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Invited Review

Regulation of the MAPK family members: Role of subcellular localization and architectural organization

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Summary. The members of the mitogen-activated protein kinase (MAPK) family are regulated by a diverse array of extracellular cues ranging from cytokines, growth factors and neuropeptides, which activate cell surface receptors, to stresses such as cold, heat, osmolarity changes and irradiation. The MAPK pathways control genetic expression by modifying transcription factor activity and cue important cell fate decisions including survival, proliferation, and programmed cell death (apoptosis). One interesting feature of the MAPK pathways is that the components are evolutionarily conserved from yeast to human, and many of the pathways are similarly organized and regulated. Unlike previously imagined, architectural organization or the multimeric organization of signaling proteins into complexes which are localized to distinct subcellular regions is an important mechanism that influences the regulation of these pathways. In addition, extracellular stimuli can induce relocalization of specific signal transduction proteins. The formation of multimeric signaling complexes, as well as the dynamic movement of signaling proteins, contribute to determine signaling specificity and efficacy. This review describes what is currently known about the subcellular localization of MAPK pathway signaling proteins and the relocalization that occurs during events associated with activation of the MAPK family members.

Key words: Apoptosis, Transcription, Kinase, Localization, Signaling

Introduction

As cell biologists and biochemists who study intracellular signal transduction mechanisms are beginning to realize, the architectural organization of signaling molecules is an important regulatory component of intracellular signal transduction pathways. Architectural organization refers to the subcellular localization and multimeric complex formation of signal transduction molecules. Unlike previously imagined, intracellular signal transduction molecules are localized to distinct regions of the cell and organized into specific multimeric complexes of signaling proteins (Alberts, 1998). In addition, signaling molecules appear to be directed to specific cellular locations following certain extracellular stimulations. The appropriate localization and organization is important for signal transduction proteins to influence the enzymatic properties of other proteins. Since proteins are organized into distinct multimeric signaling complexes which have certain positional cues, if two molecules are not in proximity to one another then they have no ability to directly influence enzymatic activity. Thus, multimeric organization and cellular localization provide an important regulatory mechanism that contributes to signaling efficacy and specificity. This is best illustrated by the serine/threonine kinases protein kinase A (PKA) and protein kinase C (PKC) in which adaptor proteins such as AKAPs (A kinase adaptor proteins) and RACKs (receptors for activated C kinase) mediate dramatic substrate specificity simply by controlling subcellular localization and protein-protein interactions (Faux and Scott, 1996; Newton, 1996; Pawson and Scott, 1997; Mochly-Rosen and Gordon, 1998).

Any cell, whether it be yeast or a highly specialized neuron of the central nervous system of the human, is subjected to a wide range of extracellular stimuli which need to be detected, interpreted and to effect cellular changes accordingly. Disregulation of these pathways can lead to adverse phenotypic consequences. In yeast, if the cell does not detect or is unable to react to adverse conditions such as nutritional and osmolarity changes, it is at a survival disadvantage. In humans, disregulation of certain signaling pathways can lead to tumor formation, autoimmunity and cell death. One way in which biology has evolved to integrate and effect the necessary changes mediated by extracellular stimuli is by modulating the activity of sequential kinase cascades in which activation of one kinase leads to changes in activity of another and then another. Activation of intracellular signal transduction pathways can cause very rapid changes within

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the cell cytoplasm that affect a number of responses such as cytoskeletal changes, alterations in ion channel activity and stability/degradation of preexisting proteins. In addition, activation of signaling pathways can lead to long term cellular changes by influencing genetic expression via modulating transcription factor activity. One family of kinases that responds to extracellular cues and effects very rapid cellular changes, as well as long term changes associated with differences in genetic expression, are the mitogen-activated protein kinase (MAPK) family members (Treisman, 1996).

Extracellular-regulated kinase (ERK), c-jun N-terminal kinase (JNK) and p38, are three of the best characterized MAPK family members. These MAPKs are regulated by a wide range of extracellular stimuli and control a diverse array of biological events (Fig. 1). Other MAPK family members, ERK3, 4 and 5, have been cloned but their regulation and biological roles are not well understood. With regard to regulation of the MAPK family members, kinases known as MAPK kinases (MAPKKs) activate the MAPKs by phosphorylating on tyrosine and threonine residues. There are currently seven different MAPKKs that have been discovered. The first MAPKKs to be cloned were MAPK/ERK kinase 1 and 2 (MEK1/2) which phosphorylate and activate ERK. MKK4 (also referred to as JNK kinase or SEK-1) and MKK7 phosphorylate and activate JNK. MKK3 and 6 specifically phosphorylate and activate p38, whereas MKK5 activates ERK5. The MAPKK family members are activated by a rapidly expanding group of kinases called MAPKK kinases (MAPKKKs), which activate the MAPKKs by phosphorylating on serine and threonine residues. The MAPKKKs include Raf-1, A-Raf, B-Raf, MAPK/ERK kinase kinases 1-4 (MEKK1-4), apoptosis-stimulating kinase-1 (ASK-1), and mixed lineage kinase-3 (MLK-3). Although not well defined, the MAPKKKs may be regulated by tyrosine and/or serine/threonine phosphorylation by MAPKKK kinases (MAPKKKKs). One kinase, p21-activated kinase (PAK), is perhaps the best characterized of the MAPKKKKs. In addition to being regulated by phosphorylation, other factors such as binding to low molecular weight GTP-binding (LMWG) proteins also influence the activity of MAPKKKs and MAPKKKKs. As we are beginning to discover, multimeric organization and subcellular positional cues are critical components that control the specificity and the efficacy of the MAPK signaling pathways.

Localization of low molecular weight GTP-binding proteins

One evolutionarily conserved family of proteins that is important for regulating the MAPK pathways are the low molecular weight GTP-binding (LMWG) proteins. Hydrolysis of guanine triphosphate (GTP) to form guanine diphosphate (GDP) by the intrinsic GTPase activity of the LMWG proteins is a key regulatory event acting as a switch altering the molecular shape of the LMWG protein and the ability to interact with and regulate effector proteins (Satoh et al., 1992). The GDP vs. GTP bound state of the LMWG proteins is influenced by at least three different classes of proteins, GTPase activating proteins (GAPs) which activate intrinsic GTPase activity, guanine nucleotide exchange factors (GEFs) which promote GTP loading by facilitating dissociation of GDP and guanine dissociation inhibitors (GDIs) which prevent GDP dissociation. There are several different families of LMWG proteins, two of which include the Ras (N-Ras, K-Ras and H-Ras) and Rho (Rac1, 2 and 3, Cdc42 and RhoA, B and C) families. Ras activity is required for ERK activation and may play some role in JNK activation (Maruta and Burgess, 1994). Rac and Cdc42 are not important for ERK activation, but are required for JNK activation and in some instances for p38 activation (Bagrodia et al., 1995; Minden et al., 1995). Like Cdc42 and Rac, under some but not all circumstances Rho has been shown to be important for JNK activation (Teramoto et al., 1996).

In mammalian cells, LMWG proteins are specifically targeted to distinct intracellular membrane regions. For example, RhoB is found in late endosomes, whereas highly related family members RhoA and C localize to submembraneous actin networks (Cussac et al., 1996; Seabra, 1998). Rac2 is found at the plasma membrane compared to Cdc42 which has been reported to be associated predominantly with the Golgi apparatus (Erickson et al., 1996; Seabra, 1998). Ras associates with the plasma membrane (Hancock et al., 1990). There are multiple mechanisms that mediate membrane association. One important mediator of LMWG membrane localization is prenylation (Seabra, 1998). The Ras family members are prenylated with farnesyl, whereas the Rho family members Cdc42, Rac and Rho are geranylgeranylated. Prenylation occurs at the extreme C-terminal cysteine residue and localizes LMWG proteins to lipid membranes. Prenylation is critical for appropriate function as prenylation mutants of activated forms of Ras (V^{12}Ras) are no longer oncogenic like normally prenylated V^{12}Ras (Willumsen et al., 1984). In addition to prenylation, other membrane localization motifs also appear to be important for appropriate membrane targeted localization. Just N-terminal to the prenylation site K-Ras contains a stretch of basic residues known as a polybasic region and H-Ras contains a palmitylation site which are both important in mediating membrane localization (Hancock et al., 1990, 1991). In addition to these mechanisms, it is likely that protein-protein interactions also play a significant role in mediating appropriate membrane localization of LMWG proteins, and membrane localized GEFs such as Rho GDP dissociation inhibitor (RhoGDI) may assist in stabilization of a membrane associated LMWG complex (Seabra, 1998). Thus, even though they contain no transmembrane spanning sequences, localization of LMWG proteins to specific membrane regions is key for biological activity.

In the yeast *Saccharomyces cerevisiae*, Cdc42 is a

component of the pheromone response pathway that induces mitotic growth known as budding in a polarized fashion (Leberer et al., 1997). The mechanism by which polarized growth is regulated is not well characterized. However, numerous proteins localize to the bud site, one of which is Cdc42. It was recently demonstrated that actin-mediated pheromone receptor clustering is required for bud formation which suggests that receptor clustering may consolidate Cdc42-mediated signaling to a specified region of the cell (Ayscough and Drubin, 1998). Subcellular localization of signaling proteins dictates the spatial cues for bud emergence and polarized cell growth in yeast. Thus, receptor clustering and preferential localization of signaling proteins including Cdc42 likely dictates the spatial cues of morphogenesis.

Localization of MAPKKKKs and MAPKKKs is a critical regulatory component

One of the best characterized families of effector kinases that associates with and is regulated by LMWG proteins is Raf (Raf-1, A-Raf and B-Raf; Seger and Krebs, 1995). The MAPKKK Raf is a serine/threonine kinase which binds to GTP-loaded Ras; this interaction is required for Raf activation. Raf then phosphorylates MEK1/2 which in turn activates ERK. Typically Raf localizes to the cytoplasm of the cells. However, Raf rapidly translocates to the peripheral membrane when Ras is activated by stimuli such as epidermal growth factor (EGF) (Marais et al., 1995). Translocation to the peripheral membrane is critical and perhaps sufficient for Raf activation since Raf that is modified with a CAAX box which mediates association with the peripheral membrane is constitutively active (Leevers et al., 1994; Stokoe et al., 1994).

PAK is a serine/threonine kinase that has been characterized as a MAPKKKK based upon the role of the yeast *S. cerevisiae* homologue Sterile20 (Ste20) and its inability to phosphorylate specific MAPKKs. Unlike Raf, PAK does not associate with Ras, but binds to and is activated by the GTP-bound forms of Cdc42 and Rac and under some circumstances is important for JNK activation (Bagrodia et al., 1995; Brown et al., 1996). PAK is localized to submembraneous vesicles in resting cells, and following stimulation with platelet-derived growth factor (PDGF) a fraction of PAK translocates to the peripheral membrane and more specifically to membrane ruffles (Dharmawardhane et al., 1997). PAK translocation to the peripheral membrane may be important in mediating morphological changes since overexpression of PAK elicits actin restructuring and morphological changes (Manser et al., 1997; Sells et al., 1997). However, PAK interacts with Nck, a Src homology 2 (SH2) containing protein which associates with proteins containing the appropriate phosphotyrosine motif. Nck mediates PAK association with the PDGF receptor, an important event required for PAK activation (Bokoch et al., 1996; Lu et al., 1996). Thus, translocation of PAK to the peripheral membrane may also be important for activation. Consistent with these two hypotheses, expression of a membrane localized form of PAK by addition of a CAAX box motif results in both constitutive activation of PAK and neurite outgrowth from PC12 cells (Daniels et al., 1998). Thus, intracellular redistribution of PAK to the peripheral membrane may be important for activation, as well as its phenotypic responses associated with morphological changes.

Intracellular localization is also important in regulating the activity of the MEKKs, a family of serine/threonine kinases which can phosphorylate MKK3 and MEK1/2 and so have been categorized as MAPKKKs. The four family members, MEKK1-4, display high homology within the C-terminal kinase domains and divergence in the N-terminal regulatory domains (Fanger et al., 1997a). The MEKK family members also display distinctly different subcellular localizations with MEKK1 localized to the cell nucleus and in vesicular-like structures in the cell cytoplasm, MEKK2 in the Golgi apparatus and cytoplasmic vesicular-like structures and MEKK4 in the Golgi (Fanger et al., 1997b). The differential localization of the MEKK family members suggests that these kinases are differentially regulated and may play different biological roles. In support of this hypothesis, overexpression of MEKK1, but not MEKK2, 3 and 4 will induce apoptosis (Xia et al., 1995; Lassignal-Johnson et al., 1996).

Of the MEKK family members, the regulation and biological role of MEKK1 is best characterized, and subcellular localization is a critical determinant of biological activity (Fig. 2). Full length 196 kDa MEKK1 is cleaved by caspases, a family of proteases which are required for apoptosis, to form a 91 kDa fragment which contains the kinase domain and a 105 kDa N-terminal form (Fig. 2A; Cardone et al., 1997; Widmann et al., 1998). Only the 91 kDa form and not the 196 kDa form of MEKK1 is proapoptotic. Caspase-mediated cleavage

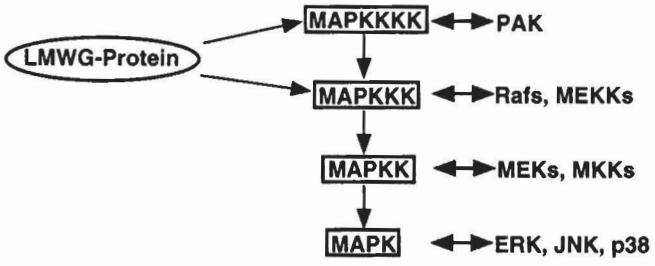

Fig. 1. Regulation of sequential kinase pathways that control MAPK *activity*. MAPKs such as ERK, JNK and p38 are activated by tyrosine and threonine phosphorylation by the MAPKKs, MEKs and MKKs. MAPKKs are activated by serine and threonine phosphorylation by the MAPKKKs such as the Rafs and MEKKs. MAPKKKK such as PAK are believed to regulate MAPKKKs. MAPKKKKs and MAPKKKs are regulated by LMWG proteins which directly associate with these kinases. MAPK: mitogen-activated protein kinase; MAPKK: MAPK kinase; MAPKKK: MAPKK kinase; MAPKKKK: MAPKKK kinase; LMWG protein: low molecular weight GTP binding protein; PAK: p21-activated kinase; MEK: MAPK/ERK kinase; MEKK: MEK kinase; ERK: extracellular-regulated kinase; JNK: c-Jun N-terminal kinase.

of MEKK1 potentiates apoptosis by a currently ill-defined mechanism which may include upregulation of Fas ligand and activation of "death receptors" (Faris et al., 1998). The 196 kDa full length form of MEKK1 is membrane associated and following caspase activation, the 91 kDa cleavage product localizes to the soluble fraction of the cytoplasm and is no longer tethered to membrane (Fig. 2B; Deak et al., 1998). Tethering the 91 kDa form of MEKK1 to the cell membrane via a CAAX box modification prevents MEKK1-mediated apoptosis (Fig. 2C; Schlesinger et al., 1999). It is possible that a putative pleckstrin homology (PH) domain found at the N-terminus of 196 kDa MEKK1 may mediate membrane tethering as it does for the guanine nucleotide exchange factor son of sevenless (SOS) (Jiang et al., 1996). PH domains associate with polyphosphoinositides and mediate localization to specific regions of the plasma membrane. Another potential mechanism that may mediate membrane tethering are the 14-3-3 proteins which bind to phosphorylated serine motifs and associate with MEKK1 at the extreme N-terminus (Fanger et al., 1998). 14-3-3 proteins mediate protein-protein interactions and may serve as both chaperones and adaptor molecules (Vincenz and Dixit, 1996). Upon caspase cleavage, the kinase domain of MEKK1 no longer binds 14-3-3 protein nor contains the putative pleckstrin homology domain, thus relocalizes to the cytosol and so may interact with a different pool of effectors stimulating apoptotic pathways. Caspases themselves have also been shown to be differentially localized within the cell as caspase-3 is found predominantly in the cytosol and caspase-7 is localized to the mitochondria and to microsomal fractions (Chandler et al., 1998). Thus, appropriate localization is critical for apoptosis and for determining the biological role of MEKK1.

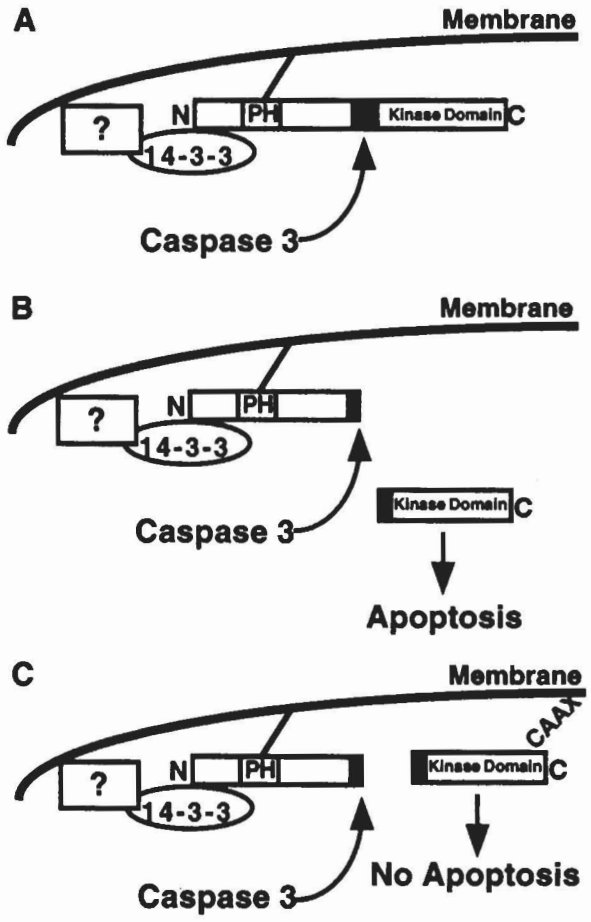

Fig. 2. Role of membrane localization in controlling the apoptotic-inducing effects of MEKK1. This figure describes the mechanism by which MEKK1 contributes to the apoptotic phenotype. **A.** The full length 196 kDa form of MEKK1 is typically associated with the membrane. Membrane association may be mediated by a putative pleckstrin homology (PH) domain located at the N-terminus of MEKK1 or via 14-3-3 proteins which bind to the N-terminus. **B.** Upon activation of caspases, MEKK1 is cleaved into a 105 kDa N-terminal fragment and a 91 kDa C-terminal fragment that contains the kinase domain. The 91 kDa form of MEKK1 is not membrane associated and unlike the membrane associated 196 kDa form is very effective at inducing apoptosis. **C.** When the C-terminus of MEKK1 is modified to contain a CAAX box which mediates membrane localization, MEKK1 is no longer able to induce apoptosis since its localization is restricted to the membrane.

MAPKs translocate to the nucleus following activation

The spatial distributions of the MAPKKs and MAPKs have also been defined. Typically ERK is located in the cytoplasm, as well as in the nucleus. Upon stimulation with growth factor, a fraction of the cytoplasmic ERK translocates to the nucleus (Chen et al., 1992; Lenormand et al., 1993). The regulator of ERK, MEK1, is located in the cytoplasm and does not translocate to the nucleus following stimulation with factors that mediate ERK activation and translocation (Zheng and Guan, 1994). MEK1 is likely restricted to the cytoplasm because of a nuclear export sequence that prevents entry (Fukuda et al., 1996). JNK is also rapidly translocated to the cell nucleus following stimulation. However, like MEK1/2, the MAPKK that activates JNK, MKK4, does not translocate to the nucleus and remains in the cytoplasm. Since MKK4 associates with actin via the adaptor protein actin-binding protein-280 (ABP-280), it is likely restricted to the cytoplasm by this mechanism (Marti et al., 1997). Nuclear translocation of ERK and JNK is important for transcription factor activation, whereas cytosolic activated MAPKs may play a different role such as mediating cytoskeletal rearrangement.

MAPK modules in yeast: a model for architectural organization

The yeast *S. cerevisiae* pheromone mating response pathway provides an ideal example of how MAPK pathways are organized into multimeric signaling protein complexes and how the spatial localization of

these complexes is important for regulation and effector functions. The pheromone response pathway requires the scaffolding protein Ste5 which organizes the MAPK module by binding to Ste11, Ste7 and KSS1/Fus3 and mediating complex formation (Fig. 3A,B; Herskowitz, 1995). In addition to mediating complex formation thereby preventing cross-talk between other MAPK modules, Ste5 has two other important regulatory functions that control the activation state of this MAPK pathway. Ste5 contains a LIM domain that mediates dimerization of Ste5. Dimerization of the Ste11-Ste7-Kss1/Fus3 kinases is sufficient for pathway activation (Inouye et al., 1997). Secondly, the LIM domain of Ste5 also interacts with the Gßγ subunits of the pheromone receptors and thus mediates spatial localization of the Ste11-Ste7-Kss1/Fus3 signaling complex to the plasma membrane where Ste11 is activated by Ste20 (Jiang et al., 1997). Ste20 is localized beneath the plasma membrane via its interaction with Cdc42. Thus, Ste5 provides a mechanism to account for spatial organization and complex formation and ultimately plays a key role in regulation of this MAPK pathway. However, the regulation of other MAPK pathways in yeast does not require Ste5, and no mammalian homologue for Ste5 has been discovered indicating that other mechanisms exist to mediate architectural organization of these MAPK pathways.

Role of Caveolae as organizers of signal transduction complexes in mammalian cells

Caveolae may provide a mechanism to account for spatial localization and complex organization of signaling molecules in mammalian cells. Caveolae are distinct invaginations of the cell surface membrane that represent a microdomain within the plasma membrane (Harder and Simons, 1997). Caveolae are enriched with caveolins, a family of 21-24 kDa proteins. In addition to being responsible for cellular transport processes, caveolae also appear to be important for the organization of signal transduction complexes (Anderson, 1998). A variety of different signal transduction molecules localize to the caveolae region. For example, a number of different cell surface receptors including the tyrosine kinase receptors for insulin, EGF and PDGF, as well as several heterotrimeric G protein couple receptors are concentrated at the caveolae region (Anderson, 1998). Caveolin interacts directly with the LMWG protein Ras (Song et al., 1996). In addition, the kinases Raf, MEK and ERK are also enriched in purified caveolae preparations (Mineo et al., 1996). Like Ste5, caveolae appear to be an architectural organization center for the assembly and spatial localization of signal transduction molecules associated with the regulation of the MAPK pathways.

Architectural organization of signal transduction proteins within caveolae leads to MAPK pathway activation allowing for a cell surface expressed receptor to effect changes in genetic expression. This is perhaps best illustrated by the EGF receptor-mediated activation of ERK. EGF induces receptor dimerization stimulating tyrosine autophosphorylation of the receptor. Phosphorylated tyrosines act as recruitment sites for other proteins that contain SH2 domains which rapidly associate with the EGF receptor (Wandless, 1996). Grb2, an adaptor protein with multiple SH2 domains, associates with the EGF receptor and recruits the guanine nucleotide exchange factor son of sevenless (SOS) to associate with and become activated by the receptor. Ras, already in association with the membrane, is activated by SOS which in turn recruits the serine/threonine kinase Raf to translocate to the plasma membrane promoting activation of this kinase. Raf activates MEK1/2 resulting in activation of ERK which may require localization to caveolae. Activated ERK phosphorylates proteins located in the cytoplasm and

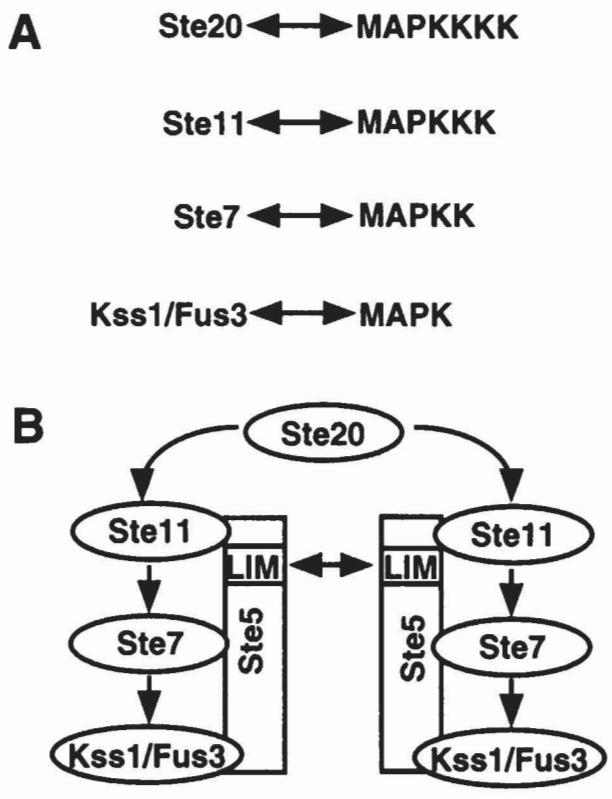

Fig. 3. Organization of the pheromone-regulated MAPK pathway in the yeast *Saccharomyces cerevisiae*. Yeast contain several different MAPK pathways, one of which responds to the pheromone receptor. **A.** Identification of specific yeast kinases as either, MAPKs, MAPKKs, MAPKKKs or MAPKKKKs. **B.** The MAPKs Kss1 and Fus3 are phosphorylated and regulated by the MAPKK, Ste7. Ste7 is phosphorylated and activated by the MAPKKK, Ste11. Ste11, Ste7 and Kss1/Fus3 bind to Ste5 which organizes these kinases into a multimeric signal transduction protein complex. Ste5 contains a LIM domain that mediates dimerization, an important mechanism sufficient for pathway activation. LIM domains of Ste5 also mediate interaction with the Gßγ subunits of the pheromone receptor which localizes the Ste11-Ste7-Kss1/Fus3 complex to plasma membrane making Ste11 susceptible to regulation by the MAPKKKK, Ste20.

following translocation to the nucleus influences the activity of various transcription factors.

Conclusion

Evolution has provided numerous mechanisms to control the activity of signal transduction pathways which include association of guanine nucleotides that affect the activity of LMWG proteins and phosphorylation events typically observed in sequential kinase pathway. One critical regulatory mechanism that is only beginning to be understood is the role of architectural organization of effector proteins in the regulation of these pathways. As discussed in this review, there are a number of mechanisms that have evolved to control the multimeric organization and subcellular localization of signaling complexes including prenylation, pleckstrin homology domains, SH2, SH3 and LIM domains. In addition, proteins such as Nck, Grb2 and 14-3-3 proteins also contribute to conferring specificity of protein-protein interactions and subcellular localization by acting as molecular adaptors and chaperones. With regard to regulation of signaling pathways other than that of the MAPK family members, the *Drosophila* protein InaD which contains five PDZ (post synaptic density protein, disc-large, zo-1) domains plays a general role in organizing multimeric signaling complexes and mediating subcellular localization (Ranganathan and Ross, 1997; Tsunoda et al., 1997). Furthermore, other families of proteins known as the AKAPs (A kinase anchoring proteins) and RACKS (receptors for activated C kinase) are critical for the regulation of protein kinase A (PKA) and protein kinase C (PKC) because they restrict subcellular localization of these kinases and hence access to effector proteins (Mochly-Rosen, 1995; Newton, 1996; Pawson and Scott, 1997; Mochly-Rosen and Gordon, 1998). The role of subcellular localization and architectural organization in regulating the MAPK pathways is still not well defined, yet appears to also be of critical importance.

There are several reasons why subcellular localization and signaling complex formation contribute to controlling the regulation of signal transduction pathways. Restricted movement and access to other proteins increases the organization of signaling pathways within the cellular melieu, as well as contributes to signaling specificity and likely the amplification of certain pathways. Thus, controlling architectural organization allows for a relatively few number of molecules to be incorporated into a diverse array of signaling pathways. It is unnecessary to have a different series of molecules that respond to each and every extracellular cue. The signaling molecules simply need to be organized differently, ready to respond to extracellular stimuli. This is why some proteins mediate the effects of very diverse cellular stimuli such as MEKK1 which is activated by both irradiation and growth factor receptors. Thus, subcellular localization and signal complex formation adds an additional layer of regulation to signaling pathways. With regard to regulation of the MAPK pathways, this additional layer of regulation only adds more complexity to an already complicated biological process.

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