Fungal Lipopeptide Mating Pheromones: a Model System for the Study of Protein Prenylation

GUY A. CALDWELL,† FRED NAIDER, AND JEFFREY M. BECKER*

INTRODUCTION

The continually expanding literature on the molecular basis of intercellular communication has highlighted the great interest in specific cellular factors in various metabolic responses. Common mechanisms of signal transduction, in which extracellular signals mediate intracellular events, continue to be discovered. The fundamental means by which cells interact in this manner often involves the recognition of small secreted molecules, typically peptides, by specific receptor proteins. In this regard, analysis of the mating process in a variety of fungal species has yielded numerous insights into the biochemistry of the signalling molecules and the machinery of signal recognition and transduction. Furthermore, the elegant simplicity of these fungal systems has allowed for genetic and biochemical dissection of the structural and functional requirements of conjugation. Therefore, fungal mating processes provide a powerful paradigm for understanding mechanisms that govern cell-cell interactions throughout the biosphere.

The transition from haploid to diploid states, as it occurs in the life cycle of many conjugative fungi, is a process dependent upon the reciprocal action of peptide pheromones. These peptides initiate an intracellular cascade of events that eventually lead to mating between cells of the same species (most recently reviewed in references 91 and 163). This group of fungal peptides comprises the pheromones (from the Greek, pherin, to transfer or spread wide, and hormon, to excite), which are diffusible substances released by an individual member of a species that elicit a biological response by another individual of the same species (80). In most fungi, peptide pheromones appear to serve a functionally equivalent role in initiating events leading to the fusion of haploid nuclei. All of these peptides seem to induce similar responses via interaction with their respective receptors, but they are significantly different in structure, presumably to maintain species specificity and mating-type recognition. Despite the inherent differences in amino acid sequence, many fungal pheromones share a common structural characteristic: alkylation of a cysteine by a polyisoprene (14, 36). Noted exceptions to lipid-modified pheromones are the unmodified tridecapeptide α-factor of the genus Saccharomyces and the recently identified P-factor of Schizosaccharomyces pombe (67, 148, 166). The identification of lipid moieties on many fungal pheromones has subsequently led to the investigation of their biosynthesis, secretion, and activity as a novel class of peptide ligands. These studies have contributed to our general knowledge of such biologically important and diverse phenomena as protein isoprenylation, secretory pathways, multidrug resistance, fungal virulence, and receptor-mediated signal transduction.

STRUCTURAL CHARACTERISTICS OF FUNGAL LIPOPEPTIDE MATING PHEROMONES

The existence of structural and functional similarities among hormones and peptides of distantly related eukaryotes has been noted previously (123). This relationship was demonstrated dramatically by the ability of the Saccharomyces cerevisiae α-factor mating pheromone, an unmodified tridecapeptide, to mimic the activity of mammalian gonadotropin-releasing hormone in stimulating the release of luteinizing hormone from cultured gonadotrophs (101). Likewise, similar-
ities in structure exhibited among fungal lipopeptide mating pheromones from diverse fungal species reflect an established theme of peptide hormone evolution, a conservation of structure with function.

In general, the fungal lipopeptide pheromones described to date contain a variable amino acid sequence of 9 to 15 residues and a hydrophobic carboxyl terminus including a methyl ester and a farnesyl group, a 15-carbon unsaturated isoprenoid lipid (Fig. 1). Variations on this theme exist and are discussed below. The modification of fungal lipopeptide pheromones with farnesylation is of general interest, since farnesylation of the Ras oncogene product was shown to be a requirement for its ability to transform cells to a cancerous state (10, 25, 45, 151). A major insight into the relationship between pheromones and Ras came from the isolation of mutants of S. cerevisiae which exhibited both a lack of functional Ras activity and an inability to mate (45, 53, 132). This observation suggested a common biochemical link between yeast mating and Ras activity and was substantiated by a report on the structure of the S. cerevisiae a-factor mating pheromone that showed that it contained a farnesyl moiety (3). Additionally, Ras, a-factor, and several lipopeptide mating factors of other fungi also contain a carboxyl-terminal methyl ester modification, thereby further establishing the biosynthetic connection between these polypeptides (3, 14, 27, 33, 36).

The isolation and purification of peptide mating factors from heterobasidiomycetous yeasts were the basis for subsequent analyses of other fungal lipopeptides. A-mating-type cells of the heterobasidiomycetous yeast Rhodotorula toruloides were found to produce a lipopeptide mating pheromone which acts in mediating the transition from the unicellular yeast stage to a dikaryotic mycelial phase of its life cycle (78, 79, 172). This activity, termed rhodotorurcine A, was purified and found to consist of an undecapeptide modified at its C-terminal cysteine residue through a thioether linkage to a farnesyl group (77). Likewise, another group of lipopeptide mating pheromones, the tremerogens, are necessary for conjugation in at least two species of the jelly fungus Tremella spp. Pheromones from the heterobasidiomycetes Tremella mesenterica and T. brasiliensis were also among the first purified fungal lipopeptides and are farnesylated pheromones that contain slight variations on the basic fungal lipopeptide structure (144–146).

There are subtle differences that affect the hydrophobicity of the fungal mating factors (Fig. 1). Many of these fungal mating pheromones contain a carboxyl methyl ester modification as well as a farnesyl group. This type of posttranslational modification increases the overall hydrophobicity of these peptides and eliminates the charge normally present with the free carboxylate of an unmodified peptide (36, 50). Among those fungal pheromones that contain both farnesyl and carboxyl methyl ester modifications are tremerogen A-10 (144, 146), tremerogen A-9291-I (69), M-factor of S. pombe (31), pheromones a1 and a2 of Ustilago maydis (15, 161), and S. cerevisiae a-factor (3). While the T. mesenterica tremerogen a-13 and rhodotorurcine A retain the polyprenyl moiety, these lipopeptides do not have carboxyl-methylated C termini. Furthermore, tremerogen A-10 of T. mesenterica and tremerogen A-9291-I of T. brasiliensis were found to have an alcohol group present on the farnesyl modification of their terminal cysteine residue (69, 70). In contrast, cells of the T. mesenterica a-mating type produce a 13-residue lipopeptide containing an unsubstituted farnesyl group (147). Additionally, a lipopeptide pheromone has been putatively identified in Cryptococcus neoformans, a major pathogenic fungus of humans (119). Although the actual pheromone has not yet been isolated, its prenylated structure has been predicted on the basis of the gene sequence found at the C. neoformans mating locus. In all, despite an apparent functional equivalence in triggering mating, small structural dissim-
ilarities at the carboxyl terminus appear in different fungal lipopeptide pheromones and are at present of undefined significance.

For many years, the precise chemical structure of the mating pheromone secreted by *S. cerevisiae* α-cells, α-factor, eluded investigators as the hydrophobicity of this lipopeptide prevented adequate purification using a variety of schemes. Nevertheless, through the use of large-scale purification schemes and mass spectrometry, Andrègg et al. (3) successfully characterized α-factor activity to consist of a mixture of farnesylated and carboxyl-methylated peptides. The size of the purified α-factors conformed to part of the amino acid sequence predicted by Brake et al. (18) on the basis of the genes encoding α-factor (see below). This led to the subsequent chemical synthesis of biologically active α-factor and α-factor analogs for use in bioactivity analyses (179). Experience with α-factor facilitated the more recent purification of M-factor from *S. pombe* by similar methods (30, 31). M-factor was determined to be a farnesylated, carboxyl-methylated peptide of 9 amino acid residues produced specifically by M (minus) cells and acting on P (plus) cells of *S. pombe*. Like α-factor, M-factor has also been synthesized and exhibits all the properties of the native pheromone (174). Given the experimental advantages associated with the budding yeast, *S. cerevisiae*, and the growing importance of the fission yeast, *S. pombe*, in the analysis of cell cycle events and pheromone-dependent signal transduction, current and future progress in lipopeptide pheromone research is likely to come from studies on α-factor and M-factor, as model pheromones for this class of extracellular effectors.

**α-FACTOR OF S. CEREVISIAE: PARADIGM FOR LIPOPEPTIDE BIOSYNTHESIS AND EXPORT**

**Overview of Mating in *S. cerevisiae***

In 1956, Levi first reported that cell-type-specific morphological changes were detectable in *S. cerevisiae*, even in the absence of cell-cell contact (96). This provided the foundation for years of subsequent investigation into the molecular basis of intercellular communication in this yeast. The vast sum of information gleaned from these studies focused on the functional requirements of mating as it is mediated by the reciprocal action of the α- and α-factor pheromones. The isolation of sterile (ste) mutants of *S. cerevisiae* provided a genetic basis for the identification of genes and gene products involved in the mating process (60). As would be expected, several of these sterile mutations were complemented by genes encoding proteins that were necessary for either pheromone production or response (91, 163).

Mating in *S. cerevisiae* is dependent on the proper expression, processing, secretion, and activity of mating pheromones (46, 91, 163). Cells of the α-mating type of *S. cerevisiae* secrete the α-factor pheromone, which is recognized at the cell surface of α-cells by a receptor protein encoded by the STE2 gene and expressed only in cells of the α-mating type (20, 121). Likewise, α-cells specifically express the STE3 gene product (58, 121), or α-factor receptor, on their cell surface as a means of recognizing the α-factor lipopeptide pheromone of α-cells. This reciprocal exchange of extracellular signals commences a series of intracellular events within both haploid cell types that directs the expression of additional mating-specific genes responsible for agglutination, cell cycle arrest, polarity and morphology changes, followed by cytoplasmic and nuclear fusion to form diploids (signal transduction in *S. cerevisiae* has been extensively reviewed elsewhere; for the most recent reviews, see references 62, 91, 99, and 163). The diploid stage of the *S. cerevisiae* life cycle may persist indefinitely; however, under conditions of environmental stress (i.e., nutrient limitation), diploids can be induced to form ascospores, which undergo subsequent meiosis and sporulate to yield haploid progeny of both mating types, thereby completing the life cycle.

**Biosynthesis of α-Factor***

Despite the apparent functional equivalence of the two *S. cerevisiae* mating pheromones, the biosynthesis and export of α-factor does not proceed along the same lines as that of its pheromone counterpart, α-factor. Two genes, *MFα1* and *MFα2*, expressed specifically in the α-mating type of this yeast, encode polypeptide precursors that contain tandem repeats of the α-factor peptide (19, 92, 158). Specifically, the *MFα1* gene product is a 165-amino-acid precursor peptide whereas the *MFα2* transcript encodes a 120-amino-acid polypeptide, both of which contain a hydrophobic signal peptide leader sequence common to most secretory proteins for transit into the endoplasmic reticulum (76, 175). These precursors undergo proteolytic processing and glycosylation following translocation into the endoplasmic reticulum, where the mature 13-amino-acid pheromone is transported out of the cell via the classical secretory pathway (76). In fact, the α-factor pheromone was one of the first substrates used in the seminal characterization of the yeast secretory pathway by Novick et al. (126, 127). Recently, the *map2* gene product of *S. pombe* has been found to encode the precursor for the unmodified peptide mating pheromone, P-factor, and would be predicted to undergo processing events similar to *S. cerevisiae* α-factor on the basis of its primary amino acid sequence as well (67). In contrast to the genes encoding α-factor, *map2* appears to be the sole gene for P-factor, because its disruption leads to sterility.

An initial clue to the mechanistic differences between the biosynthesis of the two *S. cerevisiae* mating pheromones came from the disparity between their genes and deduced amino acid sequences (18). As with α-factor, the α-factor is coded for by two functionally redundant genes (termed *MFα1* and *MFα2*); however, the α-factor genes do not encode tandem repeats of this pheromone within their primary translation products. Furthermore, the α-factor precursors do not contain the consensus signal peptide leader sequences present in the α-factor precursors and in most other secreted peptide hormones (18, 114). This initially suggested that α-factor is exported from the yeast via a novel secretory pathway. Additional evidence supporting this hypothesis came from experiments showing that yeast secretory pathway mutants, or sec mutants, retained the ability to export α-factor (111, 164). The polypeptides encoded by the *MFα1* and *MFα2* genes are precursors of 36 and 38 amino acids, respectively, each containing a single mature α-factor peptide sequence (18). While these genes seem to be functionally identical, they exhibit a single amino acid residue difference in the portion encoding α-factor (the *MFα1* gene product contains a valine instead of the leucine coded for by *MFα2* at position 6 of the mature α-factor). In the case of both *MFα1* and *MFα2*, the proteolytic processing and posttranslational modification events that occur are identical, and each gene accounts for approximately half of the total mature pheromone produced by α-cells (103).

The most significant feature of the primary α-factor gene products is the presence of a specific C-terminal motif, found in all known farnesylated proteins, representing a signal for modification of polypeptides with an isoprenoid group (130, 132, 152). In the case of both α-factor precursors, this specific sequence of amino acids is -CVIA. However, the general motif
TABLE 1. Genes involved in the biosynthesis, export, and bioactivity of the \textit{S. cerevisiae} a-factor lipopeptide mating pheromone

| Gene  | Protein encoded | Protein encoded |
|-------|----------------|----------------|
| MFa1  | a-Factor precursor (36 aa) | a-Factor precursor (36 aa) |
| MFa2  | Carboxyl methyltransferase | a-Factor precursor (38 aa) |
| RAM1  | Farnesyltransferase, \(\beta\)-subunit | Carboxyl methyltransferase |
| RAM2  | Farnesyltransferase, \(\alpha\)-subunit | N-terminal protease 1 |
| STE14 | Carboxyl methyltransferase | N-terminal protease 1 |
| STE22 | N-terminal protease 2 | N-terminal protease 2 |
| STE6  | N-terminal protease 3 | a-Factor receptor |
| STE3  | Carboxyl methyltransferase | N-terminal protease 4 |
| STE5  | Carboxyl methyltransferase | a-Factor receptor |

\(\text{AA} \) amino acids.

\(\text{a} \) Evidence suggests that two independent N-terminal proteolytic processing events occur during a-factor biosynthesis. The \(\text{STE}22 \) gene product has recently been found to possibly represent one of the proteases involved in this processing.

\(\text{b} \) Although an activity corresponding to \(\text{CAAX} \) protease has been identified, the gene has not been reported yet.

\(\text{c} \) An N-terminal protease that specifically cleaves farnesylated a-factor has been reported, but the gene has not yet been cloned.

is referred to as a CAAX box, since the consensus sequence of amino acids present at the C terminus of isoprenylated proteins consists of an invariant cysteine (C) residue followed by two aliphatic (A) amino acids and ending in a carboxyl-terminal residue of almost any (X) type (it should be noted that some slight amino acid preferences exist at the aliphatic and terminal positions; these differences are discussed elsewhere [130, 152]). The specific CAAX sequence has also been shown to target the peptide for either farnesylation or geranylgeranylation (130, 152). The availability of mutant strains of \textit{S. cerevisiae} that exhibit an inability to mate because of a lack of a-factor production led to the subsequent identification of several genes required for a-factor biosynthesis (61, 65, 89, 111, 114, 132, 151, 153, 178) (Table 1). Biochemical and genetic analysis of these mutants has led to the elucidation and ordering of the various steps in the biosynthesis of this fungal lipopeptide.

As previously noted, the discovery of specific mutants of \textit{S. cerevisiae} that were deficient in both a-factor and Ras function served to suggest the common biogenesis of these molecules (132). These were termed \textit{ram} mutants, since cells of this type were defective in ras- and a-factor modification. In these mutants, yeast Ras was rendered nonfunctional by a lack of localization to the plasma membrane. Likewise, \textit{ram} mutants were found to accumulate a-factor precursors intracellularly. Depletion of the farnesyl precursor, mevalonate, also resulted in a nonfunctional Ras and sterility in \textit{S. cerevisiae} (151). Finally, experiments performed by Schafer et al. conclusively established the link between mating and cell growth via Ras to involve the \textit{RAM1} gene product, now known to be a component of the yeast enzyme farnesyltransferase (151, 153). More recently, the \textit{RAM2} gene product has been shown to be necessary for farnesyltransferase activity as well (61). The enzymatic relationship between mating pheromone polyprenylation and Ras oncogenicity provided an impetus for further investigation into the biochemical basis of lipopeptide biosynthesis (27, 51, 130).

The in vitro maturation of a-factor synthetic precursor peptides was performed to determine the effect of specific \textit{a}-cell mutations on lipopeptide biosynthesis (8, 65, 105, 151). These studies served as a basis for elucidating the order of events required for a-factor processing and modification. These events include (i) attachment of the \(\text{C}_{15} \) farnesyl moiety to the sulfhydryl of cysteine in the a-factor -CAAX box, (ii) protoclysis of the three C-terminal amino acid residues (-AAX), (iii) carboxyl esterification of the newly exposed terminal carboxyl group, and (iv) removal of the N-terminal extending amino acids in the precursor to yield the mature lipopeptide pheromone.

Using the power of yeast molecular biology, several investigators have uncovered genes implicated in this pathway. Reconstitution of farnesyltransferase activity has been accomplished by coexpression of both \textit{RAM1} and \textit{RAM2} in \textit{Escherichia coli} and use of the resulting bacterial cell extract to catalyze farnesylation of a-factor and Ras peptide substrates (61). Furthermore, the requirement for cysteine at the terminus of a-factor for prenylation and subsequent export has been confirmed by site-directed mutagenesis and in vitro maturation of synthetic peptides (105, 151). Although no genes have been conclusively assigned to be either an N-terminal protease or the C-terminal CAAX protease, activities that correspond to these functions have been identified (8, 16, 102). Preliminary evidence suggests that the CAAX protease is a farnesyl-dependent membrane-bound protein, as would be expected if the a-factor is first prenylated and localized to the plasma membrane at a prior step in its biosynthesis (8, 102, 113). Likewise, the sequence of the \textit{STE14} gene product, the farnesylcysteine carboxyl methyltransferase responsible for methyl esterification of a-factor, encodes a protein predicted to contain multiple membrane-spanning domains (7, 107, 150). Metabolic labeling and immunoprecipitation analyses have indicated that N-terminal protoclysis is the final step in a-factor maturation prior to export of mature pheromone (112, 113). Preliminary results suggest that two proteolytic cleavage events may occur within the N terminus of pro-a-factor prior to secretion. A gene encoding a putative N-terminal a-factor protease has been recently identified and named \textit{STE22}, since accumulation of an a-factor precursor in \textit{ste22} mutants occurs and leads to sterility (16). As would be expected, haploid \textit{ste22} mutants show an a-cell-specific mating defect but, surprisingly, exhibit a bipolar budding pattern characteristic of diploid yeasts. Therefore, this protease may cleave an isoprenylated protein involved in budding as well. In this regard, DNA sequence analysis has confirmed that \textit{STE22} is identical to another gene, \textit{AXL1}, that encodes a protease implicated in bud site selection and is similar in sequence to insulin-degrading enzymes of humans and \textit{Drosophila} species (44). The combined information implies a model for a-factor biosynthesis (Fig. 2) in which the pheromone precursor polypeptides are farnesylated in the cytoplasm (where the \textit{RAM1/RAM2}-dependent activity resides) and subsequently localized to the plasma membrane via the hydrophobic interactions promoted by isoprene attachment. However, it should be mentioned that the characterization of enzymes involved in these steps in animal cells indicates they are found also in microsomal membranes, suggesting that prenylated proteins and peptides in animal cells traffic through a microsomal membrane compartment on their way to their final destination. The membrane-bound a-factor precursors are presumably modified at the plasma membrane (although microsomal membrane involvement has not been ruled out) by C-terminal cleavage of the -AAX residues, methylated by \textit{Ste14p}, and are then proteolytically processed at the N terminus (by \textit{Ste22p} and possibly another enzymatic activity?). The resulting mature a-factor is then exported by translocation via the \textit{Ste6p} across the plasma membrane of the cell (89, 111).

\textbf{Export of a-Factor: a Novel Secretory Mechanism}

The inability of peptides to passively cross membrane barriers mandates cellular mechanisms of transport accommodat-
ing the variety of peptide and protein structures produced and exported by eukaryotic cells. Fungal lipopeptide mating pheromones, which are inherently hydrophobic and act extracellularly, may represent a unique class of molecules with respect to the methods used by cells for their translocation. In this regard, the lack of a hydrophobic signal peptide sequence at the N terminus of the translated products of the \( MF_a \) and \( MF_{a2} \) genes provided initial evidence that the \( \alpha \)-factor lipopeptide may be exported by a mechanism significantly different from that of the nonfarnesylated \( \alpha \)-factor pheromone (18). Additional support for this theory was provided by the observation that several specific mutations in the classical secretory pathway, previously shown to block the secretion of \( \alpha \)-factor, had no discernible effect on \( \alpha \)-factor export (164).

Among the collection of sterile (\( ste \)) mutants of S. cerevisiae, several putative complementation groups were identified as being \( \alpha \)-cell specific (60). Within this subclass, along with the \( ram \) and \( ste14 \) mutants, a mutant strain termed \( ste6 \) was observed to block \( \alpha \)-factor export (164). McGrath and Varshavsky (111), while in the process of sequencing genes of the yeast ubiquitin-mediated proteolysis pathway, discovered an open reading frame corresponding to the previously mapped \( STE6 \) gene. Disruption of this gene led to the inability of a strain carrying this allele to secrete \( \alpha \)-factor (111). Kuchler et
al. (89) published a similar study supporting these conclusions about Ste6p function as well. Sequence analysis of STE6 furnished an intriguing outcome showing that the deduced Ste6p exhibited a general conservation of structure and significant amino acid identity (57%) to the product of the human MDR1 gene, the multiple drug resistance (MDR) P-glycoprotein (89, 111). The significance of this relationship lies in the fact that expression of human MDR has been implicated in cellular resistance to a variety of therapeutic agents in cancer patients undergoing chemotherapy (56). Because many anticancer drugs are small hydrophobic compounds designed to cross cell membranes, the mechanism by which Ste6p recognizes and exports the α-factor may be useful in understanding the resistance to specific drugs exhibited by cancer cells.

On the basis of their structural features and functional role in molecular transport, both Ste6p and MDR1 are representatives of the ABC (ATP-binding cassette) superfamily of proteins, which also includes the cystic fibrosis transmembrane conductance regulator protein (for a review, see reference 63). Members of this family are normally composed of 12 membrane-spanning helices in the form of two sets of six transmembrane domains. Each domain “half” contains a consensus cytoplasmic sequence for nucleotide binding. Some ABC members exhibit this “two-times-six” organizational structure in the form of discrete polypeptide halves instead of an intact protein unit. Alternatively, some related proteins of this family consist of just a single half domain of six transmembrane regions with a single ATP-binding fold. The transport proteins associated with antigen processing (TAP), which are thought to require dimerization in order to function in translocating peptides into the endoplasmic reticulum prior to antigen presentation, are examples of this latter type (5, 162, 170). Interestingly, “half molecules” of yeast Ste6p cannot function individually but, when coexpressed in cells, can functionally restore α-factor transport (12). Although a detailed understanding of this family of molecules awaits further investigation, it is generally thought that ABC transporters utilize the energy of ATP hydrolysis to pump substrates across cellular membranes. A major obstacle toward a comprehensive understanding of this protein family lies in the broad substrate specificity and lack of natural substrates exhibited by these transporters. In this regard, Ste6p provides a unique opportunity for investigation of this phenomenon, since it is a dedicated transport protein with an apparent specificity for export of α-factor (112).

Küchler et al. (89) performed a series of experiments indicating that α-factor export was rate limited by the level of STE6 expression in a-cells. This result is compatible with a model of α-factor biosynthesis and secretion wherein membrane translocation of α-factor occurs after or in conjunction with N-terminal proteolysis of the α-factor precursor to yield the mature pheromone. However, the structural requirements for direct recognition of α-factor by Ste6p remain a mystery. A recent study has shown that a null mutant of the ste14 gene exhibits a severe block to the export of α-factor, as judged by metabolic labeling, immunoprecipitation, and mating (150). Since STE14 encodes the membrane-bound activity responsible for the carboxyl-terminal methylation of α-factor, a strict requirement for the methyl esterification of α-factor may exist for Ste6p-mediated transport. However, C-terminal methyl modification of α-factor may simply serve to promote membrane localization or interaction of the pheromone precursor with a specific membrane-localized protease activity that is responsible for rapid N-terminal proteolysis. Such N-terminal proteolysis may play a role in substrate recognition by the Ste6p transporter (112, 151). However, this scenario remains to be proven experimentally.

The membranous surroundings in which the Ste6p protein is situated may influence the phase behavior and subsequent association of α-factor with this transporter. Clearly, farnesylation and carboxyl-terminal modification of α-factor increase the affinity of this pheromone for membranes and may act in partitioning it from an aqueous to a hydrophobic environment, where it might achieve access to a potential binding site on Ste6p. A recently published study demonstrated that another ABC family member, the mouse mdr2 gene product, functions as an ATP-dependent phospholipid transporter (160). Transgenic mice containing a disruption at this locus developed liver disease as a result of improper phospholipid export into their bile. This study also served to explain the long-sought reason for the inability of mdr2 to confer resistance to chemotherapeutic drugs, like its structural homolog in humans, MDR1. The specificity of the mdr2 gene product for phosphatidylcholine has also been demonstrated by heterologous expression in yeast cells (136). The export of α-factor by Ste6p might be similar to the transport of lipids across membrane barriers by mdr2 in that the pheromone is presented to the Ste6p protein from the cytoplasmic side and then possibly flipped by this protein to the extracellular space. Evidence in the literature appears to be mounting in support of a mechanistic model for MDR-like transporters acting as “flippases” for lipids, lipid-modified proteins, and drugs (64, 141).

Investigations into the novel secretory pathway taken by α-factor may provide insights into the mechanism of MDR-mediated export. For example, studies in which a 20-carbon geranylgeranyl group was substituted for the 15-carbon farnesyl moiety on α-factor in vivo indicated that the structural specificity of the Ste6p transporter is sufficiently broad to accept and transport pheromones containing either prenyl group (23). Additional experiments, involving metabolic labeling, immunoprecipitation, and sequence analysis, have shown that Ste6p does not discriminate against a variety of amino acid substitutions within the peptidyl portion of α-factor (24, 112, 113). Therefore, while a strict requirement for hydrophobic modification of α-factor appears to be required for export, specific changes in substrate lipid or amino acid composition do not appear to restrict peptide translocation. These results correlate well with what is known about P-glycoprotein-mediated resistance of cancer cells to drugs (56, 112).

Raymond et al. (136) have functionally complemented the mating defect associated with deletion of the S. cerevisiae ste6 gene by expressing the mouse mdr1 gene (a homolog of human MDR1) in S. cerevisiae and showing that it could restore mating of a- and a-haploid cells. In fact, functional expression of P-glycoprotein in S. cerevisiae has now been used to confer resistance to the immunosuppressive and antifungal agent FK250, thereby establishing a positive selection for screening of intragenic mutations that will provide valuable structure-function information on the transporter (137). In contrast to mdr3, expression of human MDR1 in S. cerevisiae resulted in increased resistance to some agents (90, 142) but decreased resistance to other drugs (143). Additional investigations are required to clarify the functional expression of human MDR-like proteins in S. cerevisiae.

Despite the vast amount of research performed on MDR P-glycoproteins, the precise physiological function of these transporters has remained elusive. Given the prediction that approximately 0.5 to 2% of cellular proteins contain prenyl modifications (39) and that these attachments are very stable, one putative function for this class of transporters could involve cellular detoxification by disposal of prenylcytsteine methyl esters (13, 181). In this regard, Zhang et al. (181) have published an interesting study reporting that prenylcytsteine
methyl esters compete for drug binding to the MDR1 gene product. These compounds consist of an isoprenoid moiety attached to cysteine, a carboxyl methyl group, and a free amino group, thereby resembling a putative degradation product of a prenylated protein and many substrates for MDR-like transporters, including a-factor (for Ste6p) and cationic phospholipids (for mdr2). Another structural homolog of the MDR family, the STS1 gene, has been cloned in S. cerevisiae (13). Although Sts1p does not mediate a-factor export in S. cerevisiae, it appears to be localized to intracellular membranes of the yeast cell and may function to translocate toxic metabolites through the endoplasmic reticulum into vesicular compartments for transport out of the cells via the classical secretory pathway.

At least three putatively novel genes of the ABC family in S. cerevisiae have been isolated by PCR with degenerate primers and are presently being characterized. These include MDL1/SSH1 (isolated independently but identical), MDL2, and SSH2, all of which exhibit the most similarity to the drug resistance gene MDR1 and the TAP genes implicated in antibiotic translocation (32, 55). Functional homologs of Ste6p have also recently been tentatively identified in the pathogenic yeast Candida albicans via complementation of the ste6-dependent mating defect of mutant S. cerevisiae, a surprising discovery given the asexual nature of C. albicans and its apparent lack of pheromone production (21, 135). Considering that C. albicans exhibits resistance to antifungal agents, these putative homologs and one other Candida gene (BEN), which confers resistance of this yeast to benomyl and several other agents (11), may be of clinical significance as well. Given the similarities between a-factor and the M-factor, it might be expected that an Ste6p-like protein would also be found in S. pombe to direct excretion of the latter lipopeptide pheromone. Interestingly, an S. pombe structural homolog of Ste6p has been identified by Nishi et al. (124, 125); it is not essential for M-factor export or mating but has been shown to confer drug resistance to the antifungal antibiotic leptomycin B. Disruption of this allele leads to multidrug supersensitivity in the fission yeast; however, the natural physiological function of this protein remains unknown. Clearly, as additional homologs of Ste6p are identified in pathogenic fungi and higher organisms, more work must be done to clarify the functions of and molecular mechanisms of transport utilized by these proteins. The combined information on a-factor export and related protein functions further demonstrates the similarities between the biological mechanisms associated with lipopeptides of fungi and proteins of higher eukaryotes, including humans.

PROTEIN PRENYLATION AND LIPOPEPTIDE BIOACTIVITY

Prenyltransferase Enzymes

Our current knowledge of protein prenylation owes a large debt to the initial identification and isolation of lipopeptide mating pheromones from the fungi. The pioneering biochemical studies performed on the lipopeptide pheromones of basidiomycetous fungi paved a path for the subsequent use of genetics and molecular biology in S. cerevisiae to isolate and characterize genes encoding enzymes responsible for isoprene modification of protein substrates (3, 43, 69, 70, 77–79, 144–147, 172). The prevalence of isoprene modification (see reference 39 for an approximate quantitation of prenyleneptides in a variety of species) and the variety of prenylated protein substrates in cells signify a distinctive role for isoprenylation in effecting protein function. This is best exemplified by the critical dependence of the ras oncogene product on farnesyl modification for its transforming ability (27, 40, 48, 152). Considering that approximately 90% of pancreatic cancers and 50% of colon cancers have been linked to Ras (10), the prospect of inhibiting Ras function by blocking its farnesylation has become an attractive target for drug design in the chemotherapy of cancer (48, 81).

Proteins targeted for isoprene modification contain one of three specific amino acid sequence motifs at their carboxyl terminus (reviewed in references 40, 130, and 152). These motifs act as intracellular signals calling for lipid modification of their associated proteins by prenyltransferase enzymes present in the cytosol. The CAAX box, as discussed earlier in this review, is found in many prenylated proteins, including fungal mating factors, Ras, Ras-related proteins, G-protein subunits, and nuclear lamins. The final amino acid, X, in the CAAX sequence has been shown to be a major determinant of the type of prenyl modification that is attached to the cysteine (C) sulfur of CAAX-containing proteins and peptides. Two types of prenyl modifications are naturally encountered on cellular proteins, either farnesyl (C15) or geranylgeranyl (C20), with geranylgeranyl groups being the predominant form of isoprene attachment utilized by cells in a variety of organisms (39). In protein precursors containing either an alanine, serine, glutamine, cysteine, or methionine as the final residue (X) of the CAAX box, a protein is targeted for farnesylation. An otherwise identical protein precursor in which this terminal amino acid is leucine (and in some rare cases phenylalanine), however, would receive a geranylgeranyl group posttranslationally. In some proteins that have a second type of cysteine-containing prenylation motif, CXC (where X is typically alanine, serine, or glycine), geranylgeranyl modifications are placed on either one or both of two cysteines at the carboxyl terminus, thereby bestowing additional hydrophobic character to these substrate proteins. Still other prenylated proteins terminate in a third type of isoprene-targeting motif, termed CC, that directs geranylgeranylization of specific proteins ending with two adjacent terminal cysteines. Although in vitro studies indicate that both cysteine residues can be potentially geranylgeranylated, it remains unclear whether modification of one or both cysteines occurs in vivo with CXC- or CC-type protein targets (40, 82, 83). Differences seem to exist between these carboxyl-terminal prenylation motifs with regard to their ability to direct carboxyl methylation (122). While apparently most CAAX substrates are carboxyl methylated, a recent study involved in vitro assays with a bovine brain membrane preparation containing methyltransferase activity to show that geranylgeranylated CXC proteins can be methylated whereas geranylgeranylated CC substrates are not (159). Furthermore, the block in carboxyl methylation of the terminal geranylgeranyl cysteine exhibited by CC-containing proteins appears to be attributable to interference from the immediately adjacent geranylgeranyl cysteine. Interestingly, while substitution of serine for either cysteine in CXC or CC proteins does not prevent prenylation of the remaining cysteine, it does affect methylation of the carboxyl terminus. Although replacement of the terminal cysteine (i.e., CXS or CSG) blocks methylation in all cases, a protein containing an SC terminus can be methylated in vitro. Additionally, binding competition studies indicate that CC-terminal proteins retain their ability to bind the membrane-bound carboxyl methyltransferase but are unable to form a carboxyl methyl ester (159).

Since the discovery of genes required for farnesylation of a-factor and Ras, an explosion of information has occurred regarding characterization of the various prenyltransferase enzymes that catalyze isoprene transfer (40, 130, 152). Three
specific enzymes have now been conclusively identified as being responsible for this activity in human cells, and their counterparts have also been identified in yeast cells (61, 74, 75, 86, 98, 118, 138, 140, 151, 153, 155–157). Two of these protein prenyltransferases recognize the CAAX box as the target for enzymatic transfer of either a farnesyl or a geranylglycosyl group from donor prenyl PP, molecules to the appropriate protein substrates. Farnesyl protein transferase I (FPTase I) and geranylgeranyl protein transferase I (GGPTase I) are composed of two nonhomologous subunits, termed α and β (138, 157). These enzymes have a common α-subunit but have distinct β-subunits that are required for recognition of and differentiation between CAAX motifs. In S. cerevisiae, the RAM1 gene encodes the β-subunit of FPTase I and the CDC43 gene encodes the GGPTase I β-subunit (110, 151) (RAM1 and CDC43 have also been identified as DPR1 and CAL1, respectively [54, 128]), while both of these yeast enzymes require the RAM2 polypeptide as the α-subunit for activity (41, 61). The third prenyltransferase, geranylgeranyl protein prenyltransferase II (GGPTase II), is responsible for geranylgeranylation of proteins containing either CXXC or CC carboxyl termini and is structurally different from both FPTase I and GGPTase I. While GGPTase II is also composed of α- and β-subunits that are homologous to those found in FPTase I and GGPTase I, its activity requires yet another polypeptide component to catalyze isoprene transfer (4, 74, 75, 98, 140, 155, 156). Most significantly, this secondary component of human GGPTase II has been implicated in a X-linked genetic deficiency, choroideremia, that causes blindness via retinal degeneration (4, 155, 173). The human choroideremia gene product has been recently found to be 30% identical to S. cerevisiae Mpr6p, a protein known to be required for membrane attachment of vesicular trafficking proteins in vivo (74, 173). The MRS6/MSH4 gene product, along with the yeast BET4 and BET2 gene products (the α- and β-subunits), form the catalytic activity designated as GGPTase II in S. cerevisiae.

Unlike the other two prenyltransferases, several lines of evidence suggest that GGPTase II requires additional structural elements in target protein substrates, exclusive of a terminal cysteine motif, to catalyze geranylgeranylation (120). These include the inability of C-terminal peptide competitors to block by GGPTase II prenylation of Rab5 (83, 131), a Ras-related GTP-binding protein, and the identification of both point mutations and truncations in the N terminus of Rab5 that affect geranylgeranylation (97, 149, 177). Key elements of GGPTase II substrate proteins, including a conserved N-terminal amino acid motif found in proteins of the Rab family, GDP-binding effects, and the terminal cysteines, all appear to contribute to a preferred tertiary protein structure that dictates recognition by this prenyltransferase (149).

**Farnesyltransferase as a Target for Chemotherapy**

The variety of substrates for the three types of prenyltransferases run the gamut of cellular functions and include proteins necessary for signal transduction, intracellular trafficking, nuclear lamina, growth regulation, and, of course, mating in fungi. Because of the role of Ras in cancer, particular interest in the farnesylation of Ras has prompted investigators to identify agents that may act as selective inhibitors of FPTase activity (48, 157). Ras proteins function as GDP-GTP exchange molecules that act as control switches mediating intracellular signals between kinases. Farnesylation of Ras has been shown to be necessary for its membrane localization, which, in turn, is required for the function of the Raf-1 proto-oncogene product and its control over kinases that regulate transcription and, ultimately, growth (95, 165).

Investigators studying antagonism of FPTase function are attempting to develop competitors for substrate-binding sites. For example, synthetic analogs of farnesyldiphosphate, the isochores donor molecule recognized by FPTase, have been investigated as potential FPTase inhibitors (167). A synthetic isoprenoid substrate analog of farnesyl diphosphate, (α-hydroxyfarnesyl)phosphonic acid, has been observed to inhibit FPTase-dependent processing of Ras in NIH 3T3 cells at concentrations of 1 μM, but its efficacy when considering in vivo specificity has not been revealed (48, 49). In another report, investigators have used a screen that employed the fungal mating system of S. cerevisiae for the identification of in vivo inhibitors of farnesylase (42, 59). Briefly, this approach takes advantage of the mating pheromone response pathway in which farnesylation of the γ-subunit of the yeast trimeric G protein is required for induction of growth arrest of normal cells but leads to death of mutant cells that have been disrupted in the gene encoding the G-protein α-subunit. Therefore, in vivo inhibition of FPTase by candidate drugs used to treat yeast cells will result in growth of cells that lack the G-protein α-subunit, as a result of a block in the farnesylation of the γ-subunit, a prerequisite for this form of synthetic lethality. Several potential FPTase inhibitors have been isolated with this screen. Among the most promising is a natural product of Streptomyces species, manumycin, which is a competitive inhibitor of farnesyl diphosphate and exhibits 50-fold higher inhibition of FPTase over GGPTase I.

An alternative approach to FPTase drug design focuses on the β-subunit of this enzyme and its specificity for recognition of CAAX tetrapeptides (52, 138). Initial studies involved in vitro prenylation assays to characterize substrate preferences for FPTase and led to the discovery of CAAX-based peptidomimetics (28, 47, 72, 87, 133). Several of these compounds (48) were designed as produgs that could pass across cellular membrane barriers and would be susceptible to intrinsic intracellular enzymatic processing, thereby yielding products with increased inhibitory potency toward FPTase, some in the nanomolar concentration range. As with all potential prenyltransferase inhibitors identified to date, the trade-off between prevention of cytotoxicity and effective inhibitory concentrations represents a concern with this class of molecules, and verification of any truly beneficial inhibitory compounds awaits rigorous testing in animal models.

Among the challenges faced in development of FPTase inhibitors is the identification of sufficiently potent compounds that can successfully discriminate between FPTase and GGPTases. Because geranylgeranylation of proteins is 5 to 10 times more common than farnesylation (39) and because many geranylgeranylated substrates are essential for cell viability, the selectivity of potential FPTase inhibitors is a primary concern in the design of efficacious drugs. Furthermore, there is now substantial genetic and biochemical evidence suggesting that the distinction between farnesylation and geranylgeranylation is not absolute among the protein substrates for prenyltransferases. Initially, in vitro studies with purified mammalian prenyltransferase enzymes indicated that some cross-specificity in differential prenylation occurred at specific concentrations of acceptor protein substrates (180). In other words, proteins containing a C-terminal isoprene-targeting motif that called for geranylgeranylation addition served as potential substrates for nonspecific modification with a farnesyl group. This is not surprising if one considers that prenyltransferases have a common subunit and have an additional subunit that is similar and recognizes closely related substrates.
The phenomenon of nonspecific polyisoprenyl modification by prenyltransferase enzymes has been termed crossprenylation and has now been observed to occur in vivo with a variety of prenyltransferase substrates including mammalian rhoB, the S. cerevisiae STE18 gene product (encoding a G-protein γ-subunit in the pheromone response pathway), yeast Ras, and a-factor (1, 23, 171, 176). For example, when site-directed mutagenesis was used to substitute the native alanine for leucine at the C terminus of the a-factor precursor (CVIΔ instead of CVIΔA), expression of the MFαl gene carrying this change resulted in production of both farnesylated and geranylgeranylated a-factor by a yeast strain which lacked its native a-factor genes (23). Purification, isolation, and characterization of this farnesylated protein by high-performance liquid chromatography has provided direct biochemical evidence for crossprenylation. Additional genetic evidence for the occurrence of in vivo crossprenylation comes from a report by Ohya et al. (129) showing that the lethality normally induced by a GGTase I deletion in S. cerevisiae can be suppressed by overexpression of two essential GGTase I substrates, specifically Rho1p and Cdc43p, in vivo. Rho1p and Cdc43p are yeast GTPase proteins that absolutely require C-terminal prenylation for their function. Therefore, this observation implies that in the absence of GGTase activity, these proteins are functioning via illicit farnesyl addition. In fact, additional overexpression of FPTase in these cells further enhances the growth of these mutants. Whiteway and Thomas (176) have reported that only minor effects on the function of the STE18 gene product in yeast mating are noticed in the absence of farnesyltransferase (ram1) mutant, suggesting that phenotypic rescue of these mutants is possibly dependent on crossprenylation as well. Additionally, Mitsuzawa et al. (115) have recently described the isolation and characterization of an S. cerevisiae mutant FPTase in which a single amino acid alteration in the β-subunit resulted in a decreased affinity for FPTase substrates and an increased ability to farnesylate GGTase I substrates, thereby exaggerating the natural cross-specificity of FPTase.

Obviously, crossprenylation must be considered if effective inhibitors of prenyltransferases are to be designed. This is most relevant with respect to the ability of Ras to function with a geranylgeranyl modification, even if it cannot be farnesylated by FPTase. The viability of yeast cells lacking FPTase (61, 132) has been shown to be due to prenylation of Ras proteins by GGTase I (171). However, most significantly, it has been shown that activated Ras genes, containing mutations that are linked to growth defects in yeasts and cellular transformation in mammals, exhibit a specific requirement for farnesyl modification for sufficient deleterious activity in S. cerevisiae (171). Although the molecular basis of this distinction is not clearly understood, these results indicate that farnesyltransferase remains a practical target for therapy of cancers. In the meantime, further analysis of the cellular prenylation machinery and possible variations that may exist between species, including the variety of fungi which produce farnesylated peptides, is likely to yield additional insights into the prenyltransferases and their targets.

**Functional Consequences of Prenylation**

As we further our basic understanding of the enzymology of protein prenylation, it is important to take a momentary pause and ask why various types of cells, spanning enormous evolutionary distances, utilize this form of chemical modification for influencing protein function. Moreover, it is important to ask why some proteins are modified with a geranylgeranyl moiety while others receive a farnesyl modification. While a great deal of experimental effort has been expended on the mechanism of protein prenyltransferase action, the functional significance of isoprenylation is still poorly understood. Clearly, it is known that prenylation facilitates membrane localization of proteins and bestows upon them an enhanced hydrophobic character. Nevertheless, the precise role of these modifications remains elusive with respect to their contribution to protein-protein interactions, to protein-membrane localization, and to their direct effect on protein function (109).

The biological significance of protein prenylation is implicitly derived from the production of proteins with hydropathy values that are equal to or slightly lower than those of a nonmodified protein. As previously noted, some fungal pheromones contain farnesyl groups but lack a methyl ester modification in their natural, wild-type structure and yet are biologically functional (i.e., tremerogen

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**a-Factor Bioactivity: Model for Isoprene-Dependent Protein-Target Interactions**

As described earlier in this review, studies on the a-factor of S. cerevisiae have contributed to our knowledge of substrate specificity in protein prenylation and to the discovery of a novel means of peptide export by cells. It is the recognition of an a-factor by its receptor (Ste3p, encoded by the STE3 gene) on the surface of the opposite-mating-type α-cells, however, that is of interest to investigators who desire an experimental model for the analysis of lipopeptide interaction with target proteins. Accordingly, synthetic a-factor and analogs of a-factor have proven to be excellent tools for investigating the consequences of hydrophobic modification on lipopeptide bioactivity (23, 24, 104, 179). Marcus et al. (104) have shown that synthetic a-factor analogs lacking either the carboxyl-methyl ester or farnesyl group resulted in a 100- and 1,000-fold drop in bioactivity, respectively, as measured by several bioassays. In addition, an intact peptide portion of the a-factor dodecamer lacking both of these modifications exhibited more than a 100,000-fold decrease in receptor-mediated pheromone response. These results suggest that the posttranslational modifications on a-factor have a profound impact on the ability of this peptide to interact with or be properly presented to its receptor.

Carboxyl-terminal methylation of fungal lipopeptide pheromones has been shown to be an important determinant of biological activity. In this regard, studies with synthetic peptide analogs have shown that substitution of the C-terminal methyl ester with an amide group appears to confer greater activity to both a-factor and tremerogen A-10 than does the presence of a free hydroxyl group (43, 104). However, more hydrophobic substitutions for the methyl ester on a-factor did not lead to a similar increase in activity (104). For example, placement of either a 3-methylbutyl ester or a 3-methyl-2-butenyl ester (a prenyl group) at the C terminus of a-factor yielded peptide analogs with biological activities that were equal to or slightly lower than those of a nonmethylated a-factor. As previously noted, some fungal pheromones contain farnesyl groups but lack a methyl ester modification in their natural, wild-type structure and yet are biologically functional (i.e., tremerogen
a-13, rhodotoruline A [Fig. 1]). However, for tremerogen a-13, it has been found that blocking the C terminus with an amide group actually increased pheromone activity to a level above that of the wild-type non-methyl-esterified peptide (84). The replacement of the carboxyl moiety with an amide reduces the negative charge at this terminus and might lead to a more favorable free energy for interaction with the membrane lipids of the fungal cell. It would appear that hydrophobicity at the carboxyl terminus favors lipopeptide activity. However, the results with the C5 esters of a-factor show that increased hydrophobicity alone does not always correlate with higher activity (104).

More recently, these lipopeptide structure-function studies were taken a step further in an examination of the effect on pheromone activity that would be caused by sequential removal of individual amino acid residues from the amino terminus of a-factor (24). These synthetic peptides, which retained their C-terminal modifications, exhibited only a moderate and gradual loss of pheromone activity with the removal of each successive amino acid and retained measurable bioactivity until over two-thirds of the peptidyl portion of the pheromone was truncated. These data are intriguing when one considers that an equivalent loss in bioactivity can be achieved by only the removal of both the C-terminal isoprene and methyl ester groups. If the farnesyl and carboxyl-methyl groups on a-factor interacted directly with the Ste3p receptor to trigger pheromone response in yeast cells, one would expect that a-factor analogs containing these hydrophobic groups in different spatial orientations might be drastically affected with regard to pheromone bioactivity. Interestingly, this is not the case, since synthetic a-factors in which the position of the farnesyl and carboxyl-methyl ester groups on the peptide was varied in chirality and topology did not demonstrate a dramatic effect on a-factor activity (24). Therefore, it is apparent that while posttranslational modification of the a-factor is essential for optimal pheromone function, it seems unlikely that the specific conformation maintained by these moieties is a requirement for direct interaction with the a-factor receptor protein. Perhaps the prenyl and methyl groups serve only to confer hydrophobicity.

Bioactivity studies on either synthetic a-factor analogs or biosynthetically produced a-factors containing amino acid substitutions indicate that specific residues within the pheromone contribute to a-factor activity (22, 24). Perhaps the peptidyl portion of a-factor assumes a favored conformation for ligand binding. In fact, a recent report on the identification of a hyperactive a-factor pheromone, containing a D-alanine substitution instead of glycine at position 5 of the mature a-factor, might be suggestive of a preferred functional conformation for this pheromone in vivo (22). Likewise, a-factor may be specifically presented to its receptor as a consequence of C-terminal modification. If prenylation and/or carboxyl methyl esterification serve to traffic the farnesyl to the hydrophobic membrane and subsequently direct the peptidyl portion of a-factor toward a ligand-binding pocket within its receptor, it might be suggested that posttranslational modification bestows a specific conformation on the peptide in a lipid environment. Nuclear magnetic resonance analysis of the a-factor in solution indicated, however, that the presence of the S-farnesyl moiety does not impose a particular conformational preference on the peptide (57). This observation does not preclude the possibility of such an conformational effect within a membranous context. Biophysical studies on a-factor mutated shed light on the biological role of the farnesyl moiety. In the presence of membrane vesicles in vitro, the farnesyl modification significantly influences the degree to which the a-factor peptide can penetrate membranes (38). Furthermore, sequential truncation of amino acids from the N terminus of a-factor also reduced membrane penetration of the peptide and correlated directly with decreased bioactivity for these analogs (24, 38). These results establish that a-factor associates with lipid membranes and that at least a portion of the peptide chain penetrates into the lipid. This association could suggest that a-factor manifests its biological activity by binding to a site on the Ste3p receptor that is not readily accessible to the aqueous environment, a scenario that is reminiscent of possible models for a-factor interaction and export via the Ste6p protein (64). However, a recent study has reported that several a-factor peptides containing substitutions at a variety of residues were still successfully exported yet were not biologically active (24). Therefore, although the Ste6p transporter and the Ste3p receptor must both associate with a-factor in vivo, their respective molecular determinants for pheromone recognition must be distinct. Further biophysical studies on the a-factor and model prenylated peptides should help to clarify how pheromone-membrane interactions participate in the activity of lipopeptide mating factors.

Biological Implications of Differential Isoprenylation

One aspect of protein prenylation that remains a mystery is the functional significance of differences in isoprene chain length, yet specific properties are imparted upon proteins that are dependent upon the type of isoprenoid modification they possess. Rhodopsin kinase is a protein that requires farnesylation for light-dependent translocation to cellular membranes. A mutant rhodopsin kinase, containing a CAAX box that was modified to accept a geranylgeranyl modification instead of farnesyl, exhibited native phosphorylation activity but lost its dependence on light for membrane localization (68). The presumed attachment of a C20 isoprenoid to this kinase was insufficient for maintaining its proper translocation and therefore suggests that a specific requirement for farnesyl modification exists for this prenylated protein. Cox et al. (29) have reported a distinction between geranylgeranyl and farnesyl modification of Ras and have shown that specific isoprenoid modification of normal (i.e., not “activated”) Ras is required for function. Expression of normal Ras with geranylgeranyl-targeting motif (CVLL) in place of the native farnesyl signal (CVLS) resulted in a protein that exhibited a dominant-negative phenotype of growth inhibition. Assuming that the only difference associated with this altered Ras protein was a substitution of a C20 for a C15 prenyl moiety, the replacement of an isoprenyl group accounted for a dramatic functional distinction. This growth-inhibitory effect may be associated with antagonism of endogenous Ras by normal Ras that was altered to putatively contain a geranylgeranyl modification. In contrast to these examples, changing a CAAX-targeting motif from one which directs farnesylation to one which calls for geranylgeranylation may produce a protein whose function is indistinguishable from that of the wild type. In the case of the S. cerevisiae G-protein γ-subunit, the STE18 gene product, no discernable effects on STE18-dependent mating were observed with a geranylgeranyl motif-containing Ste18p protein in an otherwise wild-type strain (176). However, it should be noted that overexpression of this mutant version of Ste18p in cells lacking the G-protein α-subunit (Gpa1p) inhibits the constitutive mating response generated by GPA1 deletion. In the case of all these above investigations into alternate isoprenyl targeting and function, definitive characterization of the actual polyisoprene attachment present on the polypeptides studied is
lacking and must be considered when interpreting these results. An investigation into the functional consequences of differential isoprenylation of α-factor has shown that geranylerganylated α-factor is successfully exported by the Ste6p protein in vivo (23). In addition, synthetic geranylgeranyl (C<sub>20</sub>) α-factor can functionally replace wild-type farnesylated α-factor in its ability to induce growth arrest and mating, although it appears slightly less efficient in this process, exhibiting approximately 25% of the activity of farnesylated α-factor. Results from another study showed that incremental increases in the length of the polyprenyl moiety on α-factor, from prenyl (C<sub>5</sub>) to geranyl (C<sub>10</sub>) to farnesyl (C<sub>15</sub>), correlated with an increase in bioactivity, as judged by assays for both growth arrest and changes in morphology (104). However, a geranylated (C<sub>10</sub>) α-factor exhibited 200% of wild-type activity in the bioassay of mating restoration. Differences between activity end points in presumably comparable bioassays with specific pheromones and receptor mutations have been reported (24, 26, 108). These may be artifacts of the physical differences between bioassays or are possibly representative of distinctions that serve to uncouple related yet different biological responses to the same ligand. The slight drop in potency observed when comparing geranylgeranylated and farnesylated α-factor may suggest that an optimal isoprenoid length has evolved with this pheromone in S. cerevisiae. This may denote a functional preference that exists and is reflected by the general prevalence of farnesyl as the predominant isoprene modification among mating pheromones of fungi (Fig. 1). Relationships between prenyl group size and activity in other fungal lipopeptide pheromones have also been studied. In *Tremella* tremerogen A-10, isoprenoid modifications up to C<sub>10</sub> continued to show an increasing ability to induce mating tube formation (43). In *Tremella* tremerogen a-13, however, tetraprenyl (C<sub>26</sub>) and pentaprenyl (C<sub>30</sub>) analogs were less active than the native farnesylated pheromone.

### Isoprene-Mediated Protein-Protein Interactions

The search for proteins that specifically bind the polyprenyl moiety has intensified in recent years, and several lines of evidence suggest a multiplicity of such molecules. Cycling between cytosol and membranes of small prenylated GTP-binding proteins has been postulated to involve binding to GTP dissociation inhibitor (GDI) proteins. It is believed that GDI-like proteins facilitate membrane release of their prenylated partners by masking the hydrophobicity of the polyprenyl and carboxyl methyl ester groups in a binding pocket (6, 109). Interestingly, the choroideremia gene product, associated with GDI, and several genes encoding prenylated proteins, including the RAS genes, thereby demonstrating that some mevalonate-derived products may act in a regulatory capacity (34). These effects refer to an apparent direct or indirect transcriptional control mechanism that may be mediated by prenylated proteins. The potential for polyprenyl-modified proteins to serve as regulatory effector proteins may also extend beyond the level of transcription. A direct role for polyprenyl-mediated protein-protein interaction has been implied by results of experiments showing that farnesylated Ras is a better inducer of adenyl cyclase production of cyclic AMP than is nonfarnesylated Ras (93). Likewise, isoprenylated Ras exhibited more than a 100-fold-higher affinity for cyclase as well. These results are significant, because these experiments were performed with solubilized adenyl cyclase in the absence of membranes and therefore imply a direct interaction with and preference for a polyprenyl-modified effector. Given the variety of prenylated proteins in animal cells, any common motifs identified in the target molecules for this class of proteins may reveal an elusive isoprene recognition sequence. Clearly, the development of additional methods for probing lipopeptide-membrane interactions and the combined use of synthetic peptide chemistry and molecular biology with novel biophysical methods will facilitate our understanding of the manner by which polyisoprenes affect protein function (37).

### VARIATIONS ON A THEME: DIFFERENCES AMONG FUNGAL LIPOPEPTIDE MATING PHEROMONES

The existence of hydrophobically modified lipopeptide mating pheromones across a diverse spectrum of fungal species...
elicits inevitable comparisons based on the apparent structural and functional similarities shared by these molecules. In our desire to define common areas among organisms, however, it is easy to neglect the subtleties that often define interesting biological questions. Investigation of additional genes involved with mating pheromones of fungi is certain to yield valuable information that may hopefully delineate novel and interesting areas of pursuit. As additional aspects of lipopeptide pheromone biosynthesis, secretion, and activity are uncovered among fungi, existing hypotheses may become validated or unanticipated cellular mechanism may be revealed. The ensuing molecular genetic analyses on \( \alpha \)-factor have been fruitful; however, until recently, limited progress with fungal species other than \( S.\, cerevisiae \) has been made toward the identification of mating pheromone-related genes. Over the past few years, however, reports have appeared of pheromone identification in a variety of fungi including \( S.\, pombe \), \( U.\, maydis \), and \( C.\, neoformans \) (31, 119, 161).

The diversity of gene structures known for the different mating pheromones of fungi is intriguing in light of the common aspects of structure exhibited among these secreted lipopeptides (Table 2). An interesting feature of fungal pheromone genes is their apparent redundancy in many species. For instance, mutational analysis has revealed that three separate genes in \( S.\, pombe \) M-mating type cells, termed \( mfm1 \), \( mfm2 \), and \( mfm3 \), must be rendered nonfunctional to result in loss of mating (31, 85). Mutants lacking all three of these genes are sterile because of their inability to produce the lipopeptide M-factor. With the exception of the presence of a common intron sequence, these \( S.\, pombe \) genes share a similar precursor structure to the \( S.\, cerevisiae \) \( \alpha \)-factor genes, as all terminate with identical residues (CVIA), implicit to posttranslational farnesylation, proteolytic processing, and carboxyl methyl esterification. As noted previously, \( \alpha \)-factor is also encoded by two functionally redundant genes, \( MF\alpha 1 \) and \( MF\alpha 2 \) (18). Likewise, rhodotoruricine \( A \) is coded for by three genes in \( R.\, toruloides \) (2). The redundancy of fungal mating pheromone genes may serve to ensure the production of sufficient amounts of hydrophobic pheromones for effective mating in their natural milieu. Alternatively, gene duplication might protect the species against mutation to sterility and allow for evolutionary diversification in the pheromone structure.

\( U.\, maydis \), the fungus responsible for corn smut, contains structural genes encoding short polypeptides of 40 and 38 amino acids that terminate with a CAAX motif (15, 161). In contrast to the above, the \( Ustilago \) pheromone genes are arranged in a single locus that encodes many of the components required for cell-cell recognition in the form of two nonhomologous alleles (9, 15). The \( a1 \) allele contains the gene for the \( a1 \) mating pheromone, termed \( mfa1 \), and the \( pra1 \) receptor gene, whose product is responsible for recognition of the \( a2 \) pheromone. Likewise, the \( a2 \) allele contains the \( mfa2 \) and \( pra2 \) genes, necessary for production of the \( a2 \) pheromone and the reciprocal receptor protein for the \( a1 \) lipopeptide pheromone. The amino acid sequences of the \( pra \) receptor genes share structural homology to both the \( S.\, cerevisiae \) \( \alpha \)-factor receptor and the M-factor receptor of \( S.\, pombe \) (15, 58, 168). Functionally, the \( U.\, maydis \) pheromones are required for morphological changes that result in filamentous growth, a prerequisite for the pathogenic development of this fungus (9). Recently, a functional assay for monitoring pheromone activity in \( U.\, maydis \) was described and both the \( a1 \) and \( a2 \) pheromones were purified and characterized (161). \( a1 \) was found to contain 13 amino acid residues, and \( a2 \) contained 9 residues. Both the pheromones were farnesylated and contained carboxyl methyl esters. These results were expected on the basis of the gene
sequences coding for $a1$ and $a2$ (Table 2). Given these findings, the relationship between pheromone induction of morphological change and virulence is significant, because specific inhibitors of pheromone production or activity (e.g., FPTase inhibitors) would be potential candidates for preventing the undesirable effects of this fungal pathogen on corn plants.

Another fungal pathogen in which virulence is linked to mating-type functionality is the dimorphic basidiomycetous yeast C. neoformans. One of several factors genetically linked to virulence in C. neoformans is the mating type, because haploid MAT$a$ strains of this fungus exhibit a greater propensity for induction of pathogenesis than do MAT$a$ strains (94). Two sets of mice infected separately with one of two congeneric C. neoformans strains, differing only in mating type, were found to die considerably earlier if they were infected with C. neoformans of the MAT$a$ mating background. The molecular basis for this is poorly understood. However, a gene sequence that would be predicted to code for a small open reading frame with similarity to lipopeptide pheromones of other fungi has been reported to be specific to the MAT$a$ locus and may begin to provide a clue to this phenomenon (119). The $MPa1$ gene of C. neoformans encodes a putative peptide, 38 amino acids in length, that terminates in a CAAX box (Table 2). It is interesting that the $MPa1$ gene is only a small region within the MAT$a$ locus that spans 35 to 45 kb of DNA specifically found in MAT$a$ strains. This form of genetic organization is similar to that of the U. maydis loci. However, C. neoformans appears to require only one large mating locus and therefore may be functionally representative of a fusion of the U. maydis $a$ and $b$ loci. It is likely that this large region encodes additional genes necessary for mating (a receptor, transcription factors, etc.) and possibly virulence. Since C. neoformans is a major causative agent of opportunistic infections that result in the death of immunocompromised patients with AIDS (139), any progress toward understanding its life cycle and potential virulence factors is clinically significant.

The genetic organization of the lipopeptide mating pheromone genes of R. toruloides is unique among fungal pheromones. As with other fungi, three functionally redundant genes encode rhodotorurcine $A$ precursors without an N-terminal hydrophobic signal sequence for directing secretion (2). However, each precursor contains multiple tandem copies of the amino acid sequence for this pheromone, with each copy separated by a spacer sequence that corresponds to a CAAX motif. Therefore, processing of rhodotorurcine $A$ precursors presumably involves an initial cleavage event to yield multiple peptides with exposed CAAX termini that would subsequently undergo farnesylation and proteolysis. It is interesting that the mature, purified rhodotorurcine $A$ pheromone is not carboxyl methylated; this may somehow be related to the atypical processing mechanism used for the biosynthesis of this lipopeptide. When compared with the budding and fission yeast mating pheromone genes, the mixture of tandem pheromone sequences separated by CAAX-like spacers in R. toruloides represents an apparent union of the two separate forms of pheromone gene structure adopted by S. cerevisiae for the $a$- and $a$-factor genes (18, 114, 163) or by S. pombe for the M- and P-factor genes (31, 85).

More detailed structural and biochemical investigations are needed to elucidate the variety of enzymatic mechanisms underlyng the production of different fungal lipopeptide pheromones and their raison d’être. Clearly, the isolation of additional genes needed for pheromone production in different fungi will facilitate our understanding of the distinctions that exist among these fascinating organisms.

### PERSPECTIVES

As the depth of our knowledge of fungal lipopeptide mating pheromones increases, the discovery of homologs by sequence comparison among fungi may establish common modes of biosynthesis, secretion, and activity. The information obtained from the identification and sequence comparisons of prenyltransferases, proteases, transporters, and receptors for mating pheromones of different fungi will be invaluable for understanding the physiology of these cells and eukaryotes in general. Among the remaining challenges to be confronted in this research is to further define the structure-function relationships of lipopeptides and their molecular interactions with protein and membrane receptors.

One facet of fungal lipopeptide activity that is poorly understood is the value imparted by hydrophobic modification of these peptides for their function in natural environments. The prevalence of hydrophobic modification among mating pheromones of fungi presupposes that an evolutionary advantage is conferred by these posttranslational additions. Jackson and Hartwell have proposed a theory of mating-partner discrimination for haploid cells of S. cerevisiae in which haploid yeast cells select a partner of the opposite mating type on the basis of the level of pheromone they secrete (71). If cell fusion is promoted by high concentrations of mating pheromone, the hydrophobicity associated with lipopeptide pheromones might serve to maintain such a concentration gradient in the vicinity of the source cell by limiting the diffusibility of these peptides. It is interesting to consider whether localized high concentrations of mating pheromone might impart a selective advantage to these yeasts in their natural habitat.

The $a$-factor export protein in S. cerevisiae cells that have been treated with $a$-factor (i.e., stimulated for mating) has been shown to be localized to the tip of these cells as they elongate toward their partner (88). The presence of Ste6p has also been shown to be required for the restoration of mating between $a$-cells deleted for the $a$-factor structural genes with supersensitive $a$-cells, in the presence of high concentrations of exogenously added synthetic $a$-factor (104). This requirement is intriguing and may suggest that Ste6p serves in a capacity other than its role as the exporter of $a$-factor. It has been proposed that hydrophobic mating pheromones remain bound to the plasma membrane at export and are, in effect, “presented” to the mating cell partners (104, 112, 114). In this regard, Ste6p shares structural homology with the TAP antigen presentation protein of mammalian cells and may have an analogous function in yeast cells. The combination of pheromone hydrophobicity with directed export or presentation may contribute to cell polarity and act to restrict partner selection in vivo. Haploid cells of U. maydis which form hyphae in response to pheromone have been observed to adhere to polystyrene surfaces, whereas normal cells do not (161). In the natural environment of this fungus, such as the surface of a corn plant, this adherence mechanism may increase the chances of mating with an identified partner that acted to induce the hyphal formation. The analysis of mating-pheromone influence on adhesion and morphogenesis may have medical implications, because these are considered putative virulence factors in some fungi. This represents an intriguing and significant area for future study.

The relationship between virulence, morphology, and mating genes may not be limited exclusively to the “sexual” fungi. The dimorphic asexual yeast C. albicans has recently been shown to contain a homolog of the S. cerevisiae transcription factor STE12, a gene directly involved in pheromonal gene expression and response (100). This C. albicans gene, named
which show an exaggerated response to mating factors, have contrast, the population of a membrane-bound calcium ATPase (116, 117). In signal transduction and is possibly associated with the stimulation of a farnesylated cysteine hormone cysteine carboxypeptidases and the previously described intracellular protease involved in dependent proteases for fungal mating factors. In addition to virulence, fungal mechanisms of signal transduction, drug resistance, and ample of the relationship between mating genes and virulence to the discovery.

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Bioactivity studies on strains lacking a-factorase activity may contribute to our understanding of interactions between a-factor and cells. Two mutants of \textit{S. pombe},xa1 and xaa2 mutants, which show an exaggerated response to mating factors, have also been identified (66). The deduced xaa1\textsuperscript{7} gene product has sequence similarity to aspartyl proteases and the \textit{S. cerevisiae} BAR/\textit{SST1} protease protein (34.6\%, an extracellular protease that cleaves a-factor. However, the expression of the xaa1\textsuperscript{7} gene is not limited to the P-mating type, and it has not yet been localized and directly shown to degrade M-factor. While the Sxa2 protein shares homology with serine carboxypeptidases and its transcription is induced by pheromone treatment, little more is known about its substrate specificity or localization. The cloning of cell-associated protease genes in these organisms and possibly in other fungi will speed progress toward assigning the precise functional roles of these enzymes in lipopeptide-mediated activities.

Finally, as progress in this field continues, unforeseen insights into cellular mechanisms will be revealed. If, in the future, agents that lead to the specific inhibition of prenyltransferases, provide a means to elude MDR in cancer cells, or allow for an extended life span in immunocompromised patients with fungal infections are identified, the basic studies on fungal lipopeptide mating pheromones will have probably contributed to their discovery.
