The Ste5p scaffold

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Summary

An emerging theme of mitogen-activated protein kinase (MAPK) cascades is that they form molecular assemblies within cells; the spatial organization of which is provided by scaffold proteins. Yeast Ste5p was the first MAPK cascade scaffold to be described. Early work demonstrated that Ste5p selectively tethers the MAPKKK, MAPKK and MAPK of the yeast mating pathway and is essential for efficient activation of the MAPK by the pheromone stimulus. Recent work indicates that Ste5p is not a passive scaffold but plays a direct role in the activation of the MAPKKK by a heterotrimeric G protein and PAK-type kinase. This activation event requires the formation of an active Ste5p oligomer and proper recruitment of Ste5p to a Gβγ dimer at the submembrane of the cell cortex, which suggests that Ste5p forms a stable Ste5p signalosome linked to a G protein. Additional studies underscore the importance of regulated localization of Ste5p to the plasma membrane and have revealed nuclear shuttling as a regulatory device that controls the access of Ste5p to the plasma membrane. A model that links Ste5p oligomerization with stable membrane recruitment is presented. In this model, pathway activation is coordinated with the conversion of a less active closed form of Ste5 containing a protected RING-H2 domain into an active Ste5p dimer that can bind to Gβγ and form a multimeric scaffold lattice upon which the MAPK cascade can assemble.

Key words: Scaffold, MAP kinase, Signal transduction, RING-H2 domain, *S. cerevisiae*, Mating

Introduction

All eukaryotic cells use mitogen-activated protein kinase (MAPK) cascades as central cores of complex signal transduction pathways that respond to a wide variety of external stimuli and regulate numerous cellular responses (Gustin et al., 1998; Cobb et al., 1999; Schaeffer and Weber, 1999; Widmann et al., 1999). A MAPK cascade is a module of three to four serially activated protein kinases named after the last kinase of the series (Fig. 1). Eukaryotic cells typically contain multiple MAPK cascades, which contain unique or shared protein kinases. Surprisingly, the same MAPK cascade can perform different functions in response to different stimuli or different levels of the same stimulus (Marshall, 1995; Elion, 2000; Pan et al., 2000). This is particularly apparent in *Saccharomyces cerevisiae*, in which four of the six MAPK cascades use subsets of the same protein kinases (Fig. 1). All of the yeast pathways exhibit a high degree of specificity, with different effector proteins activated depending on the stimulus, even in instances where the pathways share kinases. This conclusion is largely based on phenotypic analysis of yeast strains harboring null and point mutations in the different kinases (Gustin et al., 1998; Madhani and Fink, 1998).

A major question is what ensures the fidelity of signaling, particularly in instances in which the same protein kinase functions in more than one pathway. Work over the years suggests that signaling specificity is the result of many levels of control, which include: preferred kinase-kinase interactions within the MAPK cascade (Bardwell et al., 1996; Xia et al., 1998); preferred MAPK-substrate interactions (Kallunki et al., 1996; Jacobs et al., 1999); the use of pathway-specific MAPKs (Madhani and Fink, 1998); cross-regulation between MAPK modules (Hall et al., 1996; O'Rourke and Herskowitz, 1998; Davenport et al., 1999); controlled localization of the MAPKs (Schaeffer and Weber, 1999; Cobb, 1999; Cobb and Goldsmith, 1999); and control of the strength of activation (Marshall, 1995). Another crucial level of control arises from the formation of discrete complexes involving the cytoplasmic kinases within a given MAPK cascade. These multienzyme complexes form through interactions with scaffold proteins that bind to multiple components of a single MAPK cascade. The scaffold proteins are thought to facilitate interactions between the associated kinases and can also link them to receptors, G proteins or other signaling components localized on the submembrane side of the plasma membrane (Elion, 1995; Elion, 1998; Whitmarsh and Davis, 1998; Schaeffer and Weber, 1999; Burack and Shaw, 2000). MAPK cascade scaffolds are widely divergent, which suggests that they have evolved independently for the individual requirements of subsets of kinases and upstream activating events (Elion, 1998). The compartmentalization of signal transduction pathway components using scaffold or adapter proteins is a frequently encountered form of regulation (Pawson and Scott, 1997; Burack and Shaw, 2000; Miller and Lefkowitz, 2001). In the case of MAPK cascades, scaffold proteins, in theory, provide a wealth of opportunities for protein kinase regulation through localization of upstream signaling components, allosteric control of the associated kinases, control of MAPK substrate availability and feedback attenuating mechanisms.

The first MAPK scaffold to be described was the yeast Ste5 protein (i.e. Ste5p) (Kranz et al., 1994; Choi et al., 1994; Marcus et al., 1994; Printen and Sprague, 1994), which associates with G protein, MAPKKK, MAPKK and MAPK of the mating pathway (Fig. 1). Since its discovery, a number of analogs of Ste5p have been described in yeast (Fig. 2) and

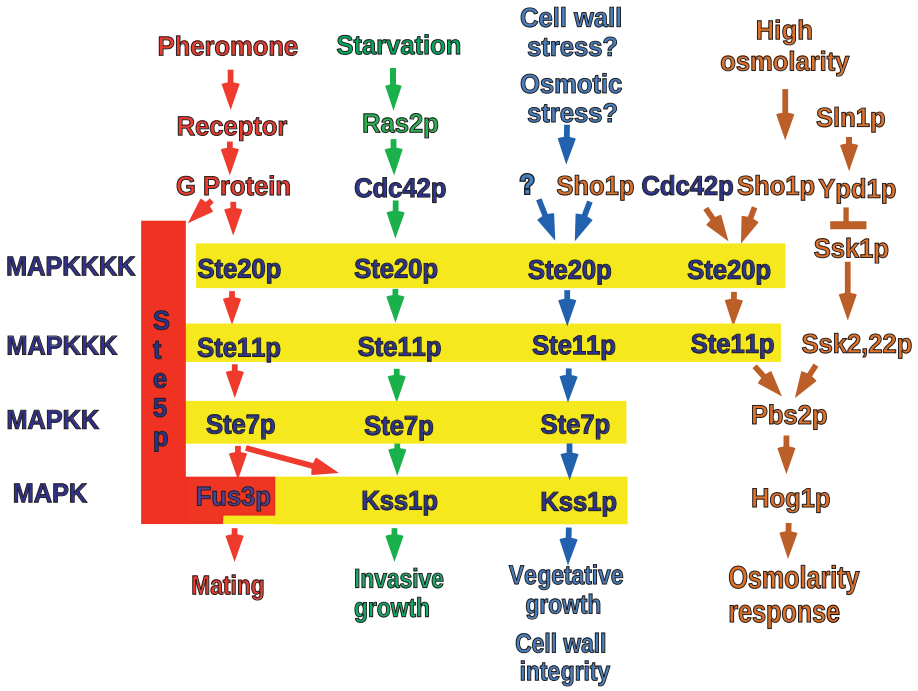

Fig. 1. Multiple MAP kinase (MAPK) cascades using shared components regulate growth and differentiation in *S. cerevisiae*. Mating, invasive growth, pseudohyphal development, high-osmolarity/glycerol response and maintenance of cell wall integrity are each regulated by structurally similar but functionally distinct MAPK cascades that are activated by different upstream signals but have in common at least three kinds of kinases: a MAPKKK, a MAPKK (or MEK) and a MAPK. Yellow highlighting indicates the kinases that are shared by the different pathways. Note that for simplicity Ste50p is not shown in this figure (but see Fig. 2), although it associates with Ste11p and is required for optimal signaling through all of the pathways shown. Details can be found elsewhere (Gustin et al., 1998; Elion, 2000; Pan et al., 2000).

mammals (Burack and Shaw, 2000; Whitmarsh and Davis, 1998). Here, I describe the multiple functions of Ste5p in the regulation of a MAPK cascade and emphasize the growing evidence that Ste5p does not play a passive role in signal transmission, highlighting its important role in spatiotemporal localization of a MAPK cascade to a G protein at the plasma membrane.

Overview of the mating MAPK cascade and identification of Ste5p

The Ste5p scaffold is required in yeast for mating, one of the best-studied examples of a cellular response to an external signal (Fig. 1) (Elion, 2000; Dohlman and Thorner, 2001). Mating is mediated by a G-protein-coupled MAP kinase cascade that is activated by peptide mating pheromones. Receptor-ligand binding is thought to activate a heterotrimeric G protein by promoting the dissociation of the inhibitory Gα subunit (Gpa1p) from stimulatory Gβγ subunits (Ste4p and Ste18p), which activate the MAP kinase cascade through binding of the Gβ subunit to key effectors. The mating MAPK cascade consists of Ste20p (a PAK-type kinase or MAPKKKK), Ste11p (a MEKK-type kinase or MAPKKK), Ste7p (a MEK-type kinase or MAPKK) and MAPKs Fus3p and Kss1p (Caffrey and Shields, 1999). Fus3p is much more crucial for eliciting mating-pathway responses than Kss1p is (Madhani et al., 1998). Ste5p is essential for mating-pathway activation and operates at multiple steps of the pathway. A second regulatory protein, Ste50p, plays a more ancillary function that involves the MAPKKK Ste11p and is not specific to the mating pathway or essential for signaling. Nearly all of the signaling components in the mating pathway were identified as mutations or overexpressed genes that affect the ability of cells to mate or undergo G_1 phase arrest in response to mating pheromone.

Many of the components of the mating MAPK cascade are used in other MAPK cascades. Ste20p (the MAPKKKK) and Ste11p (the MAPKKK) are used in the high-osmolarity/glycerol (HOG) pathway to help cells survive osmotic stress (Gustin et al., 1998; O'Rourke and Herskowitz, 1998). Ste20p and Ste11p are also used with the MAPKK Ste7p and MAPK Kss1p in both the invasive growth and pseudohyphal development pathways to promote food uptake during nutrient deprivation in haploids and diploids (Liu et al., 1993; Roberts and Fink, 1994) and in the sterile vegetative growth (SVG) pathway that promotes cell wall integrity (Lee and Elion, 1999). In these pathways, Kss1p is the key MAPK; the MAPK Fus3p does not have a positive regulatory role, but it inhibits both invasive growth and SVG pathways in its inactive form (Madhani et al., 1997; Lee and Elion, 1999). Pathway specificity is gained through the use of Fus3p, which is used solely by the mating MAPK cascade (Madhani and Fink, 1997) and is expressed only in haploids, the type of cell that mates (Elion et al., 1990). However, the use of Fus3p does not fully explain pathway specificity, because MAPK Kss1p is efficiently activated by mating pheromone in wild-type cells regardless of whether it is overexpressed (Gartner et al., 1992; Ma et al., 1995) or whether it is present at native levels (Cherkasova and Elion, 2001). Additional specificity is thought to be provided by the Ste5p scaffold, which is solely required for the mating MAPK cascade pathway and is expressed only in haploid cells.

Genetic identification of Ste5p and its placement in the MAPK cascade

Genetic experiments demonstrated that Ste5p is essential for mating-pathway signal transduction. The first *ste5* mutants were isolated in genetic screens for mutants unable to mate or undergo G_1 phase arrest in the presence of mating pheromone (Mackay and Manney, 1974; Hartwell, 1980). Subsequent cloning of the *STE5* gene and analysis of strains harboring *ste5*

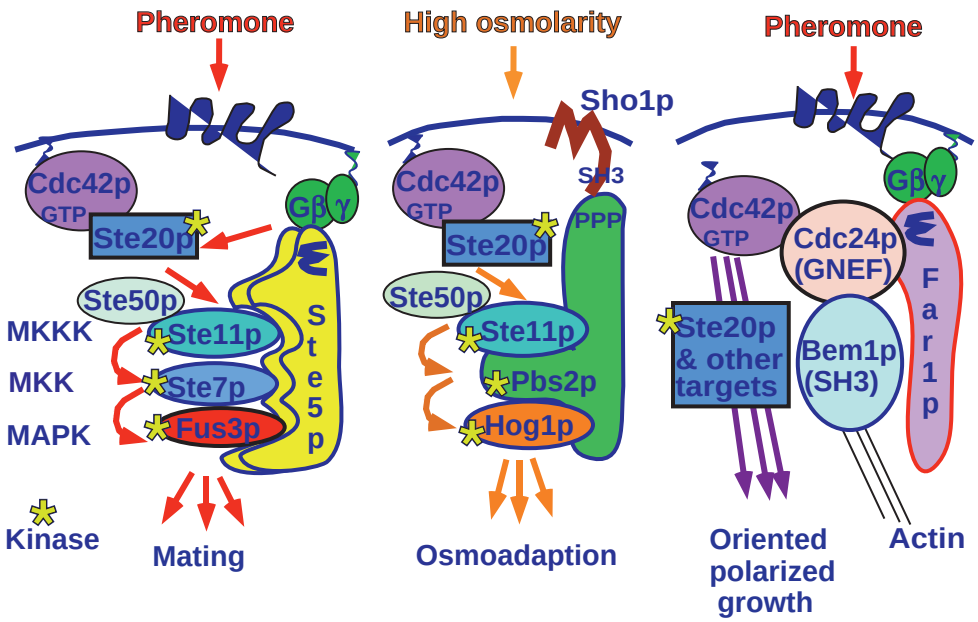

Fig. 2. Cartoon of Ste5p, Pbs2p and Far1p scaffolds. Ste5p is required for activation of the mating MAPK cascade in response to mating pheromone and does not have an intrinsic kinase activity, whereas Pbs2p encodes the MAPKK of the high osmolarity/glycerol pathway that is activated by increased osmolarity. Far1p is required for oriented polarized growth in response to mating pheromone. Pbs2p and Far1p are postulated to be analogs of Ste5p on the basis of their ability to associate with multiple components of an individual signal transduction pathway (Posas and Saito, 1997; Butty et al., 1998; Nern and Arkowitz, 1998; Nern and Arkowitz, 1999; Rait et al., 2000), but it is not known whether they simultaneously bind to associated signaling components. Similarities between Ste5p, Pbs2p and Far1p include the ability to associate with an uppermost component of a pathway that is membrane associated and senses the external signal, as well as to downstream components that regulate the activity of effectors within a pathway. In addition, all three scaffolds link signaling components that also associate with a Rho-type G protein (Cdc42p). Ste5p and Far1p share two domains of homology (Leberer et al., 1992), one of which overlaps with the RING-H2 domain that is thought to associate with the Gβ subunit Ste4p of the same heterotrimeric G protein. It is not known whether the RING-H2 domains have a function in ubiquitin-mediated proteolysis (Borden, 2001).

null mutations demonstrated that the Ste5p protein positively regulates mating (Leberer et al., 1993; Mukai et al., 1993). Although the predicted amino acid sequence of Ste5p was not informative with respect to defining its function, epistasis tests provided concrete functional evidence for its essential role in signaling and indicated that it functions between Ste20p (the MAPKKKK) and Ste11p (the MAPKKK) in the MAPK cascade (Blinder et al., 1989; Leberer et al., 1992; Leberer et al., 1993; Stevenson et al., 1992; Hasson et al., 1993).

Further genetic observations indicated that Ste5p function could not be defined in simple linear terms in a signal transduction pathway. These genetic studies suggested that Ste5p plays a complicated role in signaling and functions at multiple steps in the pathway to activate MAPK Fus3p (Kranz et al., 1994; Elion, 1995). Additional work indicated that Ste5p overexpression enhances Fus3p kinase activity and that Ste5p physically associates with and is phosphorylated by Fus3p and additional associated kinase(s) in vivo (Kranz et al., 1994). These and other observations suggested that Ste5p positively regulates the activity of Fus3p by interacting with multiple signal transduction components required for its activation.

Defining Ste5p as a MAPK cascade scaffold

Several criteria were used to define Ste5p as a tether or scaffold upon which the protein kinases Ste11p, Ste7p, Fus3p and Kss1p can assemble and be efficiently activated (Fig. 2). First, Ste5p selectively associates with Ste11p, Ste7p (Choi et al., 1994; Marcus et al., 1994) and Fus3p (Choi et al., 1994; Kranz et al., 1994). Second, Ste5p binds to the MAPK cascade kinases through separable binding sites (Choi et al., 1994). Third, Ste5p simultaneously binds the kinases; this conclusion is based on co-sedimentation in a glycerol gradient (Choi et al., 1994) and the lack of a co-sedimentation pattern upon removal of Ste5p (Elion, 1995; Choi et al., 1999). In contrast, Ste5p does not appear to have a distinct binding site for Ste20p, although the two proteins co-immunoprecipitate with the same actin-associated protein, the adaptor protein Bem1p (Leeuw et al., 1995). Thus, the two proteins are likely to be in close proximity within a complex but perhaps do not directly interact.

Further evidence argues that the association between Ste5p and the MAP kinases is a regulatory one that leads to activation of the MAPK Fus3p. Ste5p is rate limiting for activation of Fus3p (Kranz et al., 1994), and Fus3p has its highest specific activity in the 350-500 kDa glycerol gradient fractions that also contain Ste5p, Ste11p and Ste7p (Choi et al., 1999). The Ste5p-multikinase complex is detected before pheromone stimulation, especially when the kinases are inactive mutants (Choi et al., 1994). Ste5p enhances the ability of Ste11p and Ste7p to associate (Choi et al., 1994; Marcus et al., 1994) but does not appear to influence the ability of Ste7p to associate with the MAPKs (Choi et al., 1994; Marcus et al., 1994; Printen and Sprague, 1994). In addition, Ste5p preferentially co-precipitates hypophosphorylated forms of Ste7p, which are thought to represent pre-activated molecules that have not yet undergone feedback phoshorylation by Fus3p (Choi et al., 1994). These findings led to the proposal that Ste5p tethers Ste11p to a Ste7p-Fus3p dimer that dissociates upon phosphorylation of Ste7p by Fus3p (Choi et al., 1994). Subsequent work supports this model and argues that Ste7p and Fus3p form stable complexes independently of Ste5p (Bardwell et al., 1995) and that the binding of Ste5p to Ste11p and Ste7p is required for signal transmission (Inouye et al., 1997a; Bardwell et al., 2001).

Defining an activation function for Ste5p that is distinct from scaffolding

Ste5p is not a passive scaffold; it plays a direct role in the activation of the MAPKKK Ste11p. Early genetic experiments indicated that the activation of Ste11p was most likely to involve derepression of an inhibitory N-terminal regulatory domain (Cairns et al., 1992; Stevenson et al., 1992). The N-terminal regulatory domain of Ste11p is now known to bind to its C-terminal kinase domain, and this interaction is thought to inhibit catalytic activity (Wu et al., 1999; Van Drogen et al., 2000). The release of Ste11p from autoinhibition is thought to involve the phosphorylation of sites within the Ste11p regulatory domain by Ste20p (Van Drogen et al., 2000) and the binding of the Ste50p protein to Ste11p through sterile alpha motif (SAM) (Ponting, 1995) domains in the N-terminus of Ste50p and the N-terminal regulatory domain of Ste11p (Wu et al., 1999). Genetic and biochemical experiments suggest that these two events interfere with the ability of the N-terminal regulatory domain to bind to the C-terminal kinase domain. Neither event is sufficient for full activation of Ste11p in the presence of pheromone (Akada et al., 1996; Xu et al., 1996), although they activate Ste11p sufficiently for invasive growth, pseudohyphal development and survival under osmotic stress (Fig. 1).

Several lines of evidence first hinted that the binding of Ste5p to Ste11p is essential for its activation by mating pheromone. Ste5p is required for the full basal activity of hyperactive Ste11p mutants, whereas the Gβ subunit Ste4 is not (Stevenson et al., 1992; Elion, 1995). Ste5p associates with the N-terminal regulatory domain of Ste11p (Choi et al., 1994; Marcus et al., 1994; Printen and Sprague, 1994), and a larger percentage of Ste11p co-sediments with Ste5p in a glycerol gradient than with Ste7p or Fus3p (Choi et al., 1994). In addition, co-overexpression of Ste5p with Ste11p causes synergistic activation of Fus3p in the absence of pheromone (Choi et al., 1994). More recent studies in fact suggest that Ste5p plays two roles in the activation of Ste11p: (1) localization of Ste11p to the MAPKKKK Ste20p at the cell cortex; and (2) allosteric control of Ste11p or the other associated kinases.

Ste5p recruitment to Gβγ (Ste4p/Ste18p) at the plasma membrane

The Gβ subunit of the heterotrimeric G protein Ste4p transduces the pheromone signal by binding to Ste5p in addition to Ste20p (Fig. 2). During vegetative growth, Ste5p and Ste20p are both distributed throughout the cell (Leberer et al., 1997; Mahanty et al., 1999) and a pool of Ste20p is concentrated at growth sites of the emerging mother-bud neck junction (Leberer et al., 1997; Moskow et al., 2000). Ste20p is enriched at the cell cortex through a CRIB domain interaction with Cdc42p, a Rho-type G protein (Ziman et al., 1993; Simon et al., 1995; Peter et al., 1996; Leberer et al., 1997; Moskow et al., 2000). In the presence of pheromone, Ste4p binds to Ste20p through a C-terminal domain in Ste20p (Leeuw et al., 1998). Ste4p also binds to Ste5p (Whiteway et al., 1995) through a RING-H2 domain in Ste5p (Inouye et al., 1997b; Feng et al., 1998), and this interaction is also pheromone dependent (Feng et al., 1998). Genetic evidence suggests that Ste4p binds to Ste20p and Ste5p through distinct domains (Dowell et al., 1998), raising the possibility that a single Gβγ dimer could simultaneously recruit Ste20p and Ste5p. Both interactions are essential for pheromone-induced activation of Ste11p: Ste20p mutants that do not associate with Ste4p exhibit severely defective transmission of the pheromone signal (Leeuw et al., 1998); and Ste5p RING-H2 domain mutants that do not associate with Ste4p have a selective defect in transmitting the pheromone signal from the Gβγ dimer to Ste11p, although they still bind to the kinases and facilitate signaling of Ste11p to Fus3p (Inouye et al., 1997b; Feng et al., 1998).

Additional findings strongly support the view that the Gβ subunit of the Gβγ dimer (comprising Ste4p and Ste18p) recruits both Ste20p and Ste5p in the presence of pheromone and that these interactions are required for Ste20p to activate Ste11p (Fig. 2). First, the need for Ste4p and Ste18p can be bypassed by artificially targeting Ste5p to the plasma membrane using a C-terminal transmembrane domain (Pryciak and Huntress, 1998). Plasma-membrane-targeted Ste5p, with or without the first 214 residues that overlap the Ste4p-binding domain, constitutively activates the pathway, but only if Ste20p is present; this is strong evidence that Gβγ must recruit Ste5p in parallel to Ste20p activating Ste11p (Pryciak and Huntress, 1998). The Gβγ dimer appears to bind to a pool of Ste20p that is already at the cell cortex, owing to its interaction with Cdc42p-GTP, because this interaction is required for efficient signal transmission induced by the Gβ subunit Ste4p (Moskov et al., 2000). In contrast, Ste5p appears to be rapidly recruited to the plasma membrane by the pheromone stimulus (Mahanty et al., 1999). Translocation of Ste5p to the plasma membrane can be detected within a few minutes of pheromone addition in the presence of cycloheximide, which is consistent with a rapid recruitment event that would be needed for pathway activation. In co-precipitation tests, Ste5p recruits Ste11p, Ste7p and Fus3p to the cortical protein Bem1p (Lyons et al., 1996), which provides biochemical support for the idea that in vivo Ste5p recruits the kinases to targets. Thus, Ste5 appears to play an important spatial role assembling the kinases at Gβγ dimers at the plasma membrane. Currently, it is not known whether Ste5p forms a stable anchoring structure at the plasma membrane upon which the enzymes assemble, are activated and disassemble. However, the fact that membrane-targeted Ste5p can promote nuclear responses such as transcription (Pryciak and Huntress, 1998) argues that, once activated, Fus3p dissociates and translocates to the nucleus.

Oligomerization and conformational changes

A major unsolved issue is what is the stoichiometry of the complex involving Ste5p and the kinases. Genetic evidence argues that Ste5p functions as a dimer or higher-order oligomer. This conclusion is based on allelic complementation between certain pairs of Ste5p mutants that are nonfunctional when expressed on their own but functional when co-expressed (Yablonski et al., 1996) and is supported by the biochemical detection of Ste5p oligomers and by two-hybrid analysis (Yablonski et al., 1996; Feng et al., 1998). Ste5p self-associates through two domains: the RING-H2 domain, which binds to the Gβγ dimer; and a distal domain that overlaps with a potential leucine zipper and the Ste11p-binding site (Yablonski et al., 1996). The Ste5p RING-H2 domain is a member of a

family of cytseine-rich Zn-binding domains that homo-oligomerize and promote the assembly of macromolecular structures (Borden, 2000), and it can homo-oligomerize independently of the second oligomerization domain (Feng et al., 1998). The N- and C-terminal halves of Ste5p also associate when expressed as separate fragments (Sette et al., 2000), which raises the possibility that Ste5p forms a folded structure. It is currently not known whether the serial phosphorylation reactions involving the kinases occur in cis within a monomer, in trans across a dimer interface or in trans across dimers that have multimerized. Interestingly, the JNK-interacting protein (JIP) group of structurally related scaffold proteins that mediate signaling by the JNK MAPKs and specific MAPKK and MAPKKK activators also oligomerize (Whitmarsh et al., 1998; Yasuda et al., 1999), which suggests this is a conserved feature of this class of proteins.

Ste5p might translocate to Gβγ (Ste4p and Ste18p) as a dimer or as an oligomer that binds to the kinases. Ste5p oligomerization occurs constitutively and is not influenced by mating pheromone (Feng et al., 1998); it might therefore occur before binding to Gβγ. In addition, mutating the RING-H2 domain simultaneously interferes with its ability to oligomerize and associate with Gβγ (Feng et al., 1998). In a glycerol gradient, the fraction of Ste5p that co-sediments with Ste11p, Ste7p and Fus3p has an estimated mass of 350-500 kDa (Choi et al., 1994), a size that is consistent with a Ste5p dimer associated with two molecules each of Ste11p, Ste7p and Fus3p, and the interactions within this complex occur independently of pheromone (Kranz et al., 1994; Choi et al., 1994). MAPKs form stable dimers (Cobb and Goldsmith, 2000), but this has not been demonstrated for Fus3p. However, the available evidence suggests that Ste11p forms dimers (Printen and Sprague, 1994; Rad et al., 1998). Thus, a large complex consisting of a Ste5p dimer and two molecules each of Ste11p, Ste7p and Fus3p might translocate en masse to the plasma membrane.

Additional unsolved issues are how does Ste5p regulate the activity of the associated kinases and does it directly control kinase activity. Several lines of indirect evidence led to the suggestion that Ste5p positively regulates the activity of the associated kinases independently of its ability to recruit them to Ste20p at the plasma membrane (Elion, 1995; Lyons, 1996; Feng, 1998). A hyperactive Ste11-4p mutant that has significantly elevated basal activity requires Ste5p for 98% of this signaling capacity but does not require Ste4p (the Gβ subunit) (Stevenson et al., 1992) or Ste20p (Feng et al., 1998). In addition, Ste5p can promote the activation of the MAPK cascade in the absence of Ste20p (Lyons et al., 1996) and stimulate basal activity of Ste11-4p when it is unable to bind to Ste4p (Feng et al., 1998). Recent work strongly argues that Ste5p directly regulates the activity of the associated kinases by undergoing conformational changes (Sette et al., 2000). Three Ste5-GST gain-of-function mutants that stimulate Ste20p-dependent basal signaling when overexpressed in the absence of Ste4p and a functional RING-H2 domain have been isolated and characterized. Two of the mutations enhance the ability of full-length Ste5p to associate with the N-terminal half of Ste5p - the strength of the effect on binding correlating with the increase in basal signaling (Sette et al., 2000). These results led to the interesting proposal that Ste5p exists as either a folded dimer of parallel strands or as a dimer of anti-parallel strands, and that the binding of the Gβγ dimer alters the conformation of either closed structure in such a way that Ste20p, Ste11p, Ste7p and Fus3p are optimally aligned for serial phosphorylation (Fig. 3).

An oligomerization model for recruitment

The proposed conformational models (Fig. 3) do not fully explain all of the published data on Ste5p. First, RING-H2 domain mutations increase the steady-state level of Ste5p oligomers (Feng et al., 1998), and this increase is accompanied by greater basal signaling from Ste11 to Fus3 (Inouye et al., 1997b; Feng et al., 1998). This observation led to the suggestion that the RING-H2 domain negatively regulates oligomerization in the absence of mating pheromone in order to dampen basal signaling (Feng et al., 1998). Second, deletion of a functional nuclear localization sequence (NLS) in Ste5p that overlaps with the N-terminal region involved in the interaction with the C-terminal region (Ste5Δ49-66p) blocks the ability of Ste5p to be recruited to Gβγ (Ste4 and Ste18) at the plasma membrane. However, it fails to block Ste5p from binding to Gβγ in vitro or promote signal transduction when artificially targeted to the plasma membrane (Mahanty et al., 1999) or overexpressed (Mahanty and EAE, unpublished data).

An alternative model that reconciles these (Feng et al., 1998; Mahanty et al., 1999) and other data (Lyons et al., 1996; Yablonski et al., 1995; Inouye et al., 1997b) proposes that Ste5p exists in two forms: a less active, folded or closed monomer or dimer in which the RING-H2 domain is not available to bind Gβγ and basal signaling is repressed (Fig. 4A); and an open, active dimer of parallel strands (Fig. 4B) in which the RING-H2 domain binds to Gβγ and interactions involving the N- and C-termini drive the formation of head-to-tail multimers (Fig. 4C). In this model, the binding of the RING-H2 domain to Gβγ would be predicted to block intramolecular interactions between the N- and C-termini and open the folded structure, driving head-to-tail multimerization of adjacent Ste5p dimers. This event might cause a global rearrangement that could position the kinases for recognition by Ste20p. Moreover, simple concentration of the kinases by multimerization of Ste5p might also be sufficient to help drive activation of the MAPK independently of any contribution by Gβγ and Ste20p (Robinson et al., 1998; Kieran et al., 1999).

A virtue of this model is that it provides a simple mechanism for the formation of a Ste5p lattice at the plasma membrane that would be stabilized by intermolecular interactions. Ste5p recruitment would thus be determined by the forces that drive Ste5p to Gβγ as well as those that keep it there. A Ste5p scaffold lattice could underlie the formation of a stable signaling structure whose position and size is dictated by the pheromone gradient and visualized as Ste5p recruitment at the plasma membrane. In addition, a regulatory step to control signaling is provided through an equilibrium between folded and open structures. The model is potentially applicable to other recruited proteins and makes a number of predictions. The first is that mutations or conditions that open the Ste5p structure by preventing intermolecular interactions or mimicking the effects of Gβγ binding to Ste5p should cause enhanced multimerization [e.g. RING-H2 domain mutations (Feng et al., 1998) or removal of metal from the RING-H2 domain (Yablonski et al., 1995)]. The second is that mutations

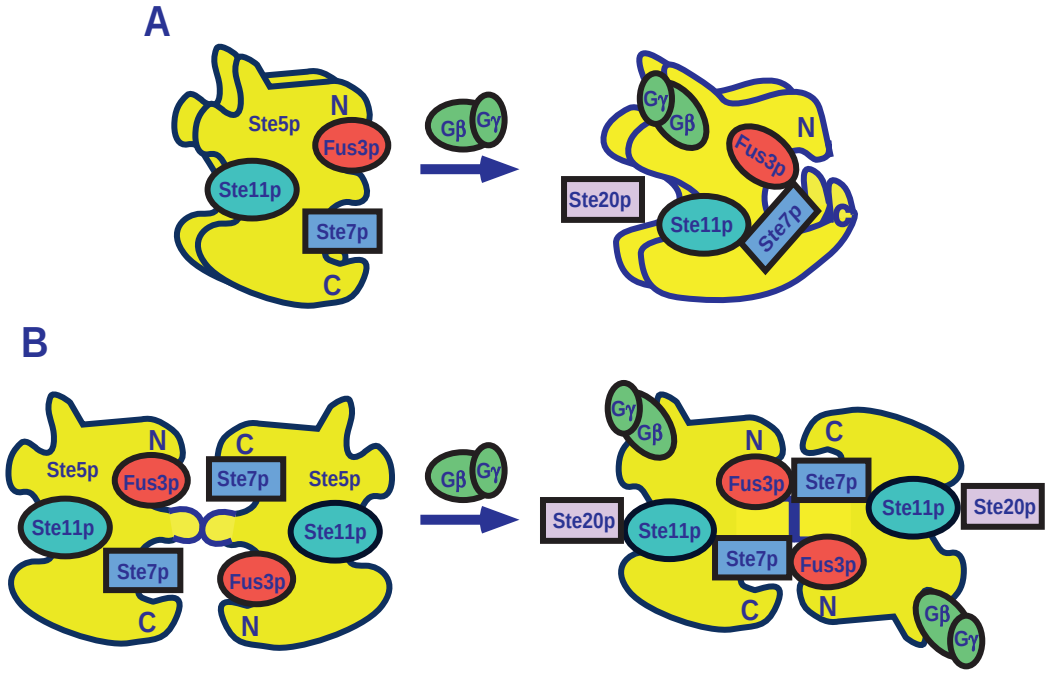

Fig. 3. Ste5p conformational models. Two models for how the binding of Gβγ to Ste5p induces a conformational change in either (A) a folded dimer or (B) an anti-parallel dimer that is formed through interactions between the N- and C-terminal halves of Ste5p. The binding of the Gβγ dimer (Ste4p and Ste18p) is postulated to align the associated kinases so as to permit serial phosphoryation. The models are adapted from (Sette et al., 2000); see text for details. Note that serial phosphorylations would occur in cis in model A and in trans in model B.

that interfere with the formation of head-to-tail multimers, but not binding to Gβγ, should prevent the formation of a stable Ste5p lattice at the plasma membrane (i.e. recruitment) and be rescued by artificial tethering of the protein to the plasma membrane or by its overexpression (perhaps, for example, of Ste5Δ49-66p) (Mahanty et al., 1999). Further work is needed to clarify the nature of the Ste5p oligomer and its relationship to recruitment and signal transmission.

Nuclear shuttling of Ste5p and links to pathway activation

The recruitment of Ste5p to Gβγ at the plasma membrane is regulated and involves the prior shuttling of Ste5p through the nucleus. Localization studies show that Ste5p is both cytoplasmic and nuclear (Pryciak and Huntress, 1998; Mahanty et al., 1999; Kunzler et al., 2001) and undergoes rapid recruitment to the plasma membrane in the presence of mating pheromone and cycloheximide (Mahanty et al., 1999), followed by accumulation at the tip of the emerging projection (Pryciak and Huntress, 1998; Mahanty et al., 1999). The cytoplasmic pool of Ste5p shuttles continuously through the nucleus, and nuclear shuttling requires the Kap95p (Rsl1p) import receptor and the Msn5p (Ste21p) export receptor (Mahanty et al., 1999). Mating pheromone induces nuclear export of Ste5p (Mahanty et al., 1999). Currently, it is not clear whether mating pheromone increases the rate of export of Ste5p or the amount of Ste5p that is exported from the nucleus. Functional analysis of a variety of Ste5p mutants that have varying abilities to be imported into the nucleus indicates that the shuttling of Ste5p is required for its efficient recruitment to Gβγ and activation of MAPK Fus3p (Mahanty et al., 1999). This led to the proposal that transit through the nucleus makes Ste5p competent to be recruited to Gβγ (Mahanty et al., 1999).

The available evidence suggests that nuclear shuttling controls the amount of Ste5p that is available to bind to Gβγ. Overexpression of Ste5p is sufficient to activate Ste11p (the MAPKKK) in the absence of pheromone (Choi et al., 1994), which suggests that the access of Ste5p to Gβγ must be carefully controlled. Hyperactivation of Ste11p is lethal, presumably because of its participation in multiple MAPK cascades. Enhanced nuclear shuttling correlates with better recruitment and pathway activation (Mahanty et al., 1999) (Y. Wang and E.A.E., unpublished data), which suggests that this regulatory device controls how much Ste5p is available to bind to Gβγ. Indeed, partial blocking of reimport of Ste5p enhances the activity of the pathway (Mahanty et al., 1999; Kunzler et al., 2001) (Y. Wang and E.A.E., unpublished data) and promotes membrane recruitment of Ste5p; (Mahanty et al., 1999) (Y. Wang and E.A.E., unpublished data).

Currently it is not known how Ste5p is affected by nuclear shuttling. This process could co-localize Ste5p with nuclear modifying enzymes or alter its conformation through interactions with shuttling factors or other proteins that are concentrated in the nucleus. The major NLS of Ste5p overlaps with sequences involved in interactions between its N- and C-termini (Mahanty et al., 1999; Sette et al., 2000), and putative NES sequences lie near the leucine-zipper oligomerization domain (Y. Wang and E.A.E., unpublished data). This suggests that nuclear shuttling and oligomerization are coordinated, an idea that is supported by a variety of evidence (Y. Wang and E.A.E., unpublished data). Nuclear shuttling could co-localize Ste5p with other proteins involved in signaling; for example, with Fus3p (the MAPK) and Ste7p (the MAPKK), which are both nuclear and cytoplasmic (Choi et al., 1999) (Mahanty and E.A.E., unpublished data). Ste11p (the MAPKKK), however, is cytoplasmic (Posas et al., 1998) and does not concentrate in the nucleus (Mahanty et al., 1999). This raises the possibility that nuclear shuttling concentrates Ste5p with at least two kinases in the pathway and possibly guides them to the plasma membrane. Interestingly, the Fus3p-binding site in Ste5p lies

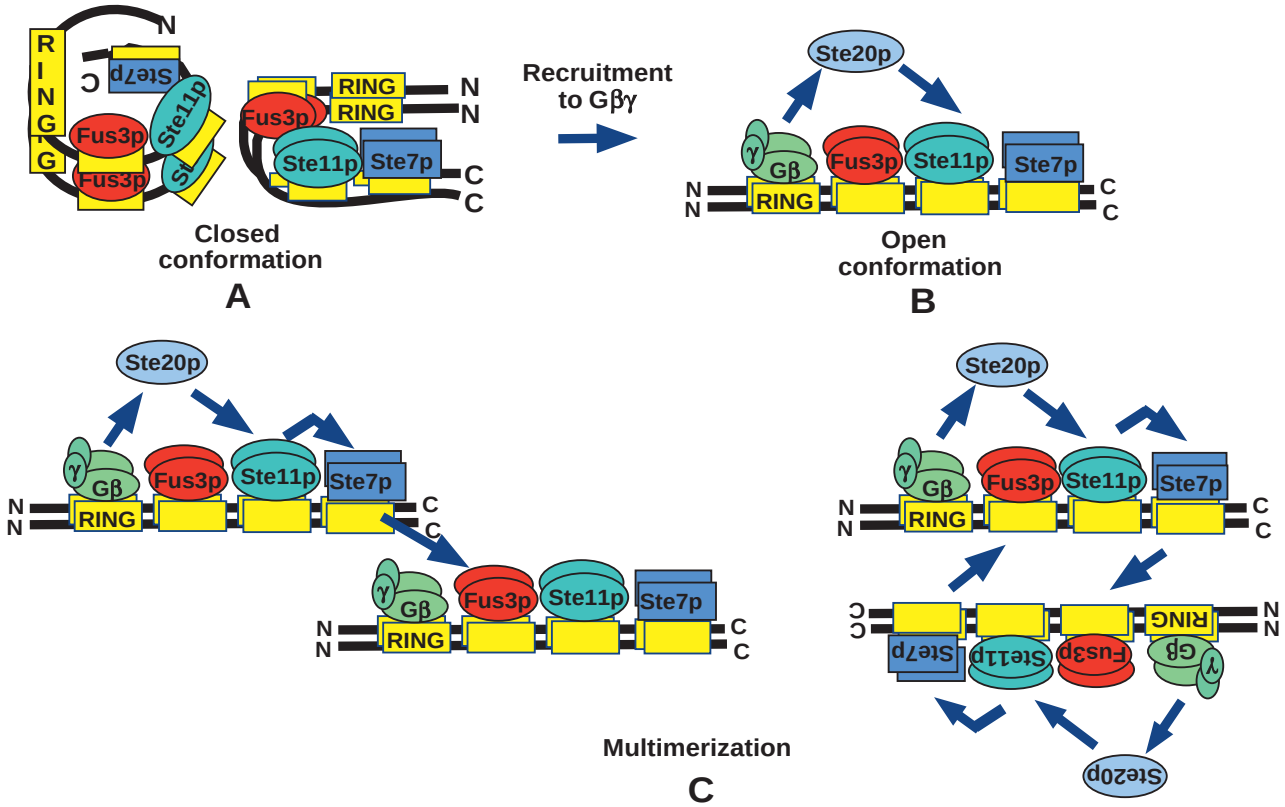

Fig. 4. Ste5p oligomerization/recruitment model. An alternative model, in which a Ste5p dimer forms higher-order oligomers by binding to Gβγ. In this scenario, a Ste5p dimer can exist with a protected RING-H2 domain as a folded dimer of parallel strands or dimer of anti-parallel strands (A) or as an open dimer of parallel strands in which the RING-H2 domain is accessible to homo-dimerize and bind Gβγ (B). Higher-order oligomers can form through N- to C-terminal interactions, the binding of Gβγ driving the formation of higher-order oligomers at the plasma membrane by interfering with intramolecular interactions. Two types of higher-order oligomer are shown: tandem head-to-tail oligomers and stacked anti-parallel oligomers. The higher-order oligomers could conceivably promote serial phosphorylation in trans.

next to the RING-H2 domain; phosphorylation of Ste5p therefore has the potential to alter the nature of this domain (Elion, 1995). As I will discuss below, it is also possible that nuclear shuttling links Ste5p to morphogenesis proteins required for polarized growth and recruitment to the plasma membrane.

Interactions between Ste5p and regulators of polarized morphogenesis

Mating cells sense the direction of the pheromone source and undergo polarized growth towards it, forming a pear-shaped projection (Segall, 1993), in a process termed chemotropism (Dorer et al., 1995; Arkowitz, 1999). Chemotropism is thought to involve the generation of an internal landmark that reflects the axis of the external pheromone signal (Herskowitz et al., 1995; Pringle et al., 1995; Arkowitz, 1999; Chant, 1999). It is mediated by the actin cytoskeleton (Ayscough and Drubin, 1996) and many other proteins that normally control bud emergence, such as the Rho-type G protein Cdc42p, its guanine-nucleotide-exchange factor (GNEF) Cdc24p and Bem1p, an adapter molecule that links Cdc24p to the actin cytoskeleton (Herskowitz et al., 1995; Pringle et al., 1995). The receptor-activated Gβγ dimer is thought to recruit a complex of polarity-establishment proteins through Far1p, a substrate of Fus3p that is a cyclin-dependent kinase (CDK) inhibitor and cell polarity adapter molecule (Chang and Herskowitz, 1990; Elion et al., 1993; Tyers and Futcher, 1993; Peter et al., 1993; Peter and Herskowitz, 1994; Valtz et al., 1995). The current view is that, in the presence of mating pheromone, Far1p functions similarly to the Ste5p scaffold and binds to Gβγ through a homologous RING-H2 domain and also binds to Cdc24p. Cdc24p binds to both Cdc42p and Bem1p, the latter protein helping to localize the polarity establishment proteins to the actin cytokeleton (Butty et al., 1998; Nern and Arkowitz, 1998; Nern and Arkowitz, 1999; Nern and Arkowitz, 2000a; Nern and Arkowitz, 2000b; Shimada et al., 2000). Locally activated Cdc42p-GTP is then predicted to bind to effectors such as the MAPKKKK Ste20p and a variety of actin-associated proteins to promote polarized growth (Chant, 1999).

During vegetative growth, Far1p is thought to be predominantly nuclear, whereas Cdc24p shuttles continuously through the nucleus and cytoplasm, a pool of Cdc24p being sequestered in the nucleus through an interaction with Far1p (Toenjes et al., 1999; Nern and Arkowitz, 1999; Shimada et al., 2000). Both proteins are exported from the nucleus to Gβγ at the projection tip in the presence of pheromone. The nuclear pool of Far1p might thus control the access of Cdc24p to Gβγ (Blondel et al., 1999; Toenjes et al., 1999; Nern and Arkowitz, 2000; Shimada et al., 2000). Such a model is attractive, but we

currently do not fully understand how the cell-polarity proteins are able to localize to the site of polarized growth in the absence of Far1p (Elion, 2000).

A key aspect of chemotropism is likely to be the asymmetric accumulation of the receptor and G protein at the internal landmark of the emerging projection tip (Ayscough and Drubin 1998; Arkowitz, 1999). The site for growth in the presence of pheromone may initially be chosen stochastically, before being biased to the correct location through a process that might involve receptor recycling (Arkowitz, 1999; Nern and Arkowitz, 2000b). Reinforcement of this bias might occur, in part, through signal amplification, perhaps through joint recruitment of the Ste5p–MAPK-cascade complex and the Far1p-morphogenesis machinery (Fig. 5). Although genetic evidence argues that Cdc24p (a GNEF), Cdc42p (a G protein), Ste20p (a MAPKKKK), Bem1p (an adapter protein) and Far1p (another adapter protein) promote polarized growth independently of the mating MAPK cascade (Schrick et al., 1997), it is also clear that the morphogenetic response requires sufficient signaling through the MAPK cascade. Dose-response analysis shows that projection formation requires the highest level of signaling through the MAPK cascade, much more than transcriptional activation (Moore, 1983; Farley et al., 1999). Furthermore, functional analysis demonstrates that Fus3p is specifically required to promote projection formation and that it promotes polarized growth independently of its ability to promote transcription (Farley et al., 1999). The recruitment of Ste5p, similar to the morphogenesis proteins, is asymmetric and enriched at growth sites and suggests that the localization machinery reinforces the bias leading towards localized activation of the MAPK cascade and might also localize Fus3p to relevant substrates (such as Far1p). Bem1p complexes contain both Ste5p and Far1p (Lyons et al., 1996), and Bem1p is required for efficient activation of Fus3p (Lyons et al., 1996). Furthermore, the recruitment of Ste5p to the projection tip requires both Cdc24p and Cdc42p (Pryciak and Huntress, 1998) and Cdc24p can still be recruited to the cortex in the absence of Far1p (Nern and Arkowitz, 2000a; Shimada et al., 2000). Thus, it is conceivable that the Ste5p and Far1p complexes physically and functionally interact in the nucleus as well as at the plasma membrane. Indeed, the links between Ste5p and Bem1p raise the intriguing possibility that Ste5p and Far1p could perform redundant functions with respect to localizing cell-polarity proteins to Gβγ (Fig. 5).

Ste5p and pathway specificity

The existence of Ste5p as a mating-pathway-specific component for kinases that function in multiple MAPK cascades (Fig. 1) has led to the general speculation that Ste5p provides pathway specificity (Widmann et al., 1999; Schaeffer and Weber, 1999; Whitmarsh and Davis, 1999; Burack and Shaw, 2000). This specificity has been hypothesized to lie at the level of selective binding of Ste5p to the MAPK Fus3p (Madhani and Fink, 1998), localization of MAPK Fus3p to specific targets (Lyons et al., 1996; Choi et al., 1999), the maintenance of Fus3p in a state of high specific activity (Choi et al., 1999) and the ability to restrict the kinases to the site of pathway activation (Elion, 1998; Pryciak and Huntress, 1998). In addition, it has been hypothesized that Ste5p serves an insulating function that segregates the associated kinases away from their participation in other pathways (Yashar et al., 1995).

Currently, no evidence proves or disproves any of these possibilities. The notion that Ste5p selectively binds MAPK Fus3p in vivo is particularly attractive because of its simplicity. However, it is not yet clear why Fus3p and Kss1p are both able to interact with the same small domain of Ste5p (Choi et al., 1994) or how Kss1p is activated by mating pheromone (Gartner et al., 1992; Ma et al., 1995; Cherkasova and Elion, 2001). One possibility is that the MAPK Kss1p resides in stable complexes along with multiple nuclear transcription factors of the invasive growth pathway (Cook et al., 1996; Madhani et al., 1997; Bardwell et al., 1998) but can be activated by a pool of MAPKK Ste7p that has dissociated from the Ste5p complex at the plasma membrane (Fig. 1). This fits with the observation that mating pheromone activates genes in the invasive growth pathway and the sterile vegetative growth pathway (Lee and Elion, 1999;

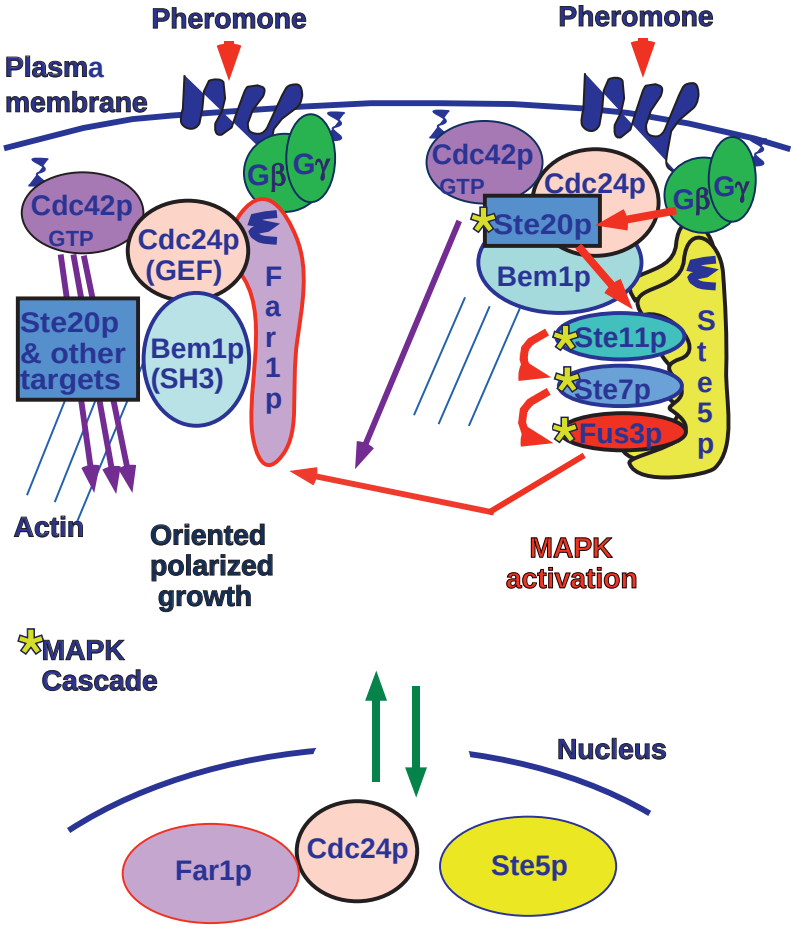

Fig. 5. Far1p-Ste5p interactions. Summary of potential related functional and physical interactions for both proteins, showing the fact that Far1p, Ste5p and Cdc24p all shuttle through the nucleus with the nuclear pool able to translocate to the cell periphery. Here it is pointed out that Far1p is a Fus3p substrate and that Ste5p could potentially target Cdc24p through its own interactions with Bem1p. See text for details.

Roberts et al., 2000), but it does not explain the interaction between Kss1p and Ste5p.

Evidence indicates that the activation of Fus3p, and not Kss1p, determines whether cells mate (Elion et al., 1990; Elion et al., 1991; Madhani et al., 1997; Farley et al., 1999). This specificity may be determined in part at the level of phosphorylation of specific targets involved in transcription, G_1 phase arrest, morphogenesis, partner selection and fusion (Elion et al., 1990; Elion et al., 1993; Tyers and Futcher, 1993; Peter et al., 1993; Farley et al., 1999). Fus3p is both nuclear and cytoplasmic (Choi et al., 1999) and concentrates at the base and tips of mating projections in live cells (A. Levchenko, personal communication; M. Qi, P. Maslo and E.A.E., unpublished), which is consistent with its multiple roles in mating. Thus, it is conceivable that Ste5p helps target Fus3p to substrates, given their overlapping localization patterns. However, Ste5p might instead provide pathway specificity simply by ensuring efficient activation of Fus3p, the activation of Kss1p not being a deciding factor in whether a cell mates. Time-course analysis shows that Fus3p is active only in the presence of mating pheromone and is rapidly dephosphorylated upon pheromone removal, which suggests that it is constitutively inactivated by phosphatases (Choi et al., 1999). This is consistent with the fact that at least three phosphatases are involved in inactivation of Fus3p (Doi et al., 1994; Zhan et al., 1999). Biochemical evidence suggests that Fus3p is not active as a monomer or dimer and only maintains high specific activity in the Ste5p complex (Choi et al., 1999). Thus, Ste5p may provide pathway specificity by ensuring that a pool of Fus3p is kept active.

Another way in which Ste5p might promote pathway specificity is to prevent the activation of kinases that participate in other pathways. For example, Ste5p might segregate or insulate the kinases so that they are not activated by stimuli other than mating pheromone. This possibility is supported by the observation that a hyperactive form of MAPKK Ste7p is better at suppressing a protein kinase C pathway mutant when Ste5p is not present (Yashar et al., 1995) and glycerol gradient analysis that shows that the presence of Ste5p sequesters the MAPKKK Ste11p and Ste7p, preventing them from forming alternative complexes (Choi et al., 1999). If Ste5p does segregate kinases, its removal from a cell should elevate signaling in the other pathways that use the segregated kinases (Fig. 1). The available evidence does not suggest that this is a major form of control, because *ste5* null mutants do not have obviously enhanced signaling through these pathways, although a minor increase has been detected (Lee and Elion, 1999). However, it is conceivable that the effect is masked by simultaneous release of inactive Fus3p, which inhibits the other pathways. Another possibility is that Ste5p promotes localized activation of the associated kinases so that they are less available to function in other pathways. For example, feedback attenuation by Fus3p could prevent further activation of the upstream kinases and limit their activation to the MAPK molecules associated with Ste5p. This possibility is supported by evidence that Fus3p feedback phosphorylates Ste7p, Ste11p and Ste5p (Errede et al., 1993, Kranz et al., 1994; Choi et al., 1994), although the significance of the feedback phosphorylation has not been established.

Conclusions and perspectives

We do not know how Ste5p propogates the pheromone signal or whether it serves as an amplifier or restrictor of the signal. All of the data to date argue that Ste5 does not function as a passive scaffold. A major function of Ste5 is to recruit the kinases to the right place at the right time for their activation; with additional allosteric roles that include direct activation of Ste11p (the MAPKKK) and transmission of the signal to Fus3p (the MAPK). It is possible that Ste5p translocates with some or all of the kinases to the plasma membrane. The heavy regulatory emphasis on proper recruitment of Ste5 to the plasma membrane suggests that this event is a key determinant of signal transmission. Plasma membrane recruitment of Ste5p involves prior nuclear shuttling, an event that could alter the absolute concentration of Ste5p and expose it to proteins that could alter its ability to be recruited to the plasma membrane and activate the MAPK cascade.

We also do not know whether Ste5p forms a transient or stable anchoring platform for the kinases at the plasma membrane. A consideration of the available data leads to a plausible model for the formation of a stable lattice through intermolecular multimerization and suggests that there is a tight link between Ste5p oligomerization and recruitment. Ferrell and co-workers have suggested that the existence of a multi-tiered processive MAPK cascade allows ultrasensitivity or switch-like responses to stimulus (Huang and Ferrell, 1996; Ferrell and Machleder, 1998). Although the presence of a scaffold is counterintuitive for maintaining a switch-like response, co-localization of proteins might be sufficient to enhance a response, depending on the relative concentrations of the scaffold and kinases (Ferrell, 1998; Burack and Shaw, 2000; Levchenko et al., 2000). The links between Ste5p and Cdc24p, and Cdc42p and Bem1p, raise the interesting possibility that these proteins play direct roles in the recruitment of Ste5p to the plasma membrane. Further work is needed if we are to clarify the nature of the recruitment event and to clarify whether simple concentration of Ste5p with its associated enzymes at specific sites at the plasma membrane underlies how Ste5p activates a MAPK cascade and whether Ste5p simultaneously regulates a morphogenetic response to a gradient of stimulus.

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