of both half-molecules in the same cell leads to functional reconstitution of STE6-mediated a-factor transport.

Key words: a-factor mating pheromone transporter/ATP binding protein/CFTR/MDR/yeast *STE6*

Introduction

The *STE6* gene product of *Saccharomyces cerevisiae* mediates export of the **a**-factor mating pheromone, a prenylated and methylated oligopeptide (Betz *et al.*, 1987; Anderegg *et al.*, 1988; Kuchler *et al.*, 1989; McGrath and Varshavsky, 1989). STE6 is a member of a superfamily of transport proteins, designated the ATP binding cassette (ABC) proteins (Hyde *et al.*, 1990) or traffic ATPases (Mimura *et al.*, 1991), that share significant sequence homology and a similar overall structure. In addition to STE6, the best studied eukaryotic members of the ABC superfamily include the mammalian P-glycoprotein, or multidrug resistance (MDR) protein, whose overexpression in tumor cells facilitates drug resistance by increasing drug efflux (Chen *et al.*, 1986; Gros *et al.*, 1986; Horio *et al.*, 1988; Endicott and Ling, 1989), and CFTR, the protein defective in patients with cystic fibrosis (CF) (Kerem et al., 1989; Riordan *et al.*, 1989; Rommens *et al.*, 1989). The STE6, MDR, and CFTR proteins are comprised of two homologous halves, each encoding six predicted membrane spanning segments and a putative ATP nucleotide binding fold (NBF) domain (Figure 1a). Certain ABC proteins, such as the bacterial hemolysin transporter, HlyB, appear to be 'half-molecule' versions of STE6 with only a single set of six membrane spanning segments and a single NBF domain (Felmlee *et al.*, 1985; Mackman *et al.*, 1986). In other ABC transporters, including bacterial nutrient permeases, the membrane spanning regions and NBF domains reside on separate polypeptides (Ames, 1986; Hyde *et al.*, 1990). All members of the ABC family whose functions are known are involved in transporting small molecules or proteins across membranes. The considerable level of homology among the ABC proteins suggests that they likely share common mechanistic features.

We have undertaken a structure–function analysis of the STE6 transporter. To assess whether both halves of STE6 are essential for **a**-factor export and to examine the functional significance of residues conserved among ABC proteins, we introduced substitutions or deletions into *STE6*, many of which are analogous to CF mutations. This analysis revealed that both NBF domains of STE6 are critical for function and that STE6 is inactivated by some, but not all CF mutations; interestingly, a single amino acid deletion in *STE6* analogous to the most prevalent CF mutation, ΔF508 in CFTR (Kerem *et al.*, 1989), has no apparent effect on STE6 activity. To examine further the contribution of each half of STE6 to function, the two halves of the transporter were expressed as separate polypeptides. Co-expression of both 'half-molecules' in the same cell led to reconstitution of a functional STE6 transporter.

Results

Construction and evaluation of mutations in STE6

Figure 1 shows *ste6* mutations generated in this study together with an alignment of the most highly conserved regions of the N-terminal and C-terminal NBF domains of STE6, MDR1 and CFTR. The segment designated 'A Region' contains the Walker A motif (GX_4GKS/T) (Walker *et al.*, 1982), which is common to a wide variety of nucleotide binding proteins, including many non-ABC proteins, and is thought to form a nucleotide phosphate binding loop (Saraste *et al.*, 1990). The 'B Region' includes the Walker B motif ($RX_{6-8}hyd_4D$) (Walker *et al.*, 1982), which is proposed to lie near the Walker A motif in the folded protein and also to contact ATP (Hyde *et al.*, 1990; Mimura *et al.*, 1991). In addition, the B Region contains residues predicted to lie outside of the ATP binding core, notably a 'signature' sequence LSGGQ, which is diagnostic for members of the ABC superfamily (Higgins *et al.*, 1986). The region designated here as 'Center' lies approximately midway between the A and B Regions. Homology within the Center Region is somewhat sparse among family members (Figure 1b). Many different CF mutations lie in the A and B regions (Davies, 1990); however, the most common CF mutation, ΔF508, lies in the Center Region of

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a

N-terminal NBF (A, B) — C-terminal NBF (A, B)

b

		A REGION	CENTER	B REGION
STE6	(N)	385-GQFTFIVGKSGSGKSTLS	435-ITVVEQRCTLFNDTLRKNILLG	506-LSGGQQQRVAIARAFIRDTPILFLDEAVSALDI
STE6	(C)	1080-GQTLGIIGESGTGKSTLV	1130-ISVVEQKPLLFNGTIRDNLTYG	1190-LSGGQAQRLCIARALLRKSKILILDECTSALDS
CFTR	(N)	451-GQLLAVAGSTGAGKTSLL	488-ISFCSQFSWIMPGTIKENIIFG	548-LSGGQRARISLARAVYKDADLYLLDSPFGYLDV
CFTR	(C)	1237-GQRVGLLGRTGSGKSTLL	1286-FGVIPQKVFIFSGTFRKNLDPY	1346-LSHGHKQLMCLARSVLSKAKILLLDEPTSAHLD
MDR1	(N)	420-GQTVALVGNSGCGKSTTV	470-IGVVSQEPVLFATTIAENIRYG	531-LSGGQKQRIAIARALVRNPKILLLDEATSALDT
MDR1	(C)	1063-GQTLALVGSSGCGKSTVV	1113-LGIVSQEPILFDCSIAENIAYG	1176-LSGGQKQRIAIARALVRQPHILLLDEATSALDT

		G ↓	K ↓	K ↘	Q ↓	F ↓	L/Y ↓	G ↓
c STE6 mutations	(N)	V_{392}	R_{398}	A_{398}	N_{440}	ΔF_{445}	ΔL_{455}	D_{509}
	(C)	V_{1087}	R_{1093}	A_{1093}	N_{1135}	ΔF_{1140}	ΔY_{1150}	D_{1193}
d Mating efficiency	(N)	0.8	1	25	87	95	105	0.5
	(C)	0.3	15	26	88	102	85	6
e CFTR mutations	(N)	V_{458}					ΔF_{508}	D_{551}
	(C)	E_{1244}						D_{1349}

Fig. 1. Alignment of highly conserved regions of the NBF domains of eukaryotic ABC proteins; mutations introduced into *STE6* and their impact on mating efficiency. (**a**) Structural organization of the STE6 protein (Kuchler *et al.*, 1989; McGrath and Varshavsky, 1989). Predicted transmembrane segments (stippled) and N-terminal and C-terminal NBF domains (solid) are shown to scale. Positions of the A and B Regions within the NBFs are indicated. The Center Region lies between A and B. The linker region (residues 609–716) connects the two halves of STE6, which is 1290 residues long. The arrow indicates the site in the linker between residues 694 and 695 where STE6 was separated for the half-molecule experiment described in Figure 5. (**b**) Alignment of highly conserved regions of the NBF domains of three ABC proteins. The amino acid sequences shown are from the N-terminal (N) and C-terminal (C) NBF domains of the yeast STE6 (McGrath and Varshavsky, 1989; Kuchler *et al.*, 1989), human CFTR (Riordan *et al.*, 1989), and human MDR1 (Chen *et al.*, 1986) proteins and are numbered according to their positions in those proteins. Residues which are identical among at least five of the six sequences are shaded. Segments designated 'A Region' and 'B Region' contain residues that are highly conserved among ABC proteins and include, but extend beyond, the Walker A and B motifs (overlined). The region designated 'Center' contains the site (*) of the most common CF mutation ΔF508 (Kerem *et al.*, 1989). This alignment was generated using the 'local homology' algorithm of Smith and Waterman as implemented in the University of Wisconsin GCG Bestfit program (Devereux *et al.*, 1984). It is essentially similar to that reported by Riordan *et al.* (1989), except that here the C-terminal Center region of CFTR is shifted upstream 10 residues relative to other members of the family. (**c**) *STE6* mutations. Residues in STE6 that were changed by site-directed mutagenesis are indicated above the corresponding amino acid alterations that were made. Subscripted number indicates position in the STE6 protein sequence. The residues L_{455} and Y_{1150} are not conserved, but occupy analogous positions in the Center region, hence the designation L/Y. (**d**) Effect of NBF mutations on STE6 function. The impact of each mutation on STE6 function was measured by the quantitative mating assay described in Materials and methods. The number shown represents the percent mating efficiency of each mutant, as normalized to wild type (100%). The Δste6 deletion mutant SM1646, into which plasmids containing the indicated point mutations were transformed, mates at <0.0001% the wild type level, as does the SM1646 derivative transformed with pRS315 used as the Δ*ste6* control strain throughout this study. Mating is proportional to the amount of **a**-factor secreted (compare with Figure 2 and Figure 3) and thus mating activity directly reflects the level of STE6 function. (**e**) Mutations which result in the disease CF. Positions of CF mutations G458V (Cuppens *et al.*, 1990), G1244E (DeVoto *et al.*, 1991), ΔF508 (Riordan *et al.*, 1989), G551D (Cutting *et al.*, 1990), and G1349D (Beaudet *et al.*, 1991) are indicated. Only the subset of CF mutations in residues corresponding to the STE6 alterations examined in this study are shown.

the N-terminal NBF of CFTR (Kerem *et al.*, 1989; Riordan *et al.*, 1989) (Figure 1e). Several modeling studies predict that the Center Region of ABC proteins lies outside the ATP binding core (Hyde *et al.*, 1990; Mimura *et al.*, 1991), although a role for this region in ATP binding has also been proposed (Thomas *et al.*, 1991).

We generated mutations in conserved residues in the A, B, and Center Regions of *STE6* on a low copy-number *CEN* plasmid; analogous changes were made in the N- or C-terminal NBF coding regions of *STE6* (Figure 1c). Many of the alterations we introduced correspond to CF mutations (Figure 1e). Mutant plasmids were transformed into the *ste6* deletion strain SM1646 and STE6 activity was evaluated by measuring **a**-factor export in several assays, including mating (Figure 1d), immunoprecipitation of extracellular **a**-factor (Figure 2), and the **a**-factor halo bioassay (Figure 3). Mating relies on the ability of cells to secrete **a**-factor (Michaelis and Herskowitz, 1988), and, as observed in the experiments reported below, mating efficiency directly reflects the level of extracellular **a**-factor as measured by immunoprecipitation and halo assays. An advantage of the mating assay is its sensitivity, which permits evaluation of low amounts of **a**-factor, below the level readily detectable by other methods.

Mutations in the NBF A Region

Alteration of the first glycine of the Walker A motif (*G*X_4GKS/T) in either NBF of CFTR results in CF (Cuppens *et al.*, 1990; DeVoto *et al.*, 1991) (Figure 1e). This glycine is also critical for STE6 transport function, since mutations in the corresponding glycine residues in STE6, G392V or G1087V, dramatically reduce **a**-factor secretion, as revealed by the quantitative mating assay (Figure 1d) and by immunoprecipitation of extracellular **a**-factor (Figure 2, lanes 3 and 10). It is notable that although STE6 transport activity is dramatically impaired in the G→V mutants, considerable residual function remains. Whereas mating is undetectable in the *ste6* deletion strain SM1646 (<0.0001% of wild type), the G→V mutants mate at low but detectable efficiency (0.8% and 0.3% that of wild type), indicating that their activity is reduced but not abolished. This residual mating activity has facilitated comparison of the severity of different *ste6* mutations and indicates that the G→V

mutations are among the most severe reported here (Figures 1d, 2, and 3).

Structural and biochemical data suggest that the conserved lysine residue of the Walker A motif plays a key role in many nucleotide binding proteins (Saraste *et al.*, 1990). Azzaria *et al.* (1989) have mutated this lysine to arginine in both halves of the mouse MDR1 protein and demonstrated a loss of drug resistance. We generated the analogous *ste6* mutations, K398R and K1093R. In both cases the ability of STE6 to transport **a**-factor is impaired. Interestingly, however, while the N-terminal mutation K398R substantially reduced mating efficiency to a level of 1% that of wild type, the C-terminal mutation K1093R had a more modest effect, leaving 15% residual activity (Figure 1d). This difference is also observed by immunoprecipitation of **a**-factor (Figure 2, compare lanes 4 and 11). Although the disparate effects of these K→R alterations could suggest that the two NBF domains of STE6 make unequal contributions to function, the comparable extents of STE6 inactivation caused by the N-terminal and C-terminal G→V alterations argue against this view. Instead, it seems more likely that the effect of changing a particular residue may depend on the contributions of its neighbors and thus is likely to be highly context-dependent.

These K→R mutants, like the other mutants in this study, were expressed from a low copy number *CEN* plasmid. To determine the effect of copy number on apparent function, *STE6* bearing the K398R mutation was cloned into a 2μ plasmid. In contrast to the low level of mating (1%) observed with K398R on a *CEN* plasmid, expression of K398R from a high copy number 2μ plasmid resulted in a greatly increased mating efficiency (16%). Thus, the level of observed STE6 function depends on both the intrinsic activity of a particular ste6 mutant protein and on its level of expression.

The substitution of arginine for lysine can be considered a conservative alteration because it preserves positive charge. We also examined the effect of a seemingly more drastic alteration, replacing lysine with alanine, which has a small, uncharged side chain. The K398A or K1093A alterations, although impaired in STE6 function, were found to retain an even higher level of residual activity (~25% for each) than the corresponding K→R mutations (1% and 15%) (Figure 1d; Figure 2, compare lanes 5 and 12 to lanes 4 and 11). While the more dramatic phenotypes of K→R mutants as compared to K→A mutants were unexpected, these results suggested that changes in local structure resulting from substitution of different residues may be complex, and could cause interference and not just inactivation of function.

Mutations in the NBF B Region

Modeling studies of the ABC proteins suggest that the highly conserved sequence motif LSGGQ lies outside the core ATP binding structure, although its influence on nucleotide binding or hydrolysis is not known (Hyde *et al.*, 1990; Mimura *et al.*, 1991). The phenotype of several *hisP* mutations in this region together with modeling studies suggests a role for this motif in energy transduction (G.F.-L.Ames, personal communication; Hyde *et al.*, 1990). Several CF mutations are clustered within the LSGGQ sequence (Cutting *et al.*, 1990; Beaudet, *et al.*, 1991), including, for example, G→D mutations in the second G (G551D and G1349D) in either NBF of CFTR (Figure 1e). We generated the corresponding *ste6* substitution mutations,

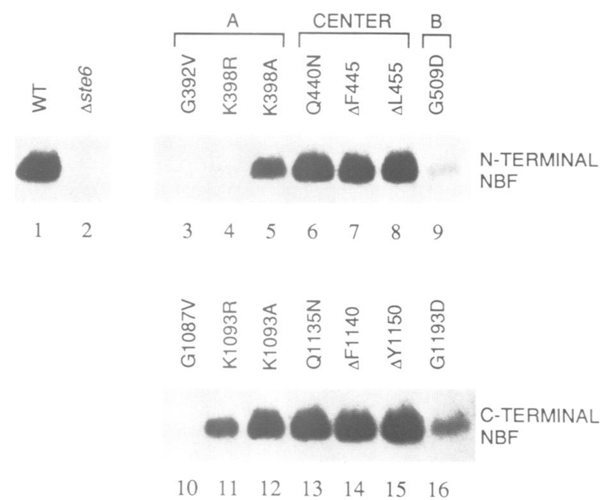

Fig. 2. Immunoprecipitation of **a**-factor secreted from wild type and *ste6* mutant strains. Cells were radiolabeled with [^{35}S]cysteine under steady-state conditions. Cell-associated and extracellular fractions were prepared and immunoprecipitated with **a**-factor antiserum 9−137 as described in Materials and methods. Immunoprecipitates were subjected to electrophoresis in a 16% SDS−polyacrylamide gel and proteins were visualized after fluorography and autoradiography. The mature extracellular species of **a**-factor is shown for wild type STE6, Δste6, and the indicated N-terminal and C-terminal NBF A, B, and Center Region mutants. Immunoprecipitation of intracellular **a**-factor (not shown) indicates that the cell-associated level of **a**-factor is comparable for all mutants examined.

G509D and G1193D, and found that they result in dramatically reduced STE6 function (0.5% and 6% of wild type mating efficiency, respectively) (Figure 1d; Figure 2, lanes 9 and 16). Like the K→R alterations described above, G→D mutations in different NBF domains differ in the degree to which they inactivate STE6.

The view that emerges from the mutational analysis described above is that conserved residues in the A and B Regions of STE6 are highly sensitive to mutation. Furthermore, our results indicate that each NBF domain plays a critical role in **a**-factor transport, since a mutation in either NBF is sufficient to inactivate STE6.

Mutations in the Center Region

The ABC proteins show only limited and patchy homology in the Center Region. Nonetheless, this region is of particular interest because the CF mutation ΔF508 occurs in this region of CFTR. As can be seen in the alignment shown in Figure 1b, this phenylalanine is not conserved among ABC proteins. However, STE6 and MDR1 each contain a hydrophobic residue at the analogous position. Accordingly, we generated the corresponding *ste6* deletion mutations, ΔL455 in the N-terminal NBF and ΔY1150 in the C-terminal NBF. Neither of these STE6 variants is detectably impaired for transport activity, as determined by mating (Figure 1d), **a**-factor immunoprecipitation (Figure 2, lanes 8 and 15) or halo assay (Figure 3). We also examined the effect of deleting a nearby phenylalanine that is conserved among many ABC family members. Neither deletion mutation, ΔF445 or ΔF1140, detectably impairs STE6 function (Figure 1d; Figure 2, lanes 7 and 14). In addition, the conserved glutamine in the Center Region of both NBF domains was altered to generate the substitution mutations, Q440N or Q1135N, which likewise had no significant effect

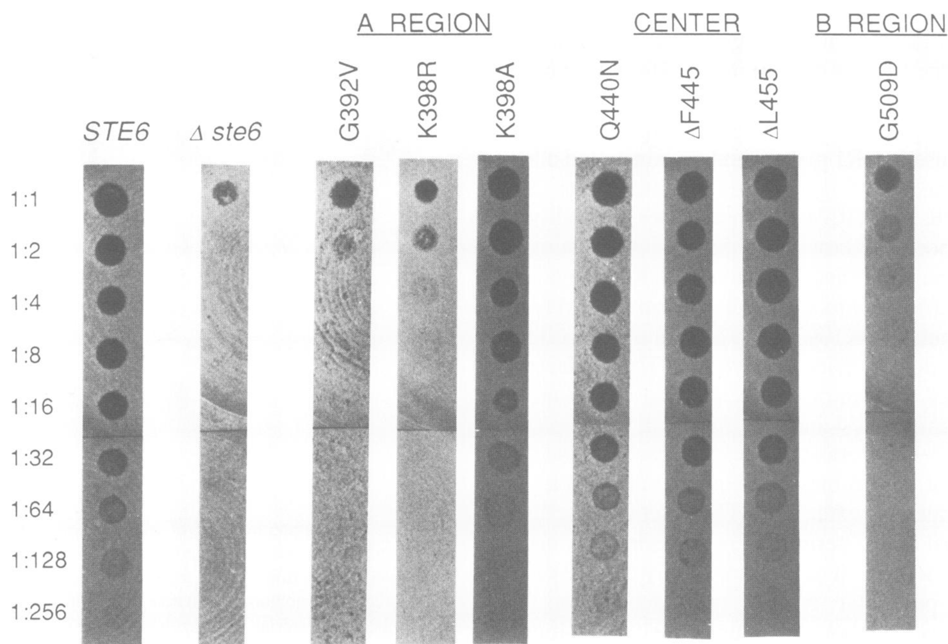

Fig. 3. Quantitative **a**-factor halo assay of wild type and *ste6* mutant strains. A 2-fold dilution series of concentrated culture fluid prepared from wild type and the indicated mutant strains is shown. The zone of clearing (halo) reflects G_1 arrest of the underlying *MATα* lawn due to the presence of **a**-factor. The final dilution at which growth inhibition occurs defines the **a**-factor titer. Titers determined here are: wild type *STE6*, 128; Δ*ste6*, 1; G392V, 2; K398R, 2; K398A, 32; Q440N, 128; ΔF445, 128; ΔL455, 128; G509D, 2. The observed degree of deviation for this assay is one dilution spot. It should be noted that in the Δ*ste6* strain containing no plasmid-borne copy of the *STE6* gene, a residual halo is produced. This residual halo could be due to a liberation of internal **a**-factor as a result of a low amount of cell lysis, or alternatively, it may reflect export of **a**-factor via a *STE6*-independent pathway (S.Sapperstein and S.Michaelis, unpublished results). The residual halo produced by the Δ*ste6* deletion mutant is not always visible and varies with the thickness of the lawn.

on STE6 function (Figure 1d; Figure 2, lanes 6 and 13).

Thus, although the A and B regions of STE6 are highly sensitive to mutation, changes in several positions of the Center Region have little effect on **a**-factor export. Even deletions in this region, which might be expected to have a dramatic impact on protein folding and structure, do not appreciably perturb STE6 function. A comparison of the markedly different effects of A and B versus Center Region mutations is illustrated in Figure 3, which shows quantitative halo assays for the N-terminal NBF mutations described above.

Metabolic stability of STE6 mutant polypeptides

It has been reported that when CFTR is altered by certain mutations, for instance ΔF508 and K464M, it is mislocalized and consequently degraded (Cheng *et al.*, 1990; Gregory *et al.*, 1991). To examine the effects of mutations on STE6 stability, we compared wild-type and mutant STE6 polypeptides by metabolic labeling and immunoprecipitation, using antiserum that recognizes the STE6 protein (Figure 4a; preparation of this antiserum is described in Materials and methods). The amount of STE6 protein present in cells 1 h after a pulse-label was not significantly reduced in any of the mutants, as shown in Figure 4b, which compares the protein levels among wild-type STE6, ΔL455, and the N-terminal NBF mutants most defective in activity. An essentially wild-type level of STE6 was also seen for all other mutants generated in this study (data not shown). Since STE6 is not degraded in any mutant we examined, loss of function is most likely due to a decrease in STE6 activity *per se*, though the possibility that these mutations cause aberrant cellular localization cannot be disregarded.

Co-expression of half-molecules of STE6 results in a functional transporter

The results described above with A and B Region mutations suggest that both halves of STE6 make a distinct and necessary contribution to function. We examined the relative importance of the two halves by another approach, asking whether half-molecules of STE6, each with six membrane spanning regions and an NBF, could promote **a**-factor export. Plasmids containing the N-terminal and C-terminal halves of STE6 (Figure 5a), designated pSM415(*N-half*) and pSM434(*C-half*), respectively, were constructed and transformed into a Δ*ste6* strain. The coding regions for both half-molecules are flanked by the same 5′ and 3′ regulatory regions, and therefore are expected to be expressed at equivalent levels. (These regulatory regions are those of native *STE6*.) The two half-molecule plasmids contain different nutritional markers.

Transformants harboring either pSM415(*N-half*) or pSM434(*C-half*) alone did not secrete **a**-factor or mate (Figure 5b,c). However, when both halves were co-expressed in the same cell, a strikingly different result was observed. In this case, substantial **a**-factor transport occurred, as seen by halo and mating assays (Figure 5b,c), suggesting that the two STE6 half-molecules form a functional STE6 transporter. Indeed, **a**-factor transport mediated by the co-expressed half-molecules occurs very efficiently, at a level >50% that of wild type, as assayed

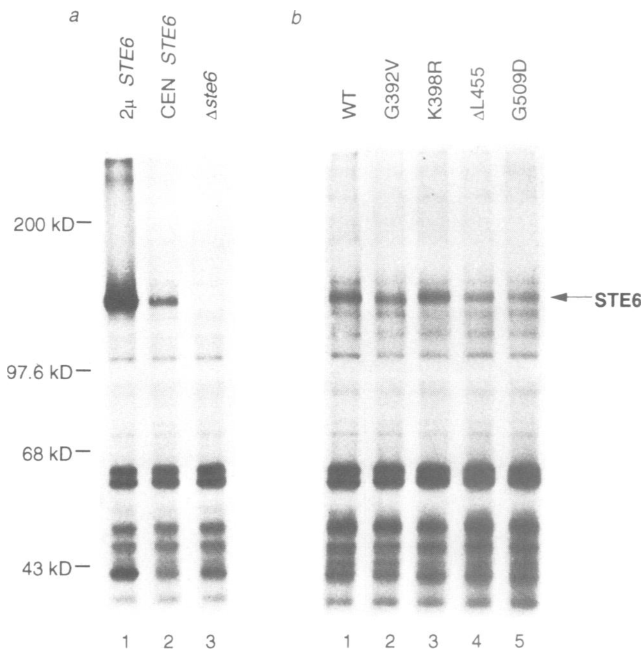

Fig. 4. Immunoprecipitation of STE6 protein from wild type and *ste6* mutant strains. **(a)** Identification of the STE6 protein in yeast extracts. Strains were radiolabeled with Tran^{35}S-label for 5 min, cell extracts were prepared, and immunoprecipitation was carried out using STE6 antiserum C12-JH210 prepared as described in Materials and methods. SDS–PAGE was carried out using 7% Laemmli gels, followed by fluorography and autoradiography. The identity of the indicated ~145 kDa band as STE6 was established on the basis of its absence in the Δ*ste6* deletion strain SM1646 (lane 3) and its overexpression in a strain bearing a 2μ *STE6* plasmid (pSM435) (lane 1) compared to a lower level of expression from a *CEN STE6* plasmid (pSM322) (lane 2). The high molecular weight material at the top of lane 1 likely represents an aggregated form of STE6, since it is absent in the Δ*ste6* strain (lane 3). **(b)** Mutant and wild type forms of STE6 have comparable stability. Cells were labeled as described above except that the 5 min labeling period was followed by a 1 h chase. Shown here are immunoprecipitates from SM1646 containing a plasmid with wild type *STE6* or the N-terminal mutations G392V, K398R, ΔL455 or G509D, as indicated.

by quantitative mating, compared with <0.0001% for strains expressing one or the other half-molecule alone. To preclude the possibility that an unusual recombination event might have occurred between the two plasmids to reconstitute a nearly full-length *STE6* gene, each plasmid was lost separately from the double transformant by screening for loss of the *TRP1* or *LEU2* nutritional marker. We observed loss of **a**-factor transport activity simultaneously with loss of either the pSM415(*N-half*) or the pSM434(*C-half*) plasmid (Figure 5b and c; patches 2 and 3). Moreover, no full-length STE6 is detectable by immunoprecipitation with STE6 antiserum in cells expressing either half-molecule alone, or in combination (Figure 5d). Instead, a polypeptide of the approximate molecular weight predicted for the C-terminal half-molecule is present in extracts from transformants containing pSM434(*C-half*) alone (Figure 5d, lane 3) or together with pSM415(*N-Half*) (Figure 5d, lane 4) but not in transformants containing only the latter plasmid (Figure 5d, lane 2). Since the STE6 antibodies were generated against the C-terminal NBF only, they are not expected to recognize the N-terminal half-molecule. It is interesting to note that a higher level of polypeptide is detected for the C-terminal half-molecule than for full-length STE6 (compare lanes 3 and 1). Whether this difference reflects a higher expression level or greater stability of the half-molecule, or is simply due to more efficient recognition by our antibodies, remains to be determined.

Discussion

We have used mutational analysis to dissect the structure and function of STE6, the yeast **a**-factor transporter. Among eukaryotic members of the ABC protein superfamily, STE6 is particularly tractable to genetic and molecular analysis because its physiological substrate is known and export of the **a**-factor pheromone can be measured directly. Furthermore, although STE6 is necessary for mating, it is not essential for cell viability (Wilson and Herskowitz, 1984; Kuchler *et al.*, 1989; McGrath and Varshavsky, 1989), and can therefore be readily manipulated.

We have shown here that co-expression of the two halves of STE6 as separate polypeptides in the same cell results in formation of a functional STE6 transporter. It is notable that the **a**-factor export promoted by this severed transporter is quite efficient (>50% of the wild type level), although neither half of STE6 expressed alone exhibits any detectable transport activity (<0.0001%). These results demonstrate that both halves of STE6 provide a unique and essential contribution to the formation of a functional transporter. The notion that both halves of STE6 are required for function is also supported by our analysis of *ste6* substitution and deletion mutations (see below).

Reconstitution of an active complex from separated protein fragments has been demonstrated for several membrane proteins, including the *E.coli* lactose permease (*LacY*) (Bibi and Kaback, 1990), β-adrenergic receptor (Kobilka *et al.*, 1988), and the sodium channel (Stuhmer *et al.*, 1989). However, the STE6 'half-molecule' analysis reported here is the first example of *in vivo* severing and functional assembly for a protein belonging to the ABC superfamily. The ability of the separated halves of STE6 to associate and reconstitute a functional transporter may reflect an underlying modular architecture characteristic for the family. Four distinct domains (two NBFs and two sets of six membrane spanning segments) are thought to be necessary for a basic ABC transporter (Ames, 1986; Hyde *et al.*, 1990), but in a number of family members these domains are not all present on a single polypeptide. In some bacterial nutrient permeases, for instance, each domain is encoded by a separate gene (Hiles *et al.*, 1987). For the naturally occurring 'half-molecule' transporters such as HlyB, an NBF domain and a transmembrane domain are joined on a single polypeptide. Two identical HlyB polypeptides are presumed to form homodimers, thereby attaining the requisite four domains. In the case of STE6 half-molecules, our results suggest that transport function is dependent upon the formation of heterodimers since both halves are required for activity. It will be interesting to explore the possibility that artificially constructed 'half-molecules' comprising halves derived from two different ABC family members could form functional chimeric transporters. Reconstitution of a functional complex *in vivo* by protein fragments may also have potential applications for CF gene therapy.

To determine the functional significance of residues conserved among the ABC proteins, we have examined the

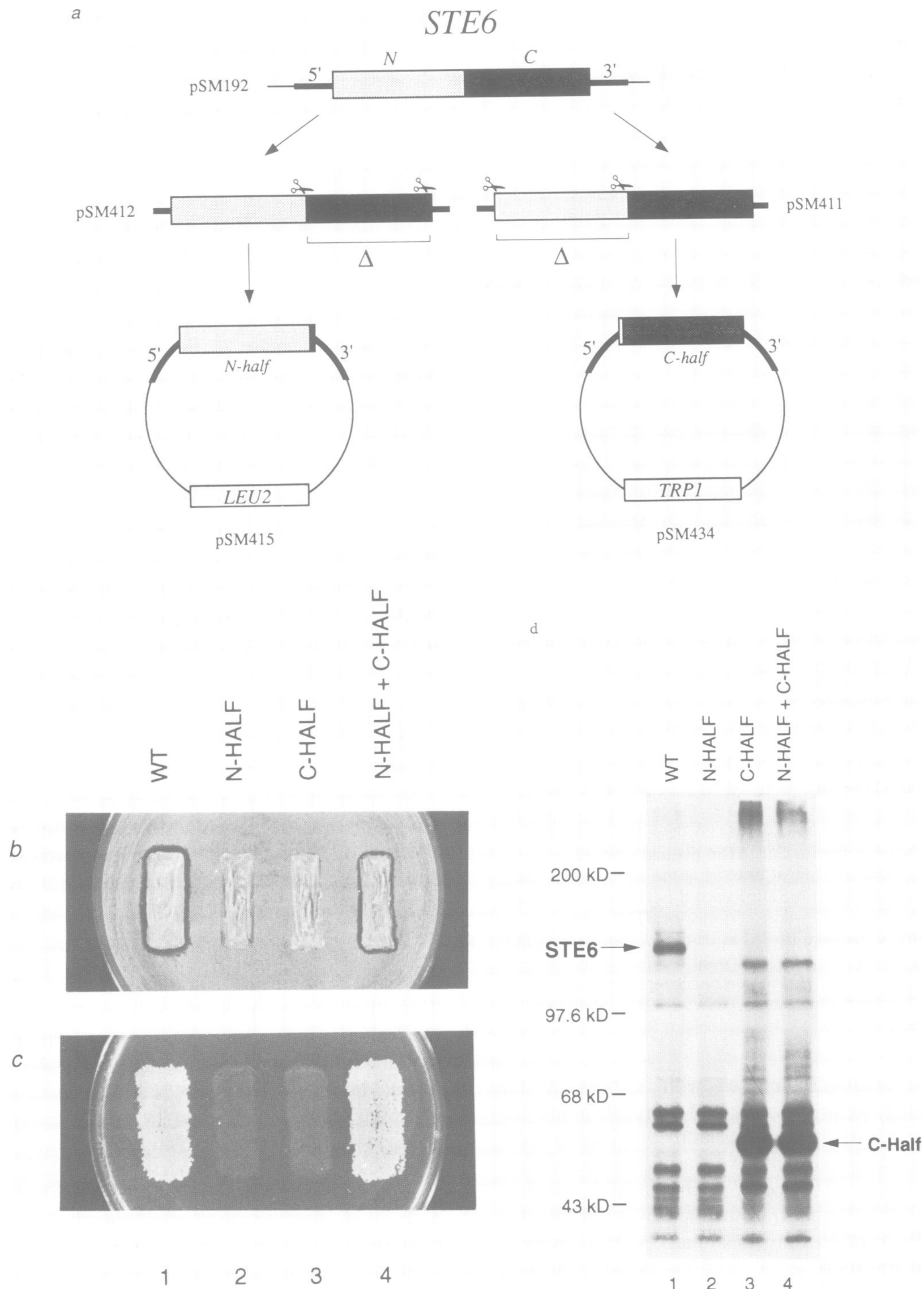

Fig. 5. Co-expression of STE6 half-molecules results in a functional STE6 transporter. (**a**) Construction of plasmids expressing STE6 half-molecules. Pairs of *Bam*HI restriction sites (scissors) were engineered into the *STE6* gene in such a way that digestion with *Bam*HI followed by re-ligation would yield derivatives containing the coding region for only the N-terminal (light gray) or C-terminal (dark gray) half of *STE6* (designated *N-half* or *C-half*, respectively). The *Bam*HI sites were placed so that each half-molecule gene would utilize the native *STE6* upstream and downstream regulatory regions (bold), ribosome binding site, translation start site and stop codon. (**b**) and (**c**) Co-expression of *N-Half* and *C-Half STE6* half-molecules results in export of **a**-factor. Plasmids pSM415 (*N-Half*) and pSM434 (*C-Half*) were transformed together into the Δ*ste6* deletion strain SM1646, and Leu^+ Trp^+ co-transformants were selected. A cell patch of one such co-transformant, designated SM1817, is shown (patch 4). Leu^- and Trp^- 'cured' derivatives were obtained by random plasmid loss after propagation of SM1817 in non-selective media. The resulting strains, SM1839 Leu^+ Trp^- (patch 2) and SM1840 Trp^+ Leu^- (patch 3), contain the individual half-molecule plasmids, pSM415(*N-Half*) and pSM434(*C-Half*), respectively. The strain SM1783, containing a wild-type *STE6* plasmid, is also shown (patch 1). The phenotype of the transformants was assessed by qualitative halo (b) and patch mating (c) assays. (**d**) Absence of full-length STE6 protein in cells co-expressing *STE6* half-molecules. Strains were radiolabeled with Tran^{35}S-label for 5 min, extracts were prepared, and proteins immunoprecipitated using antiserum C12-JH210. Lane 1, SM1783; lane 2, SM1839; lane 3, SM1840; lane 4, SM1817. The C-terminal half-molecule migrates at a position slightly lower than its expected molecular weight of 68 kDa. Similarly aberrant rapid mobility has been detected for the *E.coli* LacY permease which, like STE6, has multiple membrane spanning segments (Bibi and Kaback, 1990). Migration of full-length STE6, which is absent from strains expressing half-molecules, is indicated.

consequences of mutating these residues in STE6. Interestingly, every mutation we examined in the A and B Region of either NBF domain of STE6 significantly impaired function, although the precise extent of inactivation varied. Such deleterious changes include mutations in the glycine and lysine residues of the Walker A motif and alteration of a glycine residue of the LSGGQ motif in the B Region. It will be of interest to directly determine the effects of these mutations on ATP binding and hydrolysis. However, regardless of the specific mechanisms involved, the finding that a single substitution mutation in either NBF can inactivate STE6 provides further evidence that both halves of STE6 make an essential contribution to function. CFTR and MDR are also debilitated by mutations that disrupt only one NBF (Azzaria *et al.*, 1989; Currier *et al.*, 1989; Cuppens *et al.*, 1990; Cutting *et al.*, 1990; DeVoto *et al.*, 1991). Presumably, two distinct functional steps carried out by ABC proteins involve utilization of ATP (Higgins *et al.*, 1985; Mimmack *et al.*, 1989).

In contrast to our findings with mutations in the A and B Regions, none of the STE6 Center Region mutations described here appeared to have any significant effect on **a**-factor transport. Perhaps most intriguing was the wild type level of activity observed in the ΔL455 deletion mutant. This leucine residue occupies a position in the N-terminal NBF of STE6 analogous to that of F508 in CFTR, site of the prevalent CF mutation, ΔF508. Similarly, deletion of Y1150, the C-terminal counterpart of L455 (for which there is no corresponding mutation thus far identified in CFTR), did not impair STE6 function. Two other *ste6* deletion mutations in the Center Region, ΔF445 and ΔF1140, also transported **a**-factor at essentially wild type levels. In light of the suggestion that single residue deletions are likely to profoundly affect the structure of this region (Thomas *et al.*, 1991), the negligible impact of these deletions on STE6 function is interesting.

What can account for the lack of effect of Center Region mutations on STE6 function? One possibility is that the Center Region, unlike the A and B Regions, is functionally duplicated in STE6 (and not in CFTR) so that only by mutating the Center Regions of both halves simultaneously can a phenotype be revealed. Preliminary results suggest that the *ste6* double mutant ΔF445 ΔF1140 is significantly impaired in STE6 activity (C.Berkower and S.Michaelis, unpublished). Yet another possibility is that the function of residues in the Center Region is highly 'context dependent' so that only a small subset of the possible changes are detrimental. In this regard, it is interesting to note that while the ΔF508 mutation of CFTR results in disease, a different alteration, F508C, is silent (Kobayashi *et al.*, 1990). Alternatively, the Center Region may play a special role in CFTR, one that it does not have in common with STE6; for instance, CFTR has been proposed to function as a chloride ion channel instead of, or in addition to, mediating active transport (Anderson *et al.*, 1991a,b; Kartner *et al.*, 1991) and several mutations including ΔF508 appear to result in metabolic instability of CFTR (Cheng *et al.*, 1990; Gregory *et al.*, 1991). While further characterization of the function of the NBF Center Region will require direct structural analysis, our finding here that alterations in this region have negligible impact on STE6 function lends further support to the possibility (Hyde *et al.*, 1990) that the Center Region may not be directly involved in ATP binding or hydrolysis.

It has previously been established that yeast *MAT***a** cells must secrete **a**-factor in order to mate (Michaelis and Herskowitz, 1988). Our studies here have revealed that the efficiency of mating is directly proportional to the level of secreted **a**-factor. This direct correlation between mating and the level of secreted **a**-factor allows the use of quantitative mating assays as an accurate and highly sensitive indicator of STE6 function. STE6-mediated export of **a**-factor represents a novel mechanism for protein secretion, distinct from the classical secretory pathway (Schekman, 1985; McGrath and Varshavsky, 1989). **a**-Factor itself is somewhat unusual among secreted molecules in that it is prenylated and methylated (Anderegg *et al.*, 1988; He *et al.*, 1991; Hrycyna *et al.*, 1991), and consequently extremely hydrophobic. Interestingly, hydrophobicity is also a common feature of the drugs capable of being transported by the MDR protein. The mechanistic basis for recognition of a wide variety of substrates by MDR is not understood. Further mutational analysis of STE6, including the construction of chimeras with other ABC family members, may be useful in revealing specific regions within these proteins responsible for substrate recognition.

Materials and methods

Yeast strains, media and growth conditions

The *ste6* deletion strain SM1646 was generated by one-step gene replacement of the wild-type *STE6* gene in SM1060 (*MAT***a**/α *trp1 leu2 ura3 his4 can1*) followed by sporulation dissection and selection of a haploid MATa *ste6* spore. Transformation was carried out using an *Sph*I−*Fsp*I fragment from pSM280, a derivative of pBR322-*STE6* (Wilson and Herskowitz, 1984) obtained by deleting the segment of *STE6* between the *Stu*I and *Sna*B1 sites (Kuchler *et al.*, 1989; McGrath and Varshavsky, 1989) and replacing it with the selectable marker *URA3*. This deletion mutation, designated Δ*ste6*::*URA3*, removes most of the *STE6* coding sequence (amino acids 39−1167 of 1290 total). The gene disruption was verified by Southern hybridization. Strains SM1086 (*MAT*α *sst2−1 his6 met1 can1 cyh2*) and SM1068 (*MAT*α *lys1*) were used for **a**-factor halo and mating assays, respectively. Construction of the transformants designated SM1817, SM1839, and SM1840 is described in Figure 5. Complete media (YEPD), SD drop-out media, and SD minimal media were prepared as previously described (Michaelis and Herskowitz, 1988). Where necessary, SD medium was supplemented with histidine (20 μg/ml), tryptophan (20 μg/ml), uracil (20 μg/ml) or leucine (100 μg/ml). All experiments were performed at 30°C.

Plasmid constructions

The vectors used for constructions in this study are pRS314 (*TRP1*), pRS315 (*LEU2*) and pRS316 (*URA3*), which are all single-copy *CEN* plasmids that can be propagated in yeast and *E.coli* and that contain the M13 replication origin (Sikorski and Hieter, 1989). The parental plasmid for site-directed mutagenesis is pSM322 (*STE6 CEN6 LEU2*), which contains the *Sal*I−*Hin*dIII *STE6* fragment from pBR322-*STE6* (Wilson and Herskowitz, 1984) cloned into pRS315-1, a PRS315 derivative in which a small region including the *Bam*HI site was removed from the linker. In some cases, mutations were initially created in a similar plasmid, pSM192 (*STE6* in pRS316), and then subcloned into pRS315-1 prior to transformation into SM1646. The high copy number plasmid pSM435 was constructed by cloning the *Sal*I−*Hin*dIII *STE6* fragment from pSM192 into the polylinker of pSM218 (2μ LEU2), which is a derivative of pRS305 (Sikorski and Hieter, 1989).

For construction of the *N-half* plasmid pSM415 (see Figure 5), the *STE6* gene derived from pSM192 was used to construct pSM412, which has *Bam*HI sites at positions corresponding to residues 694 and 1290 of STE6, in the vector pRS315-1. Plasmid pSM412 was digested with *Bam*HI and re-ligated, yielding the deletion derivative pSM415(*N-half*), which encodes the first six membrane spanning segments, N-terminal NBF, and linker region of *STE6*, and contains the *LEU2* marker. The *C-half* plasmid pSM434 construction involved a similar strategy, except that *Bam*HI sites were introduced at positions corresponding to codons 7 and 694 of *STE6* in plasmid pSM192 and the modified gene was recloned into the vector pRS315-1, yielding pSM411. Deletion of the segment between the *Bam*HI sites, followed by re-ligation and cloning into the vector pRS314, yielded the deletion

derivative pSM434(*C-half*), which encodes the C-terminal half of *STE6* (residues 695–1290) preceded by residues 1–7 and contains the *TRP1* marker. A native *Bam*HI site normally present in *STE6* was removed by oligonucleotide-directed mutagenesis (resulting in a silent codon change in *STE6*) at an early step of construction and thus is absent from the plasmids used here. For plasmid loss experiments, the double transformant SM1817 was subjected to three rounds of re-passaging in non-selective medium (YEPD). Single colonies cured of one or the other plasmid were identified by replica-plating onto SD-LEU and SD-URA drop-out media.

Oligonucleotide-directed mutagenesis

Single-stranded template DNA was generated from the *STE6* plasmid pSM322 (or pSM192) using the helper phage VCS-M13. Mutagenesis was performed using the Muta-Gene Phagemid *in vitro* Mutagenesis kit (Bio-Rad). Mutagenic oligonucleotides ranged in size from 26 to 36 bases. Codon usage was taken into account to avoid introducing particularly rare codons into the *STE6* sequence. After mutagenesis, plasmids were propagated in *E.coli* strain DH5α and screened by DNA sequencing for the presence of the desired mutation. Two independent isolates were obtained for most mutations. Plasmids bearing mutations were transformed into the Δste6::*URA3* yeast strain SM1646, and were subsequently rescued from yeast and resequenced to verify the mutation.

Preparation of STE6 antibodies

A gene fusion was constructed between the *E.coli trpE* gene and a portion of *STE6* using the pATH vector system (Koerner *et al.*, 1990). The 1.8 kb *Nco*I–*Hin*dIII fragment from pBR322-*STE6* was made blunt-ended using Klenow fragment and cloned into pATH2 that had been digested with *Sma*I. The resulting plasmid, pSM253, contains a *trpE-STE6* fusion encoding the C-terminal portion of the STE6 protein, including residues 1043–1290. Induction of the *trpE–STE6* fusion was carried out as previously described (Hrycyna *et al.*, 1991). Four hours after induction, cells were collected by centrifugation, lysed and insoluble proteins were collected essentially as described by Koerner *et al.* (1990). The fusion protein band was excised from a preparative 10% SDS–polyacrylamide gel, stained with 0.3 M copper chloride, rinsed with water, macerated, frozen at −80°C, and lyophilized. Rabbits were injected with 100 μg of the fusion protein and boosted at 2 week intervals (Hazelton Labs, PA). Polyclonal antiserum from one rabbit, C12-JH210, was shown to recognize the STE6 polypeptide in yeast (see Figure 4).

Metabolic labeling and immunoprecipitation of STE6

For immunoprecipitation of STE6, cells were grown to log phase (OD_{600} 0.4–0.8) in SD medium containing required supplements. Five OD_{600} units of cells were resuspended in 0.5 ml fresh medium together with 300 μCi Tran^{35}S-label (ICN) and incubated at 30°C. Labeling was terminated after 5 min by addition of cells to ice-cold 2× azide stop mix (40 mM cysteine, 40 mM methionine, 20 mM NaN_3, 500 μg/ml BSA). In some cases, as stated in the text, cells were subjected to a 1 h chase period after radiolabeling. The chase was initiated by addition of unlabeled methionine and cysteine to a final concentration of 40 mM each and terminated by addition of cells to 2× azide stop mix. Cells were lysed in 0.24 M NaOH/1% β-mercaptoethanol and proteins were precipitated with 6% TCA at 4°C and resuspended in sample buffer for TCA pellets (3.5% SDS, 14% glycerol, 80 mM Tris base, 80 mM Tris pH 8, 8 mM EDTA, 120 mM DTT, 0.01% bromophenol blue). The soluble portion of this whole cell lysate was added to immunoprecipitation buffer [1.0% Triton X-100, 150 mM NaCl, 5 mM EDTA, 50 mM Tris–HCl pH 7.5, 1 mM PMSF, 0.5% Trasylol (FBA Pharmaceuticals)] and STE6 was immunoprecipitated in the presence of an excess amount of lysate from unlabeled Δ*ste6* cells using STE6 antiserum C12-JH210. Antigen–antibody complexes were collected on protein A–Sepharose beads, released by addition of Laemmli sample buffer, and incubated at 37°C for 20 min prior to electrophoresis. SDS–PAGE was carried out using 7% Laemmli gels, followed by fluorography and autoradiography.

Metabolic labeling and immunoprecipitation of a-factor

Log phase cells (five OD_{600} units) were labeled under steady-state conditions for 1 h in SD-LEU drop-out medium with 300 μCi of [^{35}S]cysteine (New England Nuclear). Cell-associated and extracellular fractions were prepared and immunoprecipitated with **a**-factor antiserum 9-137 as described elsewhere (Hrycyna *et al.*, 1991). Immunoprecipitates were subjected to electrophoresis in a 16% SDS–polyacrylamide gel and proteins were visualized after fluorography and autoradiography.

Physiological assays

Quantitative mating assays were performed by the plate mating procedure (Michaelis and Herskowitz, 1988). Mutants to be tested were grown to saturation in SD-LEU medium and serially diluted in 10-fold increments into YEPD. An aliquot of each dilution (0.1 ml) was spread on an SD plate together with ~10^7 cells of the *MAT*α mating tester SM1068 in YEPD. All mutant *MAT***a** strains tested were His$^-$ and the *MAT*α tester SM1068 was Lys$^-$. Diploids resulting from mating are prototrophic and can therefore form colonies on SD. After 3 days, diploids were counted and normalized to the total number of *MAT***a** cells plated. The frequency of diploid formation for the mutant as compared to wild type is designated 'mating efficiency' and is expressed as a percentage of wild type. Results represent an average of three or more separate trials. Standard deviation (SD) for values ≥87% is 27%; for values of 15–26%, SD = 7%; for the value of 6%, SD = 2.1%; for values ≤0.5%, SD = 0.2%.

For quantitative halo assays, cells grown in SD medium containing required supplements were inoculated into YEPD at OD_{600} 1.0 and propagated for 4–5 generations to saturation. The culture fluid was clarified twice by centrifugation and concentrated 8-fold using a centrifugal ultrafiltration concentrator (Centricon 30, Amicon Corp.). A 2-fold dilution series of the concentrated culture fluid was generated in 250 μg/ml BSA. Aliquots (3 μl) of each dilution were spotted onto a lawn of the supersensitive *MAT*α strain SM1086 and incubated at 30°C for 20 h. Qualitative halo tests and patch mating were carried out as previously described (Michaelis and Herskowitz, 1988).

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