The Saccharomyces cerevisiae STE14 gene encodes a methyltransferase that mediates C-terminal methylation of α-factor and RAS proteins

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Post-translational processing of a distinct group of proteins and polypeptides, including the α-factor mating pheromone and RAS proteins of Saccharomyces cerevisiae, results in the formation of a modified C-terminal cysteine that is S-isoprenylated and α-methyl esterified. We have shown previously that a membrane-associated enzymatic activity in yeast can mediate in vitro methylation of an isoprenylated peptide substrate and that this methyltransferase activity is absent in ste14 mutants. We demonstrate here that STE14 is the structural gene for this enzyme by expression of its product as a fusion protein in Escherichia coli, an organism in which this activity is lacking. We also show that α-factor, RAS1 and RAS2 are physiological methyl-accepting substrates for this enzyme by demonstrating that these proteins are not methylated in a ste14 null mutant. It is notable that cells lacking STE14 methyltransferase activity exhibit no detectable impairment of RAS function or cell viability. However, we did observe a kinetic delay in the rate of RAS2 maturation and a slight decrease in the amount of membrane localized RAS2. Thus, methylation does not appear to be essential for RAS2 maturation or localization, but the lack of methylation can have subtle effects on the efficiency of these processes.

Key words: α-factor mating pheromone/C-terminal farnesyl cysteine methyltransferase/methyl esterification/RAS processing/yeast STE14 gene

Introduction

A group of eukaryotic proteins and polypeptides that undergo post-translational modification are synthesized with a C-terminal sequence, -Cys-Xaa-Xaa-Xaa (where Xaa is any amino acid). This sequence motif, designated the CXXX motif, serves as a signal for three ordered modification events including isoprenylation of the cysteine sulfhydryl, proteolysis of the terminal three residues, and α-methyl esterification of the newly exposed carboxyl group (Clarke et al., 1988; Hancock et al., 1989; Rine and Kim, 1990; Stimmel et al., 1990). Examples include fungal mating pheromones (Ishibashi et al., 1984; Anderegg et al., 1988; Akada et al., 1989), RAS proteins (Gutierrez et al., 1989), nuclear lamins (Farnsworth et al., 1989) and the γ-subunit of trimeric G proteins (Yamane et al., 1990). All of these polypeptides are membrane associated, or pass through a membrane. While isoprenylation has been shown to play an essential role in membrane localization, the role of the methylation reaction has not been established.

Genetic analysis in the yeast Saccharomyces cerevisiae has provided a means for identifying cellular components that mediate these post-translational processing events. The yeast mating pheromone α-factor precursor terminates in a CXXX motif (Brake et al., 1985; Powers et al., 1986; Michaelis and Powers, 1988). Mature bioactive α-factor is a secreted dodecapeptide with a C-terminal cysteine that is farnesylated and methyl esterified (Anderegg et al., 1988). Because defects in the synthesis of α-factor result in a readily detectable MATA cell-specific sterile phenotype, it has been possible to identify four mutants, ste6 (Rine, 1979), ste14 (Blair, 1979), ram1 (Powers et al., 1986; Wilson and Herskowitz, 1987) and ram2 (Goodman et al., 1990), that are impaired in α-factor secretion or processing. STE6 encodes a membrane transporter responsible for the export of α-factor (McGrath and Varshavsky, 1989; Kuchler et al., 1989). RAM1, alternatively named DPR1, was originally identified on the basis of its necessity for both RAS and α-factor activity (Powers et al., 1986; Fujiiyama et al., 1987; Michaelis and Powers, 1988). In ram1 mutants, the RAS and α-factor precursors fail to become membrane localized and remain in the cytosol (Powers et al., 1986; Schafer et al., 1990; P. Chen and S. Michaelis, unpublished observations). Recently, the RAM1 gene was shown to encode a component of the farnesyltransferase enzyme responsible for isoprenylation of RAS, α-factor and the γ-subunit of a yeast G protein (Goodman et al., 1988, 1990; Finegold et al., 1990; Schafer et al., 1990). Thus, the yeast farnesyltransferase mediates modification of multiple CXXX-terminating cellular proteins. The RAM2 gene, like RAM1, appears to be critical for isoprenylation of proteins in yeast (Goodman et al., 1990). Farnesyltransferase activity has also been found in mammalian systems (Reiss et al., 1990; Schafer et al., 1990).

We have investigated the role of the STE14 gene product in the post-translational modification of yeast proteins containing a CXXX sequence at their C-terminus. DNA sequence analysis reveals that the STE14 product is 239 residues in length and appears to contain multiple membrane spanning domains (Sapperstein et al., 1989). We have recently demonstrated, using an in vitro assay, that S. cerevisiae contains a membrane-bound C-terminal methyltransferase activity specific for a peptide substrate terminating in S-farnesyl cysteine (Hrycyna and Clarke, 1990). This methyltransferase activity is present in both MATA and MATα cells and is dependent upon the presence of a wild-type STE14 gene. A mammalian counterpart of the yeast methyltransferase has been recently described that possesses similar activity (Stephenson and Clarke, 1990).

In the present report, we further examine the in vitro and
finding that the \textit{STE14}-dependent C-terminal methyltransferase from yeast efficiently recognizes N-AcFC as a substrate suggests that peptide sequences which precede the isoprenylated cysteine residue in CXXX-terminating proteins are not required as a signal for methylation.

The wild-type and \textit{ste14} null mutant strains we tested here are isogenic. Thus, the lack of significant levels of methyltransferase activity observed in these strains (Table I) can be attributed solely to the presence or absence of an intact \textit{STE14} gene, and not to unrelated strain differences. Furthermore, a \textit{ste14} mutant complemented by a high copy number \textit{STE14} plasmid produces a higher than wild-type level of methyltransferase activity (Table I; Hrycyna and Clarke, 1990). The observation that we detect essentially no methyltransferase activity \textit{in vitro} using \textit{ste14} extracts, together with the \textit{in vivo} results for \textit{a-factor} and RAS presented below, suggest that \textit{STE14} is the major yeast methyltransferase involved in post-translational modification of isoprenylated proteins.

Recent evidence from mammalian systems suggests that the methyl esterified cysteine residue of some proteins with a C-terminal CXXX motif is not modified by a farnesyl (C\textsubscript{15}) group, but rather by a geranylgeranyl (C\textsubscript{20}) group (Yamane \textit{et al}., 1990). Although this modification has not yet been detected in yeast proteins, we examined whether the substrate S-geranylgeranyl LARYKC could be recognized by the \textit{STE14} methyltransferase. We found that purified membranes from wild-type yeast catalyze the methylation of S-geranylgeranyl LARYKC at a rate of at least 50\% that of S-farnesyl LARYKC, whereas membranes prepared from \textit{ste14} mutants show no detectable activity with either substrate (data not shown). Thus it appears that the \textit{STE14} methyltransferase can recognize a C-terminal cysteine residue modified by either a farnesyl or geranylgeranyl moiety.

\textbf{STE14-mediated methylation of \textit{a-factor}}

The \textit{ste14} mutation was first identified on the basis of its defect in \textit{MATa} cell mating. To determine directly whether \textit{a-factor} is methylated by the \textit{STE14} methyltransferase, we compared \textit{in vivo} methylation of \textit{a-factor} in wild-type and isogenic \textit{ste14} null mutant strains. To enhance our ability to detect \textit{a-factor}, the strains used in these experiments contain a high copy number plasmid which over-produces \textit{a-factor}. Cells were labelled with \textsuperscript{[\textit{35}S]}cysteine and S-adenosyl-	extsuperscript{[\textit{3}H]-methyl}methionine (\textsuperscript{[\textit{3}H]-methyl}AdoMet), intracellular and extracellular fractions were separated, and immunoprecipitation was carried out using \textit{a-factor} antiserum. After SDS-PAGE, \textsuperscript{[\textit{35}S]}cysteine-labelled \textit{a-factor} was visualized by autoradiography, and \textsuperscript{[\textit{3}H]-methyl} groups were detected using the gel slice vapor phase assay (Clarke \textit{et al}., 1988). This assay specifically detects esterlinked methyl groups which are liberated as \textsuperscript{[\textit{3}H]}methanol after base hydrolysis.

In Figure 3, the position of the \textsuperscript{[\textit{35}S]}cysteine-labelled \textit{a-factor} band detected by autoradiography is indicated by an arrow. The difference in migration of extracellular and intracellular \textit{a-factor} relative to the 6.2 kd marker reflects the difference in molecular weight between mature and precursor species of \textit{a-factor}. In the intracellular sample (Figure 3, panel B), a methyl ester peak is apparent in wild-type cells at a position corresponding to the \textit{a-factor} precursor. In contrast, no peak is observed for the \textit{ste14} mutant. Since both strains appear to synthesize equivalent amounts of the \textit{a-factor} precursor based on autoradiography of the \textsuperscript{[\textit{35}S]}labelled \textit{a-factor} (data not shown), the lack of a \textsuperscript{[\textit{3}H]-methyl} peak observed for the \textit{ste14} mutant reflects a lack of methylation, and not absence of the polypeptide \textit{per se}. Thus, we can conclude that the \textit{STE14} methyltransferase is responsible for methylation of \textit{a-factor}.

In the extracellular fraction (Figure 3, panel A), a methyl ester peak is present at a position corresponding to mature \textit{a-factor} in the wild-type strain and absent in the \textit{ste14} mutant. We observe four-fold less \textsuperscript{[\textit{35}S]}labelled \textit{a-factor} in the extracellular fraction from the \textit{ste14} mutant as compared with wild-type (data not shown). Thus, the lack of methylation appears to have an adverse effect on secretion of \textit{a-factor},
in vivo activities promoted by STE14. We show that an extract from an *Escherichia coli* strain synthesizing a TrpE-STE14 hybrid protein is capable of promoting methylation in vitro. This result provides strong evidence that STE14 is the structural gene for the yeast methyltransferase enzyme. We also demonstrate that STE14 is required for the in vitro methylation of a-factor, RAS1 and RAS2. Thus, the STE14 gene product, like RAM1, is an enzyme responsible for the post-translational modifications of multiple yeast proteins. Interestingly, ste14 null mutants are not detectably impaired in RAS activity, suggesting that methyl modification is not essential for RAS function. We do observe, however, that ste14 mutants exhibit a significant kinetic delay in RAS2 maturation and a slight decrease in the amount of RAS2 that becomes membrane-bound.

**Results**

**Methyltransferase activity of TrpE-STE14 hybrid proteins synthesized in *E.coli***

We recently characterized a farnesyl cysteine methyltransferase activity in membrane extracts from *S.cerevisiae* and found that the activity is absent in extracts from ste14 mutant strains (Hrycyna and Clarke, 1990). This result raised the possibility that the STE14 gene encodes the methyltransferase enzyme. Alternatively, the STE14 product could be a rate-limiting component of this enzyme or a regulator of its synthesis. To distinguish these possibilities, we expressed STE14 as a hybrid protein in *E.coli*, an organism with no endogenous farnesyl cysteine methyltransferase activity (Figure 1). Using pATH vectors, two gene fusions were constructed, **TrpE-STE14** and **TrpE-STE14**, in which all or part of the STE14 coding sequence, respectively, was joined to the C-terminal end of the *E.coli* TrpE gene.

We assayed in vitro methyltransferase activity in membrane and cytosolic extracts from strains induced for synthesis of these fusions. A high level of methyltransferase activity was observed with the membrane fraction from a strain containing the **TrpE-STE14**, fusion (Figure 1) in which the entire coding sequence of STE14 is present. This activity was dependent upon the presence of a methyl-accepting substrate and product formation was linearly dependent on time. No activity was observed in membrane fractions from the parental strain which lacks a plasmid, nor in membrane fractions from a strain with the **TrpE-STE14** fusion, which contains only the C-terminal portion of STE14 (Figure 1). In no case was cytosolic methyltransferase activity detected. These results indicate that the STE14 gene product is a methyltransferase and provide strong evidence that the STE14 polypeptide is the sole component of this methyltransferase. The possibility that another component required for activity might be supplied by the *E.coli* membrane fraction is unlikely, but cannot be ruled out.

**Characterization of substrate recognition by the STE14 methyltransferase**

In our previous work, we showed that the farnesylated peptide, S-farnesyl LARYKC is an efficient in vitro substrate for the yeast methyltransferase (Hrycyna and Clarke, 1990). To refine our understanding of the substrate requirements for the STE14 methyltransferase and, in particular, to learn whether specific peptide sequences are required for enzyme recognition, we examined in vitro methylation of a compound lacking any amino acids except for a farnesylated cysteine residue. This compound, N-acetyl farnesyl cysteine (N-AcFC), has been reported to be a methylatable substrate in a mammalian cell extract (Stock et al., 1990). We compared the efficiency of methylation of S-farnesyl LARYKC and N-AcFC substrates using membrane extracts from a wild-type strain and from two isogenic ste14 null mutants. As shown in Table I, the wild-type extract is able to methylate both substrates with comparable efficiency. The identity of the expected product, methyl esterified N-AcFC, was confirmed (Figure 2). No methylation of either substrate was detected in the ste14 membrane extracts (Table I). The

![Fig. 1. Expression of the full length STE14 gene product as a TrpE fusion protein in *E.coli* generates C-terminal farnesyl cysteine methyltransferase activity. Crude membrane fractions (10 µl) from the parent *E.coli* strain, BSG24 ( ), and from transformants with a plasmid containing TrpE fused to the full length STE14 coding region, BSG24 (I TrpE-STE14, ), or to the C-terminal portion of STE14 BSG24 (I TrpE-STE14, ) ( ) were assayed with 50 µM [3H-methyl]AdoMet and 20 µl 100 mM Tris-HCl (pH 7.5) with or without the methyl-accepting substrate N-acetyl farnesyl cysteine (N-AcFC) (1 nmol). The samples were incubated for the times indicated at 37°C. The protein concentrations of the BSG24, BSG24 (I TrpE-STE14, ), and BSG24 (I TrpE-STE14, ) samples were 15.5 mg/ml, 17.8 mg/ml and 19.5 mg/ml respectively. Methyl esters were detected as described in Materials and methods. The small amount of methyl esters formed (less than 0.1 pmol) in the absence of N-AcFC were subtracted to give the values presented. Similar results were obtained when S-farnesyl LARYKC was used as a substrate.

**Table I. Methyltransferase activity in isogenic strains**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Relevant genotype</th>
<th>Methyltransferase activity (pmol/min/mg protein)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Substrate: S-farnesyl-LARYKC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SM1058 (STE14*)</td>
<td>STE14*</td>
<td>0.94 (±0.20)</td>
</tr>
<tr>
<td>SM1188 (ste14)</td>
<td>ste14</td>
<td>0.003 (±0.008)</td>
</tr>
<tr>
<td>SM1639 (ste14)</td>
<td>ste14</td>
<td>0.0006 (±0.0005)</td>
</tr>
<tr>
<td>SM1639 (N-SM344)</td>
<td>ste14 (2µ STE14 LEU2)</td>
<td>4.42 (±0.02)</td>
</tr>
<tr>
<td>Substrate: N-acetyl S-farnesyl cysteine</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SM1058 (STE14*)</td>
<td>STE14*</td>
<td>0.43 (±0.01)</td>
</tr>
<tr>
<td>SM1188 (ste14)</td>
<td>ste14</td>
<td>0.011 (±0.002)</td>
</tr>
<tr>
<td>SM1639 (ste14)</td>
<td>ste14</td>
<td>0.005 (±0.003)</td>
</tr>
<tr>
<td>SM1639 (N-SM344)</td>
<td>ste14 (2µ STE14 LEU2)</td>
<td>8.40 (±0.05)</td>
</tr>
</tbody>
</table>

* Activities from control incubations lacking farnesylated substrate have been subtracted. Each value represents duplicate incubations ± the observed range.
Table II. Incorporation of [3H]methyl esters and [35S]cysteine in immunoprecipitated RAS1 and RAS2 in STE14+ and ste14 strains

<table>
<thead>
<tr>
<th>Strain</th>
<th>Plasmid</th>
<th>[3H]Methyl esters in RAS1 or RAS2 (c.p.m.)</th>
<th>[35S]Cysteine in RAS1 or RAS2 (c.p.m.)</th>
<th>Relative extent of methylation ([3H]c.p.m./[35S]c.p.m. × 10)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SM1058 (STE14+)</td>
<td>(YEp-RAS2-4)</td>
<td>17.3</td>
<td>285.8</td>
<td>0.61b</td>
</tr>
<tr>
<td>SM1188 (ste14)</td>
<td>(YEp-RAS2-4)</td>
<td>0.9</td>
<td>192.2</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>SM1058 (STE14+)</td>
<td>(pADH-RAS2)</td>
<td>148.8</td>
<td>1120.8</td>
<td>1.3</td>
</tr>
<tr>
<td>SM1188 (ste14)</td>
<td>(pADH-RAS2)</td>
<td>0.1</td>
<td>1563.8</td>
<td>&lt;0.0006</td>
</tr>
<tr>
<td>SM1058 (STE14+)</td>
<td>(YEp-RAS1)</td>
<td>114.8</td>
<td>1574.6</td>
<td>0.73</td>
</tr>
<tr>
<td>SM1188 (ste14)</td>
<td>(pADH-RAS2)</td>
<td>0.1</td>
<td>1720.5</td>
<td>&lt;0.0008</td>
</tr>
<tr>
<td>SM1058 (STE14+)</td>
<td>(YEp-RAS1)</td>
<td>115.8</td>
<td>2259.2</td>
<td>0.51</td>
</tr>
<tr>
<td>SM1188 (ste14)</td>
<td>(pADH-RAS1)</td>
<td>0.1</td>
<td>1182.4</td>
<td>&lt;0.0008</td>
</tr>
</tbody>
</table>

*Background radioactivity from adjacent gel slices have been subtracted.

methyl]AdoMet, and RAS proteins were examined after immunoprecipitation and SDS–PAGE. Gel slice vapor assays were performed to detect [3H-methyl]esters, and [35S]cysteine incorporated into proteins was monitored by autoradiography and direct counting of gel slices. Figure 4 shows the data obtained with strains containing the RAS2 plasmid. In the autoradiograph shown in Figure 4 (top), a 40 kd [35S]cysteine-labelled band corresponding to RAS2 is present at similar levels in both wild-type and ste14 strains (lanes 1 and 3). In the ste14 mutant, a doublet was consistently observed at this position (see below). The identity of the 40 kd species and doublet bands as RAS2 was confirmed by their disappearance in control immunoprecipitations performed with a competitor peptide recognized by the Y13–259 RAS monoclonal antibody (Figure 4, top, lanes 2 and 4).

To determine the level of RAS2 methylation, the dried gels shown at the top of Figure 4 were examined by gel slice vapor assays (Figure 4, lower panels A–D). In the wild-type strain (Figure 4, panel A), a methyl ester peak is apparent in gel slice number 13, which corresponds to RAS2, as evidenced by its disappearance with addition of the blocking peptide (Figure 4, panel B). Strikingly, no methyl esters are detected at the RAS2 position in immunoprecipitates from the ste14 mutant (Figure 4, panels C and D). These results indicate that RAS2 remains unmethylated in the ste14 mutant and demonstrate that RAS2 is a physiological substrate for the STE14 methyltransferase. These data are quantified in Table II, together with similar results from experiments using a different ste14 null mutant.

We also examined methylation of RAS1 in wild-type and ste14 strains containing a high copy number RAS1 plasmid. The results we obtained were analogous to those described above for RAS2 (Table II). Taken together, the results in Figure 4 and Table II establish that STE14 is responsible for methylation of both the RAS1 and RAS2 proteins. Since it is known that functional RAS proteins are required for viability in yeast (Powers et al., 1984; Tatchell et al., 1984), and since ste14 mutants are not compromised for viability, our results point to the possibility that methylation is not essential for RAS function, at least under normal conditions.

**Maturation of RAS2 in strains lacking the STE14 methyltransferase**

We were interested in understanding the significance of the RAS2 doublet detected in the ste14 mutant, above (Figure 4, upper panel, lane 3). The upper band of the doublet has a mobility characteristic of the RAS2 precursor (p41), which migrates slightly above authentic RAS2 (p40) (Fujiyama et al., 1987; Tamanoi et al., 1988). Normally, maturation of the RAS2 precursor occurs quite rapidly and p41 can only be detected in pulse-labelled wild-type strains or in mutants, such as ram1, in which isoprenylation is blocked (Powers et al., 1986). Thus, the persistence of p41 here suggested that the rate of RAS2 maturation might be altered in strains lacking the STE14 methyltransferase.

To examine this possibility, we performed pulse–chase analysis of RAS2 in wild-type and isogenic ste14 strains. Cells were labelled briefly with a mixture of [35S]cysteine and [35S]methionine ([35S]Translabel), chased with non-isotopically labelled amino acids for varying lengths of time, and RAS2 proteins were immunoprecipitated and analyzed by SDS–PAGE (Figure 5). Since processing in the wild-type strain is extremely rapid, we observe that even before the chase is initiated, 50% of the RAS2 protein has already undergone processing to p40 (Figure 5). By 20 minutes, 80% of RAS2 is converted to the p40 form, and by 40 minutes, conversion to p40 is essentially complete. In
as has also been previously observed (Marr et al., 1990; S.Sappington and S.Michaelis, in preparation). In addition, the lack of methylation greatly reduces a-factor biological activity; as described in Materials and methods, we determined by halo dilution assays of concentrated culture fluid that the unmethylated species of a-factor secreted by the ste14 mutant appears to be at least 200-fold less active than a-factor produced by wild-type cells.

**STE14-mediated methylation of RAS1 and RAS2**

The RAS proteins of yeast, like a-factor, are farnesylated and carboxyl methyl esterified (Deschenes et al., 1989; Stimmel et al., 1990). Since the isoprenylation of RAS proteins and a-factor is carried out by a common mechanism involving the RAM1 and RAM2 gene products (Powers et al., 1986; Goodman et al., 1990) and since STE14 activity is not confined to cells of the MATa mating type, we were interested in determining whether RAS and a-factor are also methylated by a common enzyme. To test whether STE14 is responsible for methylation of RAS proteins, we compared in vivo methylation of RAS in wild-type and ste14 mutant strains. Cells containing a high copy number RAS1 or RAS2 plasmid were double-labelled with [35S]cysteine and [3H]-

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**Fig. 3.** Comparison of a-factor methylation in vivo in wild-type and ste14 strains of *S. cerevisiae*. A wild-type strain, SM1058 (■) and ste14 mutant, SM1188 (○) containing the a-factor plasmid pSM219 were labelled separately with [35S]cysteine or [3H]-methyl)AdoMet at 30°C and processed as described in Materials and methods. Extracellular and intracellular fractions were immunoprecipitated using a-factor antiserum. Immunoprecipitates from the extracellular (A) and intracellular (B) fractions were subjected to SDS–PAGE using a 12.5% acrylamide gel. The lanes of the dried gel containing the [3H]-methyl)AdoMet samples were cut into 3 mm slices and each slice assayed for methyl esters as described in Materials and methods. The distribution of base hydrolyzed [3H]-methyl groups released from extracellular (A) and intracellular (B) proteins is shown. The portion of the gel containing [35S]cysteine-labelled samples was subjected to autoradiography to visualize a-factor. The open arrow indicates the migration position of the mature (A) and precursor (B) a-factor species that were detected. Positions of the pre-stained molecular weight markers are marked.

**Fig. 4.** Comparison of RAS2 methylation in wild-type and ste14 strains. Wild-type (SM1058) and ste14 (SM1188) cells containing pADH-RAS2 plasmids were double-labelled with [35S]cysteine and [3H]-methyl)AdoMet at 30°C and total cell extracts were prepared and immunoprecipitated with rat anti-pan-ras monoclonal antibody Y13–259 as described in Materials and methods. Immunoprecipitates were fractionated by SDS–PAGE on a 10% acrylamide gel. The dried gel was subjected to autoradiography to allow detection of [35S]cysteine incorporation (top). To examine methylation, the lanes were sliced into 3 mm slices after autoradiography (lower panels A–D). The slicing was designed using the autoradiogram above as a guide, such that the band corresponding to RAS2 was fully contained in gel slice number 13 and the non-specific background band which appears immediately below RAS2 in all lanes was fully contained in gel slice number 14. These slices were assayed for methyl esters as base-volatile [3H]methanol radioactivity (panels A and C ( □)). Controls with the blocking peptide are shown in panels B and D. Subsequently, the same slices were quantitatively assayed for total [35S]cysteine incorporation into the RAS2 protein (■) as described in Materials and methods and shown in Table II. Migration positions of the low molecular weight standards are indicated.
independent isolates of SM1823 (ras2val19 ste14) were tested and gave results that are indistinguishable from their ras2val19 STE14 parent, TK161-R2V (data not shown). Thus, the ste14 mutation does not appear to reverse the heat shock sensitivity or lack of iodine staining of ras2val19. Apparently, although lack of methylation can lead to subtle defects in RAS processing and localization, there are no measurable consequences for RAS activity under the conditions tested here.

Discussion

Methyl esterification appears to be the final step in formation of C-terminal farnesyl and geranylgeranyl cysteine methyl esters in peptides and proteins. The results presented here demonstrate that this methylation reaction is catalyzed by the product of the STE14 gene in S.cerevisiae. We have also shown that the a-factor mating pheromone, RAS1 and RAS2 lack detectable C-terminal methylation in a ste14 null mutant, and thus are physiological substrates of the STE14 methyltransferase. Our results suggest the biochemical link between RAS and a-factor that was first suggested by studies of RAM1 (Powers et al., 1986), and extend the notion that a common machinery mediates the series of three modifications (isoprenylation, proteolytic cleavage, and methylation) proposed to occur on proteins that terminate with a CXXX motif, where the penultimate residue is aliphatic (Stimmel et al., 1990).

Where in the cell does C-terminal methylation of isoprenylated proteins occur? Interestingly, inspection of the STE14 DNA sequence reveals multiple potential membrane spanning domains, indicative of integral membrane proteins (Sapperstein et al., 1989). Moreover, biochemical studies clearly indicate that STE14 methyltransferase enzyme activity is found in the membrane fraction (Hrycyna and Clarke, 1990). It will be interesting to determine whether methylation is carried out at the plasma membrane of yeast or on an intracellular membrane such as the cytoplasmic face of the endoplasmic reticulum. One attractive hypothesis is that the three reactions involved in maturation of CXXX-terminating proteins are carried out by a higher order complex of several polypeptides. Our observation here that a defect in methylation can impede the preceding steps of isoprenylation and proteolytic processing of RAS proteins (see below) supports the idea of a processing complex. However, it should be noted that while the STE14 methyltransferase is membrane-bound, the RAM1 component of the isoprenyltransferase is apparently soluble (Schaer et al., 1990; Goodman et al., 1990) though a weak membrane association might not be easily detectable.

Methylation of a-factor by STE14

Mutants in the STE14 gene were originally isolated on the basis of the inability of MATa cells to mate, due to a defect in a-factor production (Blair, 1979; Wilson and Herskowitz, 1987). We show here that methylation of a-factor fails to occur in the ste14 mutant. The absence of methylation has dramatic consequences for a-factor. We have observed that the non-methylated a-factor produced by a ste14 mutant is at least 200-fold less active than a-factor made by a wild-type strain. In addition, others have shown that a-factor lacking its methyl group has severely decreased biological activity (Anderegg, 1988; J.Becker, personal communication). Thus the methyl ester on a-factor appears to be necessary either for binding of a-factor to its receptor, STE3, on the surface of MATα cells (Nakayama et al., 1985; Hagen et al., 1986), or for the subsequent activation step that leads to G1 arrest and mating. As observed here and elsewhere (Marr et al., 1990; S.Sapperstein and S.Michaelis, in preparation) a second consequence of the lack of methylation is a reduced amount of a-factor secretion by ste14 mutants. The basis for this apparent defect in a-factor export remains to be established.

Modification of RAS proteins by the STE14 methyltransferase

The profound methylation defect we observe for RAS1 and RAS2 in a ste14 null mutant (Figure 4 and Table II) suggests that STE14 may be the major, if not the only yeast methyltransferase that can modify the C-terminus of RAS proteins. It is surprising, however, that while mutations that eliminate RAS function cause a major disruption in cell growth and survival (Kataoka et al., 1984; Tatchell et al., 1984), the apparent lack of methylation in ste14 mutants has no impact on cell viability, nor on the more subtle phenotypes of heat shock sensitivity and low starch levels conferred by a ras2val19 mutation. In comparison with ram1 and ram2 mutants, which are defective in farnesylation of RAS and exhibit a severe growth defect (Powers et al., 1986), the ste14 mutant grows normally under all conditions that we have examined. Although we cannot rule out the possibility that the methylation of an undetectable fraction of RAS proteins by an as yet unidentified enzyme could preserve their function, we stress that we detect <1% of the wild-type methylation level of RAS1 and RAS2 in ste14 mutants (Table II). In addition, methyltransferase activity is essentially undetectable in in vitro assays using membranes derived from ste14 null mutants (Table I). In view of the essential nature of RAS function in yeast, the viability of the ste14 mutant suggests that RAS proteins retain function in the absence of methylation. Alternatively, function may be somewhat impaired, but if the amount of RAS present in cells is actually far above saturating levels, then even a dramatic decrease in RAS activity would go unnoticed under normal laboratory conditions. If this were the case, it might be possible to identify a particular carbon source, temperature or ionic condition under which cell growth becomes dependent on a functional STE14 methyltransferase activity.

Maturation and membrane localization of RAS2 in a ste14 mutant

Despite the lack of an obvious effect on cell viability, we have observed several intriguing differences in RAS2 maturation between wild-type and ste14 mutant strains. Biogenesis of RAS proteins is a complex process. In both yeast and mammalian cells, RAS is initially synthesized as a soluble precursor with a C-terminal CXXX motif. Maturation has been proposed to involve two steps (Hancock et al., 1989; Fujiyama and Tamaoki, 1990): Step 1 includes isoprenylation, proteolytic cleavage and methylation, resulting in a cytosolic species whose gel mobility differs slightly from that of the precursor. Methylation is thought to occur last in this series of reactions. In Step 2, RAS becomes membrane localized and palmitoylated. In ste14 cells we observe a striking kinetic
contrast, in the ste14 mutant, very little of the processed p40 species of RAS2 is observed at the end of the pulse period and even after an extended chase (up to 60 min) only 50% of the RAS2 present has undergone conversion to p40. Thus, the rate of RAS2 maturation appears to be dramatically reduced in the ste14 mutant. The results shown in Figure 5 also demonstrate that the RAS2 mobility shift does not rely on methylation, since the p41 to p40 conversion occurs despite the lack of methylation in the ste14 mutant. Therefore, gel mobility must reflect isoprenylation or proteolytic cleavage. The finding that maturation of RAS2 is slow in the ste14 mutant is surprising, since methylation of the cysteine carboxyl must necessarily occur after isoprenylation and proteolytic cleavage are complete. Thus, the lack of the methyltransferase would not be expected to prevent processing from p41 to p40. Possible explanations for this apparent paradox are discussed below.

**Membrane localization of RAS2 in a ste14 mutant**

Farnesylation of the C-terminal cysteine residue of RAS is known to play a critical role in membrane localization. The availability of ste14 mutants has provided us with the opportunity to assess whether methylation, like isoprenylation, plays a role in RAS2 membrane localization. To examine this possibility, total extracts from the pulse-chase experiment shown in Figure 5 were fractionated into membrane and cytosolic fractions, and RAS2 was examined after immunoprecipitation and SDS-PAGE (Figure 6). It is notable that in the ste14 mutant, a significant amount of RAS2 reaches the membrane. On the one hand, this indicates that methylation is not essential for membrane localization. On the other hand, it is evident that the total amount of membrane-bound RAS2 is significantly less in the ste14 mutant than in the wild-type strain. After 20 min, the ste14 mutant contains only 20% the amount of membrane-associated RAS2 as in the wild-type strain. After 20 min, the level of RAS2 in the membrane does not increase substantially for either strain. It is interesting that with a very long chase (60 min, Figure 6) the total amount of membrane-associated RAS2 actually decreases, perhaps due to proteolytic degradation. This phenomenon is particularly evident in the wild-type case, so that the difference between the amounts of RAS2 in wild-type and ste14 membranes is diminished at later times. We have also examined RAS2 localization under steady state conditions by Western blotting and by steady-state isotopic labelling followed by immunoprecipitation. The results of these experiments are similar to those seen in the 40 min time point in Figure 6; the ste14 mutant contains less membrane-bound RAS2 than wild-type and the unprocessed soluble species of RAS2 persists (data not shown).

The reduced level of membrane-associated RAS2 in the ste14 cells may result from an altered capacity for RAS2 either to reach the membrane, or to be retained there when the C-terminal methyl ester modification is absent. Alternatively, the low amount of RAS2 in the membrane may simply reflect the p41 to p40 maturation defect discussed above, which results in a reduction of the amount of cytoplasmic p40 substrate that is available for localization to the membrane.

**Physiological activity of RAS2 in a ste14 mutant**

Since cell viability is not impaired in ste14 mutants (S.Sapperstein and S.Michaelis, in preparation), and RAS function is required for survival (Kataoka et al., 1984; Gibbs and Marshall, 1989), there appears to be no major defect in RAS activity due to a lack of methylation. However, the partial maturation and membrane localization defect described above led us to examine more subtle phenotypes associated with RAS function. A sensitive test for decreased RAS activity makes use of the hyperactive mutation ras2_w19, which activates the RAS adenylate cyclase pathway and causes cells to become heat shock sensitive and to lose the ability to accumulate glycogen stores (Kataoka et al., 1984). Mutations such as raml and ram2 which compromise RAS function result in a reversal of these phenotypes when introduced into a ras2_w19 strain (Powers et al., 1986; Goodman et al., 1990). We tested whether ste14 could also have such an effect. The ste14::URA3 mutation was introduced into the ras2_w19 strain, TK161-R2V, by gene replacement as described in Materials and methods. The resulting double mutant, designated SM1823 (ras2_w19 ste14), was compared with the isogenic strains TK161-R2V (ras2_w19 STE14) and SP1 (RAS2 STE14) for heat shock sensitivity and glycogen accumulation. As expected, the SP1 wild-type strain is heat shock resistant and exhibits strong iodine staining, whereas TK161-R2V is heat shock sensitive and stains poorly with iodine. Six

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**Fig. 6.** Kinetics of RAS2 membrane localization in wild-type and ste14 strains. Total cell extracts from the pulse-chase experiment shown in Figure 5 were fractionated as described in Materials and Methods. The resulting soluble cytoplasmic (C) and membrane (M) fractions were analyzed by immunoprecipitation and SDS-PAGE as described in the legend of Figure 5. Times shown indicate the length of the chase.

1704
Table III. Saccharomyces cerevisiae proteins containing Cys-Xaa-Ala-Xaa C-terminal tails and related proteins

<table>
<thead>
<tr>
<th>Protein</th>
<th>Carboxyl terminal sequence</th>
<th>Lipidation on conserved Cys</th>
<th>Methylation by STE14</th>
</tr>
</thead>
<tbody>
<tr>
<td>a-factor</td>
<td>Asp-Pro-Ala-Cys-Val-Ile-Ala&lt;sup&gt;b&lt;/sup&gt;</td>
<td>farnesylation&lt;sup&gt;c&lt;/sup&gt;</td>
<td>yes&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>RAS1</td>
<td>Gly-Gly-Cys-Ile-Ile-Ile&lt;sup&gt;d&lt;/sup&gt;</td>
<td>farnesylation&lt;sup&gt;e&lt;/sup&gt;</td>
<td>yes&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>RAS2</td>
<td>Gly-Gly-Cys-Ile-Ile-Ser&lt;sup&gt;d&lt;/sup&gt;</td>
<td>farnesylation&lt;sup&gt;e&lt;/sup&gt;</td>
<td>yes&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>RHO1</td>
<td>Lys-Lys-Lys-Cys-Val-Leu-Leu&lt;sup&gt;f&lt;/sup&gt;</td>
<td>polyisoprenylation&lt;sup&gt;1&lt;/sup&gt;</td>
<td>?</td>
</tr>
<tr>
<td>RHO2</td>
<td>Ala-Asn-Cys-Ile-Ile-Leu&lt;sup&gt;f&lt;/sup&gt;</td>
<td>polyisoprenylation&lt;sup&gt;1&lt;/sup&gt;</td>
<td>?</td>
</tr>
<tr>
<td>RSR1</td>
<td>Ala-Ser-Thr-Cys-Thr-Ile-Leu&lt;sup&gt;g&lt;/sup&gt;</td>
<td>polyisoprenylation&lt;sup&gt;1&lt;/sup&gt;</td>
<td>?</td>
</tr>
<tr>
<td>STE18</td>
<td>Ser-Lys-Lys-Cys-Thr-Ile-Leu&lt;sup&gt;h&lt;/sup&gt;</td>
<td>polyisoprenylation&lt;sup&gt;1&lt;/sup&gt;</td>
<td>?</td>
</tr>
<tr>
<td>CDC42</td>
<td>Ser-Lys-Lys-Cys-Thr-Ile-Leu&lt;sup&gt;i&lt;/sup&gt;</td>
<td>polyisoprenylation&lt;sup&gt;1&lt;/sup&gt;</td>
<td>?</td>
</tr>
<tr>
<td>YPT1</td>
<td>Gly-Gly-Cys-Cys&lt;sup&gt;j&lt;/sup&gt;</td>
<td>polyisoprenylation&lt;sup&gt;1&lt;/sup&gt;</td>
<td>?</td>
</tr>
<tr>
<td>SEC4</td>
<td>Ser-Asn-Cys-Cys&lt;sup&gt;k&lt;/sup&gt;</td>
<td>polyisoprenylation&lt;sup&gt;1&lt;/sup&gt;</td>
<td>?</td>
</tr>
</tbody>
</table>

<sup>a</sup>This study; <sup>b</sup>Brake et al., 1985; <sup>c</sup>Anderegg et al., 1988; <sup>d</sup>Powers et al., 1984; <sup>e</sup>Stimmel et al., 1990; <sup>f</sup>Madaule et al., 1987; <sup>g</sup>Bender and Pringle, 1989; <sup>h</sup>Whiteway et al., 1989; <sup>i</sup>Finegold et al., 1990; <sup>j</sup>Johnson and Pringle, 1990; <sup>k</sup>Gallwitz et al., 1983; <sup>l</sup>Salminen and Novick, 1987.

Table IV. Saccharomyces cerevisiae strains and plasmids

<table>
<thead>
<tr>
<th>Name</th>
<th>Genotype</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>STRAINS</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IM5058</td>
<td>MATA trp1 leu2 ura3 his4 can1 STE14&lt;sup&gt;+&lt;/sup&gt;</td>
<td>This laboratory</td>
</tr>
<tr>
<td>SM1188</td>
<td>ste14-Δ1::TRP1, isogenic to SM1058</td>
<td>This laboratory</td>
</tr>
<tr>
<td>SM1639</td>
<td>ste14-Δ2::URA3, isogenic to SM1058</td>
<td>This laboratory</td>
</tr>
<tr>
<td>RC757</td>
<td>MATA ss2–1 rme his6 met1 can1 cyh2</td>
<td>Chan &amp; Ott, 1982</td>
</tr>
<tr>
<td>SP1</td>
<td>MATA leu2 ura3 his3 trpl ade8 can1 gal2</td>
<td>Toda et al., 1985</td>
</tr>
<tr>
<td>TK161-2V</td>
<td>ras2&lt;sup&gt;Δ206&lt;/sup&gt;, isogenic to SP1</td>
<td>Toda et al., 1985</td>
</tr>
<tr>
<td>SM1823</td>
<td>ste14-Δ2::URA3 ras2&lt;sup&gt;Δ206&lt;/sup&gt;, isogenic to SP1</td>
<td>This laboratory</td>
</tr>
<tr>
<td>PLASMIDS</td>
<td></td>
<td></td>
</tr>
<tr>
<td>pSM433</td>
<td>2μ STE14 LEU2</td>
<td>This laboratory</td>
</tr>
<tr>
<td>pCM219</td>
<td>2μ MFA1 URA3</td>
<td>This laboratory</td>
</tr>
<tr>
<td>YEp-RAS2–4</td>
<td>2μ RAS2 LEU2</td>
<td>Powers et al., 1986</td>
</tr>
<tr>
<td>pADH-RAS2</td>
<td>2μ RAS2 LEU2</td>
<td>Powers et al., 1986</td>
</tr>
<tr>
<td>YEp-RAS1</td>
<td>2μ RAS1 LEU2</td>
<td>Kataoka et al., 1984</td>
</tr>
</tbody>
</table>

Bromide (Aldrich) was added. The reaction was allowed to progress at room temperature for 30 min. After the reaction was complete, 300 μl was fractionated by HPLC using a preparative scale Econosphere C18 reversed phase column (Alltech/ Applied Science, 10 mm inner diameter × 190 mm) equilibrated in solvent A at room temperature. The column was eluted using a non-linear gradient running from 0–60% solvent B and 100–30% solvent A (solvent A is 0.1% trifluoroacetic acid in water, and solvent B is 0.1% trifluoroacetic acid−90% acetonitrile−9.5% water) at an initial flow rate of 3 ml/min over 40 min, followed by a linear gradient to 100% solvent B over an additional 5 min. The N-acetyl product eluted at 29.4–29.6 min.

C-terminal methyltransferase assay

Enzyme activity was measured in S. cerevisiae membrane and cytosolic fractions and in E. coli extracts by the method previously described by Hrycyna and Clarke (1990).

Metabolic labelling and immunoprecipitation of a-factor

To label intracellular a-factor, cells were grown in SD medium with appropriate supplements to OD<sub>600</sub> 0.7, harvested, and for each separate labelling 5 OD<sub>600</sub> units were resuspended in 0.5 ml of growth medium in a polypropylene tube. Either 150 μCi [35S] cysteine (Amersham, > 600 Ci/mmol) or 120 μCi [35S] adenosine-1-[3H]-methyl methionine ([3H]-methyl)AdoMet; Amersham, 15 Ci/mmol) was added to separate cultures. Labelling was carried out for only 6 min at 30°C, since intracellular a-factor is short-lived, and was terminated by addition of 0.5 ml ice-cold azide stop mix (40 mM cysteine, 40 mM methionine, 20 mM Na<sub>2</sub>SO<sub>4</sub>, 500 μl BSA (Miles Laboratories)). Cells were pelleted by centrifugation at 13,000 g for 1 min, washed once with H<sub>2</sub>O, resuspended in 500 μl 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 mM Trasylol (FBA Pharmaceuticals)) and lysed in the presence of 0.5 g baked zirconium beads (0.5 mm diameter, Bicspec Products) by vortexing 10 times in 1 min bursts at 4°C. The cell lysate (designated the intracellular fraction) was removed from the beads by addition of 1.3 ml immunoprecipitation buffer.

To examine extracellular a-factor, labelling was carried out as described above, except that SDS-URA media was used and labelling was allowed to proceed for 90 min. After addition of azide stop mix, cultures were moved to a new tube and centrifuged at 13,000 g for 1 min. The labelling tube was retained and a-factor adhering to the tube was recovered as described below. The cell pellet was discarded and the supernatant, which contains secreted a-factor, was retained. Extracellular proteins were concentrated from the sample by addition of an equal volume of 20% TCA (w/v), incubation at 4°C for 15 min, and centrifugation at 13,000 g for 5 min. The pellet was resuspended in 15 μl 2× Laemmli sample buffer (20% glycerol, 10% 2-mercaptoethanol, 4.5% SDS, 0.125 M Tris–HCl pH 6.8 and 0.2% bromophenol blue) and neutralized with 1 μl 1.0 M Tris base. Since a significant portion of extracellular a-factor remains bound to the polyprene labelling tube, this tube was rinsed with H<sub>2</sub>O and a-factor was eluted from the sides with 0.4 ml n-propanol. The n-propanol eluate was evaporated to dryness with heating. This sample was resuspended in 15 μl 1× Laemmli sample buffer and combined with the culture fluid fraction prepared as described above. This combined sample (designated the extracellular fraction) was heated at 100°C for 3 min and added to 1.3 ml immunoprecipitation buffer.

Prior to immunoprecipitation, intracellular and extracellular samples were centrifuged for 1 min at 13,000 g to remove insoluble debris; samples were transferred to a new tube and 10 μl of a-factor rabbit antiserum Ab9–137 (S. Saperstein and S. Michaelis, in preparation) was added and then allowed to incubate overnight at 4°C. To collect immunoprecipitates, a 45 μl aliquot of protein A-Sepharose CL-4B beads (Pharmacia) suspended in immunoprecipitation buffer (1.3 beads:total volume ratio) was added and the tubes gently agitated at 4°C for 90 min. The beads were pelleted by a 10 s spin at 13,000 g and subsequently washed four times with immunoprecipitation wash buffer A (0.1% Triton X-100, 0.02% SDS, 150 mM NaCl, 50 mM Tris–HCl pH 7.5, 5 mM EDTA, 1 mM PMSF, 0.5% Trasylol) and once with immunoprecipitation wash buffer B (150 mM NaCl, 50 mM Tris–HCl pH 7.5, 5 mM EDTA, 1 mM PMSF, 0.5% Trasylol). After the final wash, bound immune complexes were released from the beads by the addition of 30 μl 2× Laemmli sample buffer. The samples were heated at 100°C for 3 min, clarified, and the supernatant was subjected to SDS–PAGE.
delay in Step 1 of RAS2 processing, as measured by the extremely slow conversion of p41 to p40. This result is somewhat puzzling since it suggests that a step prior to methylation (either isoprenylation or proteolytic cleavage) is affected when the STE14 product is absent. Possible explanations are that the machinery which mediates Step 1 processing may be comprised of a complex of proteins that cannot properly form in the absence of the STE14 product, or that a component of the machinery itself requires methylation for full activity. It could also be that the isoprenylation machinery has a sensor that responds to an insufficient amount of membrane-bound RAS by dampening further processing. Alternatively, more complicated explanations must be invoked to explain why lack of methylation affects other steps of maturation. It should be noted that the experiments described here were performed using strains overexpressing RAS2. High levels of RAS2 might cause subtle differences between wild-type and ste14 strains to be accentuated.

Step 2 of RAS2 maturation is also in some way altered in the ste14 mutant since we observe an apparent decrease in the total amount of RAS in the membrane. It may be that the lack of the methyl group affects the ability of RAS to reach the plasma membrane or to be retained there. Alternatively, there may be less isoprenylated substrate available for membrane attachment due to the maturation defect discussed above.

**Other candidate substrates for the STE14 methyltransferase**

Characterization presented here suggests that both farnesyl (C₁₅) and geranylgeranyl (C₂₀) modified proteins might be substrates of the STE14 methyltransferase and further predicts that residues preceding the CXXX motif do not influence methylation. Other yeast proteins that terminate in a CXXX motif, where the penultimate residue is aliphatic, and are thus candidates for methylation (Stimmel et al., 1990) are shown in Table III. We also include examples from another class of *S. cerevisiae* proteins synthesized with a C-terminal -Cys-Cys motif. Indirect evidence suggests these latter proteins may also be isoprenylated (Ferro-Novick, personal communication), but their state of methylation has not been evaluated.

Many of the proteins listed in Table III are known to be localized to a membrane or to be involved in membrane associated processes. In addition, all of these polypeptides, with the exception of a-factor, RH02 and STE18, are essential for viability. Our determination here, that STE14 appears to be the major C-terminal methyltransferase in yeast predicts that the proteins listed in Table III, if methylated, would also be substrates for this activity. Were this the case, then the viability of ste14 mutants argues that methylation may not play an essential role for their function. However the methyl group may indeed be important for modulating processing or facilitating the interaction of these proteins with the membrane. We suggest that the critical role of methyl esterification for the biological activity of a-factor may thus be distinct from the function of this reaction for other proteins.

**Materials and methods**

**Yeast strains, media and growth conditions**

Yeast strains used in this study are listed in Table IV. SM1058 was formerly designated EG123 (Michaelis and Herskowitz, 1988). Strains SM1188 and SM1639 were derived from SM1058 by single-step gene replacement and contain substitution mutations in which a significant portion of the STE14 coding sequence is deleted and substituted by the selectable markers TRP1 or URA3, respectively. Construction of these strains is described elsewhere (S.Sapperstein and S.Michaelis, in preparation). A double mutant containing ste14Δ and rar2Δ::HIS3 was made by gene replacement. Briefly, a 2.5 kb BamHI–Clal fragment containing the ste14ΔΔ::URA3 substitution mutation was used to transform TK1621–R2. Ura" transformants were screened for loss of STE14 function by the a-factor halo and mating assays (Michaelis and Herskowitz, 1988), and one of the sterile transformants was designated SM1823. Complete media (YPED), synthetic drop-out media (SD) and SD minimal media were prepared as previously described (Michaelis and Herskowitz, 1988) except that drop-out media are lacking L-methionine and L-cysteine. Where necessary, SD media was supplemented with l-histidine (20 µg/ml), l-tryptophan (20 µg/ml), uracil (20 µg/ml), or l-leucine (30 µg/ml). All in vivo experiments were performed at 30°C.

**Plasmids and manipulation of DNA**

Plasmids used in this study are listed in Table IV. The TrpE–STE14 fusion plasmids were constructed using pATH vectors (Koerner et al., 1990) and inserts from pSM191, in which a 1.6 kb Clal–BamHI fragment containing the STE14 gene is flanked by polylinker sites (S.Sapperstein and S.Michaelis, in preparation). Subscripted numbers designate the STE14 codons present in each fusion. A 1.5 kb Clal fragment from pSM191 was cloned into the Clal site of pATH3 to produce pTrpE·STE14Δ290. The entire STE14 coding sequence is present in this fusion. To construct pTrpE·STE14Δ290, a 1.1 kb HindIII fragment from pSM191 was cloned into the HindIII site of pATH3 resulting in a fusion which contains only the C-terminal half of STE14. Construction of plasmids pSM433 (2a STE14 LEU2) and pSM219 (2a MA11 URA3) will be described elsewhere (S.Sapperstein and S.Michaelis, in preparation). Plasmids YEP-RAS2, pADH-RAS2, and YEP-RAS1 were kindly provided by S.Powers. Transformation of plasmids into yeast and in vitro manipulation of DNA were performed as described previously (Michaelis and Herskowitz, 1988).

**Induction of TrpE–STE14 fusions and preparation of E.coli extracts**

Fusion plasmids were transformed into E.coli strain BSG24, also designated FZ-392, which is a mutagenized derivative of L392 (Murray et al., 1977) obtained from Abbott laboratories via S.Gerratt. Media used for propagation and induction of fusion strains, 'modified M9' and 'modified M9-Trp', were prepared essentially as described (Koerner et al., 1990), except that vitamin free casamino acids (Difco no. 0288 Vitamin Assay Quality) were used and ampicillin was replaced by carbenicillin (50 µg/ml). For induction of TrpE fusion proteins, cells were grown in 50 ml 'modified M9' to OD₆₀₀ of 0.7–1.2, harvested at 11 000 g for 10 min at 4°C, washed once with 30 ml 'modified M9-Trp', and resuspended in 500 ml of this medium. After incubation for 1.5 h at 30°C with constant shaking, imidazole-acetic acid (1 M final concentration) was added to initiate induction. The cultures were allowed to grow for an additional 6 h and harvested as before. The cell pellet was weighed and resuspended at a concentration of 0.5 g/ml of E.coli lysis buffer A (5 mM sodium phosphate, 5 mM EDTA, 10% glycerol (v/v), 25 µM PMSF and 15 mM 2-mercaptoethanol, pH 7.0; the last two components added just prior to use). The cells were subjected once to French press treatment at 1000 lbs/in². Unbroken cells and large debris were removed by centrifugation at 11 000 g for 10 min at 4°C. This extract was spun at 100 000 g for 1 h at 4°C. The supernatant (cytoplasmic fraction) was stored at −20°C and the pellet (crude membrane fraction and insoluble proteins) was resuspended in 1.5 ml cold E.coli lysis buffer A and stored at −20°C.

**Preparation and purification of S.cerevisiae membrane and cytosolic fractions**

Cells were grown in SD medium containing required nutrients to an OD₆₀₀ of 0.7–1.2. Crude membranes and cytosolic fractions were prepared and the membranes were subsequently purified by sucrose gradient centrifugation as previously described by Hrycyna and Clarke (1990).

**Synthetic substrates**

1-Leu1-1-Ala1-1-Arg1-1-Tyr1-1-Lys1-S-trans-farnesyl1-L-Cys (S-farnesyl LARYRK) and 1-Leu1-1-Ala1-1-Arg1-1-Tyr1-1-Lys1-S-trans-geranylgerany1-1-L-Cys (S-geranylgeranyl LARYRK) were provided by Robert Stephenson (UCLA) and are described elsewhere (Stephenson and Clarke, 1990). N-acetyl farnesyl cysteine (N-AcFC) was synthesized by a modification of the method of Stephenson and Clarke (1990). N-acetyl cysteine (450 nmol) was dissolved in 0.35 ml of dimethyl formamide, H₂O, 0.5 M KHCO₃ (5:1:1, v/v/v) and then two equivalents (200 nmol) of trans-farnesyl


Labelling and immunoprecipitation of RAS proteins

Cells were grown to mid-logarithmic phase (OD₅₆₀ 0.4–0.6) in SD medium supplemented with appropriate nutrients. For the double labelling experiment shown in Figure 4, 5 OD₅₆₀ units of cells were harvested, washed once with 5 ml H₂O and then incubated in a total vol of 1 ml containing 150 μCi [³H]methyl-AdoMet, 150 μCi [³²P]cysteine (Amersham, >600 Ci/mmol), and 835 μl fresh growth media for 15 min at 30°C with constant shaking. Labeled cells were harvested by centrifugation at 13,000 g for 1 min, washed once with H₂O and resuspended in 50 μl of 1% SDS. 1 mM PMSF. Zinc chloride (0.2 g) were added to and samples were heated at 100°C for 2 min. Cells were lysed by vortexing for 10 min at 4°C in 1 ml of this mixture followed by heating at 100°C for 3 min. This cell extract was removed from the lysis beads and added to 45 μl 2× Laemmli sample buffer.

For immunoprecipitation, 25 μl of cell extract in sample buffer and 15 μl (1.5 μg) rat anti-pan-ras (Ab-1) monoclonal antibody Y13–259 (Oncogene Science Inc., Manhasset, NY) were added to 1.3 ml immunoprecipitation buffer. In control incubations, the monoclonal antibody was neutralized prior to addition to the antibody with a ten-fold excess (w/w) of a blocking peptide, peptide 1 (Oncogene Science Inc.), that corresponds to the binding region of the Y13–259. Immunoprecipitation was carried out as described for a-factor. Except that the protein A-Sepharose CL-4B beads were pre-washed with rabbit antibodies against IgG since rat antibodies do not bind well to protein A. To the supernatant, an 800 μl aliquot of 50 μl CL-4B beads (Sigma) was added in 5 ml cold H₂O for 2 h, 160 μg rabbit anti-rr1 IgG heavy and light chain antibodies (Cappel Laboratories) were added and the beads were incubated at 4°C for 2 h. Beads were washed twice with 1.3 ml of immunoprecipitation buffer and resuspended in 1 ml of this buffer. An aliquot of the co-cultured cell suspension (80 μl) was added to each immunoprecipitation reaction.

Pulse–chase labelling, fractionation and immunoprecipitation of RAS2

To analyze the kinetics of maturation and membrane localization of RAS2, cells were grown in SD medium containing necessary supplements to an OD₅₆₀ of 0.4–0.6. Twenty OD₅₆₀ units of cells were harvested and resuspended in 2.0 ml of the same medium. Pulse labeling was carried out by incubation of cells with 600 μCi [³²P]Translabel (ICN, >1000 Ci/mmol) for 2 min. The chase was initiated by addition of 40 μl chase mix (1.0 M l-cysteine, 1.0 M l-methionine) and was terminated after various lengths of time by removal of 0.5 μl aliquot of cells into 0.5 ml ice cold chase stop mix. Cells were pelleted, resuspended in lysis buffer (150 mM NaCl, 50 mM Tris–HCl pH 7.4, 1 mM PMSF, 0.5% Trypsol) and disrupted at 4°C by vortexing 6 times in 1 min bursts in the presence of 0.25 g zirconium beads. This cell extract was adjusted to 1 ml with lysis buffer, removed from the lysis beads, and centrifuged for 10 min at 500 g to remove cellular debris. A 0.5 μl portion of this sample (designated the whole cell extract) was directly subjected to immunoprecipitation after addition of Tris–HCl to pH 7.4 and 100 μM dithiothreitol to a final concentration of 1% each of the remaining 0.5 μl portion was separated into membrane and cytoplasmic fractions prior to immunoprecipitation.

For fractionation, lysed cellular material was centrifuged at 100,000 g for 1 h. The supernatant (cytoplasmic fraction) was transferred to a new tube and adjusted to a final concentration of 1% Triton X-100. The pellet (membrane fraction) was washed with 500 μl lysis buffer, resuspended in 1 ml of extract buffer (1% Triton X-100, 1% sodium deoxycholate, 150 mM NaCl, 50 mM Tris–HCl pH 7.4, 1 mM PMSF, 0.5% Trypsol) and sonicated for 15 s.

The total cell extract, membrane fraction and cytoplasmic fraction prepared as described above were subjected to immunoprecipitation. Prior to addition of antibody, immunoprecipitation material was removed by centrifugation at 13,000 g for 5 min, and the clarified sample transferred to a new tube. Ten microliters of anti-pan-ras monoclonal antibody 259 (kindly provided by J. Gibb) was added and samples were incubated at 4°C overnight. Immunoprecipitates were collected using coated beads as described above and resuspended in 30 μl 2× Laemmli sample buffer.

SDS–PAGE analysis, gel slice vapor phase equilibrium assay for methyl esters, and gel slice assay for total ³⁵S incorporation

SDS–PAGE was performed using the buffer system described by Laemmli (1970). Electrophoresis of a-factor immunoprecipitates was carried out on 12.5% gels with pre-stained high molecular weight markers (Bethesda Research Laboratories) as standards. RAS samples were analyzed in 10% gels using low molecular weight standards (Biorad). Gels were fixed in 10% acetic acid for 15 min, rinsed in H₂O for 5 min, and soaked in 0.7 M sodium salicylate pH 7.0 for 15 min. Gels were dried under vacuum at 80°C and subjected to autoradiography at ~80°C. For the experiment shown in Figure 4, gels were stained in 50% methanol, 10% acetic acid (w/v), 0.1% Coomassie brilliant blue for 15 min and destained in 10% acetic acid, 5% methanol for 3 h, prior to drying and autoradiography.

Radiolabeled methyl esters were assayed by a modification of the method of Clarke et al. (1988). The lanes on the dried gels were cut into 0.3 cm slices and mixed with 200 μl 1 M NaOH in a polypropylene microcentrifuge tube. The tube was placed in a 20 ml scintillation vial containing 5 ml of scintillation fluid (ASCII, Amerham) and capped. After 24 h at 37°C, the microcentrifuge tube was removed and the radioactivity that had been transferred by diffusion to the scintillation fluid was as [³H]methanol was assayed in a liquid scintillation counter. Total ³⁵S radioactivity incorporation was assayed by adding 1 ml PROTOSOL (New England Nuclear), a tissue solubilizer, to each of the microcentrifuge tubes containing the gel slices and allowing them to incubate for 6 h at 35°C. The tubes were opened and put directly into 10 ml of organic countng scintillation fluid (OCS, Amerham) with 100 μl of glacial acetic acid and subsequently counted in a liquid scintillation counter.

Physiological assays

Bioassay of a-factor: To compare the activity of extracellular a-factor produced by wild-type and stel4 cells, a-factor was collected from the culture fluid of strains SM1058 (pSM120) and SM1188 (pSM219) using polyethylene glycol beads (Beard, MacKay, 1983) and assayed by the spot halo method described previously (Michaelis and Herskowitz, 1988). Briefly, cells were grown to saturation in one liter of appropriately supplemented SD medium containing 50 g of Amberlite XAD-2 polyurethane beads (Sigma) to which a-factor quantitatively absorbs. After discarding the culture, beads were washed several times with water and a-factor was eluted from the beads with n-propanol and reduced to 5 ml. This concentrated a-factor was serially diluted into 200 g/ml bovine serum albumin and assayed by spotting 3 μl aliquots onto a lawn of the suprasensitive strain RC757. For the wild-type strain, a zone of clearing was seen up to the 1000-fold dilution. By contrast, no a-factor activity was observed for the stel4 culture fluid, even using the undiluted material. Since the culture fluid from this stel4 mutant contains only ~25% as much a-factor as the wild-type culture fluid as determined by immunoprecipitation and densitometry, the non-methylated a-factor secreted by the stel4 mutant appears to be at least 200-fold less active than authentic methylated a-factor.

Heat shock sensitivity assay: Heat shock sensitivity was performed essentially as described (Brook et al., 1983). Cells were patched onto YEPD plates and grown 36 h at 30°C. Patch plates were replica plated onto a YEPD plate that was immediately set into a shallow water bath at 53°C. After incubation for 30 min, plates were transferred to a 30°C incubator. After one day, wild-type RAS2 cells formed a confluent patch whereas no growth was observed for ras2a¹⁶¹⁹ derivatives. By 2 days, 2–15 papillae per patch were observed for the latter strains.

Starch accumulation assay: To test starch accumulation, cell patches on YEP plates were grown for 24 h on starch and 100 mM sodium dodecyl sulfate to a final concentration of 1% each of the remaining 0.5 μl portion was separated into membrane and cytoplasmic fractions prior to immunoprecipitation.

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