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MINI REVIEW

THE MITOGEN ACTIVATED PROTEIN KINASE SIGNAL TRANSDUCTION PATHWAY: FROM THE CELL SURFACE TO THE NUCLEUS

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Abstract—Activation of the mitogen activated protein kinase (MAPK) plays esential roles in many signal transduction pathways. MAPK has been demonstrated to phosphorylate and regulate numerous cellular proteins, including growth factor receptor, transcription factors, cytoskeletal proteins, phospholipase and other protein kinases. Activation of MAPK requires phosphorylation of both threonine and tyrosine residues, which are catalysed by a single protein kinase known as MAPK kinase or MEK. MEK itself is activated by phosphorylation on two conserved serine residues. Three distinct mammalian Ser/Thr kinases, including Raf, Mos and MEKK (for MEK kinase), have been demonstrated to phosphorylate and activate MEK. The MAP kinase cascade is highly conserved in all eukaryotes and involved in numerous cellular responses. Activation of MAPK is a transient event that is tightly regulated by both kinases and phosphatases. A growth factor induced dual specific phosphatase is likely to play an important role in MAPK regulation.

Key words: Kinase; phosphatase; MEK; MAPK; ERK.

Phosphorylation of cellular proteins plays an important role in the control of cell growth and differentiation induced by growth factors and oncogenes. Numerous serine/threonine kinases have been identified that may catalyse these reactions. One of the most promising candidates discovered is the family of enzymes known as mitogen activated protein kinase (MAPK) or extracellular signal regulated kinase (ERK) [1–4]. Activation of MAPK is a common event in many signal transduction pathways. The kinase cascade leading to MAPK activation is highly conserved through evolution and has been found in yeast, *C. elegans*, *Drosophila*, and mammals [1–7]. The activity of MAPK is acutely stimulated by virtually every mitogenic stimulus, including growth factors with tyrosine kinase receptors, cytokines, T cell antigens, hormones that bind to G protein coupled receptors, and phorbol esters, suggesting that this pathway is likely to represent a site of integration for common signalling mechanism in cellular regulation [1–5].

THE PATHWAY

Ray and Sturgill initially discovered that insulin stimulated a rapid activation of a cellular serine/threonine protein kinase which is widely

Abbreviations: ERK—extracellular signal regulated kinase; GAP—GTPase activating protein; MAPK—mitogen activated protein kinase; MEK—MAP kinase kinase.

known as MAPK or ERK [8]. In fact, the same kinase was activated by many extracellular signals in addition to insulin [2]. This kinase activity itself is regulated by phosphorylation. Anderson *et al.* demonstrated that MAPK activation requires both tyrosine and threonine phosphorylation [9]. These observations led to the initial excitement that activation of MAPK may integrate multiple signalling events involving activation of tyrosine and serine/threonine kinases. However, subsequent studies demonstrated that MAPK is in fact activated by a single protein kinase, MEK (for MAPK or ERK kinase), which activates MAPK by phosphorylation of both threonine and tyrosine [10–13].

Recent studies of the mitogenic growth factor signal transduction and ras function have elucidated a linear pathway from ligand binding to MAPK activation and alteration of cellular activities (Fig. 1). The best understood system is the pathway involving the mitogenic growth factors. Receptors for growth factors have intrinsic tyrosine kinase activity which is activated by binding of ligands [14]. In the case of epidermal growth factor receptors, EGF causes a dimerization and tyrosine autophosphorylation of its receptor [14]. The autophosphorylated EGF receptor then recruits several signalling molecules which bind to the growth factor receptor *via* their SH2 domains. SH2 domain is a structural motif frequently found in signalling molecules and specifically recognizes phosphotyrosine and neighbouring sequences. One of the associating molecules is a protein called Grb2 (for growth factor receptor binding protein 2) which binds to the receptor in a ligand dependent manner [15]. Grb2 not only contains a SH2 domain but also contains two SH3 domains. Experiments from several laboratories have demonstrated that the SH3 domains of Grb2 directly associate with a proline rich region in the Sos molecule which is a guanidine nucleiotide exchange protein [15–22]. Sos was initially identified in *Drosophila* to be downstream of the Sevenless receptor tyrosine kinase and upstream of ras gene in eye development [22–25]. Sos can facilitate the conversion of Ras-GDP, an inactive form of Ras, to Ras-GTP which is the active form of Ras. Genetic evidence from *C. elegans* shows that the sem-5 gene product (a Grb2 homolog) plays essential roles in the vulva induction and sex myoblast migration [26]. The vulva development in *C. elegans* is regulated by the let-23 receptor tyrosine kinase. Therefore, both genetic and biochemical studies support that Grb2 and Sos function between the tyrosine kinase and Ras protein. The discovery of Grb2 and Sos and their interaction with tyrosine kinase receptors and Ras made the direct connection of growth factor binding and activation of Ras [27].

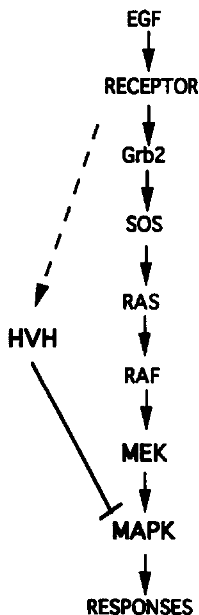

Fig. 1. Signal transduction pathway of mitogentic growth factors and MAPK.

This basic scheme is employed by other growth factor receptors although a slightly altered signal transduction pathway is used for each specific growth factor. For example, IRS1 (insulin receptor substrate 1) serves as a molecular docking adaptor rather than the tyrosine kinase receptor itself during insulin stimulation [28]. Furthermore, Shc2, a SH2 and SH3 containing molecule, appears to mediate the initial interaction with IRS1 instead of Grb2 [29]. Other SH2 containing molecules such as SYP, a SH2 containing

tyrosine phosphatase, can directly associate with autophosphorylated EGF receptor then lead to the association of Grb2 and activation of Ras [30]. Nck is another SH2 and SH3 containing molecular adaptor which binds to autophosphorylated PDGF receptor [31].

The Ras protein has been known for a long time to be a molecular switch regulating cell growth [32]. However, the connection between Ras activation and cell proliferation is not clear although the GTPase activating protein (GAP) has been suggested as a putative downstream effector molecule of Ras [33]. A significant breakthrough came from the study of Ras and Raf interaction. Moodie *et al.* reported that the immobilized Ras-GTP can serve as an affinity ligand to purify Raf protein [34]. These data provided the first evidence that Ras and Raf might interact with each other. Similar experiments have been performed in yeast using the two-hybrid system, and have shown that Raf and Ras can associate *in vivo* [35–37]. Both biochemical and genetic experiments have demonstrated that the Ras-GTP but not Ras-GDP interacts with Raf either directly or indirectly. It is the N-terminal domain of Raf required for interaction with Ras. Furthermore, Raf has been demonstrated to directly phosphorylate and activate the MAP kinase kinase (MEK) [38–40]. These observations made a direct connection from ras to the MAPK kinase cascade. However, this signal transduction pathway is far from complete. The most serious defect of this scheme is that no direct evidence is available to demonstrate that association of Raf with Ras-GTP activates the Raf kinase activity. Therefore, the significance of Ras-Raf association in Raf activation remains to be elucidated.

How the signal crosses the nuclear envelope is not fully understood. However, MAPK is likely to play an important role in this process. MAPK can phosphorylate and activate the $p90^{rsk}$ which can translocate into cell nucleus upon mitogen stimulation [41]. Several MAPK substrates are nuclear transcription factors, including $p60^{TCF}$, c-jun and c-myc [42–44]. MAPK translocates into the nucleus when cells are stimulated by mitogens [41]. Therefore, the nuclear translocation of MAPK is consistent with a role in signal transduction from the cytoplasm to the nucleus.

MAPK can be activated by numerous extracellular signals. Activation of MAPK has been observed with hormone binding to trimeric G-protein coupled receptors [45]. Stimulation of protein kinase C by phorbol ester also leads to activation of MAPK [46]. Signal transduction of trimeric G-protein coupled or PKC dependent MAPK activation is not as well understood as the growth factor response. In the budding yeast *S. cerevisiae*, the mating pheromone response pathway is similar to the mammalian MAPK pathway [5]. The yeast pheromone receptor is coupled to trimeric G-protein. Although how the activation of trimeric G-protein leads to MAPK activation is not completely understood, interestingly, many signalling molecules in the yeast pheromone response and mammalian mitogenic growth factor response are highly conserved. For example, highly related structural homologues of MAPK, MEK and MEKK (for MEK kinase) are also found in the mating pathway as *FUS3/KSS1*, *STE7* and *STE11*, respectively [5]. Genetic studies in yeast also show that multiple pathways using distinct MAPK, MEK isozymes operate in the cell (see review by Errede and Levine, ref. 5). These observations clearly suggest that the MAPK cascade is a multidimensional pathway involved in numerous cellular responses and is highly conserved through evolution.

MEK ACTIVATORS

Raf is a serine/threonine kinase which is activated upon growth factor stimulation. In raf transformed cells, the MAPK pathway was found to be constitutively activated, suggesting that raf is upstream of MEK [38–40]. Furthermore, direct biochemical evidence showed that immunoprecipitated Raf can activate MEK *in vitro* [38–40]. C-Raf as a direct activator of MEK was further proved by the observation that purified Raf can phosphorylate and activate the purified MEK [47]. Activation of MEK and MAPK can be reconstituted *in vitro* by purified recombinant proteins [47]. These experiments established that Raf is a direct

activator of MEK. However, in PC12 cells, over expression of Raf is not sufficient to cause an activation of MEK and MAPK while expression of Ras was sufficient to induce a MEK and MAPK activation [48]. These data indicated that Raf may not be the only activator for MEK.

Cooper and colleagues have shown that immunoprecipitated c-Mos, a serine/threonine kinase, can activate MEK *in vitro* [49]. However, the c-Mos protein was exclusively found in early embryonic tissues and germ cells. Therefore, mos may be important for MEK activation in restricted cell types, but unlikely to play a major role in MEK activation in general. The yeast gene *STE11*, which encodes for a putative protein kinase, has been genetically demonstrated to function upstream of the *STE7* gene which encodes for a MEK homologue in the pheromone response pathway [50]. In an effort to search for *STE11* homologue in high eukaryotes, Johnson and colleagues isolated a *STE11* related kinase from mouse, and named it as MEKK for MEK kinase [51]. MEKK has been over expressed in cultured cells and known to cause MEK phosphorylation and activation. MEKK was initially suggested to mediate the trimeric G-protein coupled MEK activation due to its analogy to the yeast mating pheromone response. However, recent data have indicated that it is Ras and Raf which are activated in the muscurinic receptor induced MAPK activation [52]. Therefore, the trimeric G-protein coupled MEK/MAPK activation is also mediated at least in part by Ras and Raf which are certainly important for receptor tyrosine kinase induced MEK/MAPK activation. The role of MEKK in MEK activation is still an unsolved puzzle. At least three pathways activating MEK/MAPK homologues in yeast [5]. These include the pheromone response pathway, and pathways involved protein kinase C and osmotic regulation. It is likely that multiple MEK activators/kinases exist in higher eukaryotes.

MAPK SUBSTRATES

Analysis of the substrate specificity of MAPK using synthetic peptides demonstrates that P-X-S/T-P (P for proline, X for any amino acid, S/T for the phosphorylation residue serine or threonine) represents the optimal sequence for MAPK phosphorylation although the first proline in this motif is not absolutely required [53, 54]. MAPK phosphorylates and regulates numerous cellular signalling molecules. These include cell surface proteins, cytoskeletal components, cytoplasmic kinases, and nuclear transcription factors. MAPK phosphorylates EGF receptor and possibly decreases the sensitivity of the receptor to EGF [55]. Phosphorylation of phospholipase A2 by MAPK increases its activity, therefore enhances the production of second message [56]. Substrates of MAPK also include cytoskeletal proteins. In the case of *Xenopus* oocytes, MAPK can cause a dramatic morphological change of cytoskeletal [57]. Perhaps the most interesting and extensively studied MAPK substrates are nuclear transcription factors, including c-jun, c-myc, $p62^{TCF}$ and ATF2 [42–44, 58]. Available evidence suggests that these transcription factors can be phosphorylated by MAPK and their transcription activities are regulated by these phosphorylations. Therefore, one of the prominent effects of MAPK activation is transcriptional activation of numerous cellular genes essential for growth. Protein kinase $p90^{rsk}$ is clearly one of the physiological substrates of MAPK [59]. Phosphorylation of $p90^{rsk}$ by MAPK is responsible for its activation and has an important role in signal transduction. MAPKAP kinase (for MAPK activated protein kinase) is another serine/threonine protein kinase whose activation requires the phosphorylation by MAPK [60]. Furthermore, other kinases, including MEK [61] and c-Raf [62] which function upstream of MAPK, are also phosphorylated by MAPK although the physiological significance of these phosphorylations is yet to be determined. However, it is certain that the list of MAPK substrates is likely to be increased as the research in this area progresses. The identification of important cellular signalling molecules as the substrates of MAPK supports the idea that activation of MAPK plays a central role in regulation of cell growth and differentiation.

BIOCHEMICAL MECHANISMS OF MEK AND MAPK ACTIVATION

One of the most interesting features of MAPK activation is that phosphorylations of both threonine and tyrosine residues are required. The MAPK can be completely inactivated by dephosphorylation with either serine/threonine specific or tyrosine specific phosphatase [9, 63]. Sturgill and colleagues identified that threonine 183 and tyrosine 185 in p42mapk are the activation phosphorylation sites (Fig. 2) [64, 65]. Mutation of either threonine 183 or tyrosine 185 using recombinant p42 mapk unequivocally demonstrated that phosphorylation of both residue is absolutely required for its activation [66].

The requirement for both threonine and tyrosine phosphorylation for MAPK activation initially suggested a very intriguing possibility that MAPK may represent an integration point of signal transduction where serine/threonine kinase pathway and the tyrosine kinase pathway are merged. However, purification and characterization of the MAPK kinase demonstrated that the threonine 183 and tyrosine 185 in MAPK were phosphorylated by a single kinase, MEK. Therefore, MEK is a dual specific kinase which can efficiently catalyse the phosphorylation of specific threonine and tyrosine in MAPK. MEK is an extremely specific kinase which shows a very high substrate selectivity toward MAPK [67]. Although multiple isozymes of MEK exist, MAPK is the only identified physiological substrate of MEK. Similarly, MEK is the only activator of MAPK. This unique relationship between MAPK and MEK suggests that MEK is a point of signal integration from different pathways such as the activation of Raf, Mos and MEKK (Fig. 2). MAPK elicits cellular responses by phosphorylation of a wide variety of target proteins. The kinase activity of MAPK can be activated by MEK as much as 1000 fold using purified recombinant proteins [66]. The tyrosine 185 is also a major autophosphorylation site of MAPK although autophosphorylation is insufficient to activate the kinase fully.

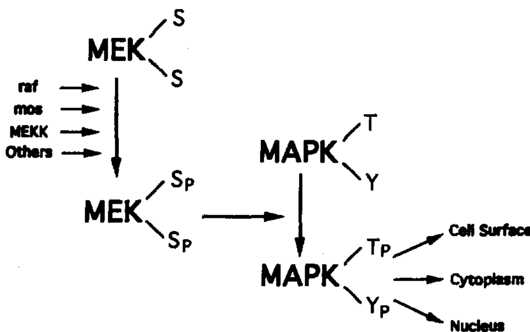

Fig. 2. Activation of MEK and MAPK.

The observation that MEK is the sole protein kinase which is responsible for MAPK activation generates an intense interest in MEK regulation. MEK activity was rapidly activated by growth factor and its activity declined rapidly afterwards. MEK activation was found to be associated with serine and maybe threonine phosphorylation. Two serine residues (Ser218 and 222) of human MEK1 are the phosphorylation sites by Raf [68]. Mutation of either residue completely eliminates the Raf-dependent MEK activation, suggesting that phosphorylations of both residues are essential for activation. These two residues are absolutely conserved in all members of the MEK family including the yeast MEK homologues. Mutation of corresponding residues in yeast *STE7* showed that both residues are essential for the biological function of *STE7* in the mating pheromone response. Other MEK activators, such as MEKK and Mos, are likely to employ the same mechanism in MEK activation although direct biochemical evidence is not available.

It is worth noting that threonine 183 and tyrosine 185 of MAPK and serine residues 218 and 222 of MEK are located between the kinase subdomain VII and VIII. Phosphorylation of residues in this region are also important for kinase activity of PKA [69], cdc2 [70], and p90rsk [71]. Crystal graphic structure of MAPK has recently been solved [72]. The three dimensional structure of MAPK predicts that tyrosine 185 in the unphosphorylated MAPK is located at a position which blocks the binding of peptide substrates [72]. Phosphorylation of this tyrosine residue and threonine 183 will likely induce a dramatic con-

formational change and allow the enzyme to bind substrates. One of the striking features for MAPK and MEK activation is the requirement of phosphorylation of two residues by a single kinase. In contrast, activation of PKA, cdc2, and p90rsk only requires phosphorylation of a single residue between subdomain VII and VIII. The activation pathway of MEK/MAPK is one of the central signal transduction pathways in the cell. This kinase cascade must be tightly and precisely regulated. The requirement for phosphorylation of two residues is clearly beneficial to guarantee a high specificity and to avoid activation by nonspecific phosphorylation.

PHOSPHATASE AND INACTIVATOR

The activation of MAPK is followed by a rapid deactivation in EGF-stimulated Swiss3T3 cells. Western blotting with anti-MAPK antibody demonstrated that the protein level of MAPK is not changed significantly during this process, suggesting that the MAPK is inactivated by post translational modification [73]. The activated MAPK displays a retarded electrophoretic mobility shift on SDS-PAGE. The mobility shift disappeared when MAPK activity declined after growth factor stimulation. This evidence suggests that MAPK is inactivated by dephosphorylation.

Treatment of cells with okadaic acid in adipocyte causes an activation of MAPK suggesting a role of protein phosphatase 2A (PP2A) in this kinase cascade [74]. However, it is unclear whether PP2A directly dephosphorylates MAPK or indirectly regulates kinases upstream of MAPK. The okadaic acid induced MAPK activation is not observed in other cell types, indicating that PP2A may not be a general MAPK inactivator. The best candidate for MAPK phosphatase is a mitogen induced dual specific phosphatase HVH1 [75] which is also known as CL100, 3CH134 or MKP [76–78].

HVH1 is an immediate early gene whose mRNA is induced by serum, growth factors and phorbol ester [76]. The HVH1 mRNA reaches a maximum approximately 30 min after stimulation and rapidly declines thereafter. The HVH1 protein is also very unstable and shows an induction pattern similar to its mRNA. Biochemical characterization of recombinant HVH1 protein shows that it specifically dephosphorylates the tyrosine and threonine residues of the activated MAPK [75–78]. In fact HVH1 dephosphorylates MAPK on the same residues which are recognized by MEK. Furthermore, the HVH1 phosphatase displays a remarkable substrate specificity toward the activated MAPK and shows little activity toward other phosphoproteins [75]. Further evidence supporting the role of HVH1 phosphatase in MAPK kinase regulation was obtained by Sun *et al.* [78]. The HVH1 phosphatase can be co-immunoprecipitated with MAPK in a growth factor dependent fashion. This co-immunoprecipitation has been substantiated by the *in vitro* association of recombinant HVH1 and MAPK. A HVH1 related protein phosphatase, PAC1, has been isolated as an immediate early gene in B-lymphocyte activation and can also inactivate MAPK [79, 80]. PAC1 was found to be localized into the nucleus. The nuclear localization of PAC1 is particularly interesting because the activated MAPK is also accumulated in the cell nucleus.

A VH1 related phosphatase gene, *MSG5*, has been isolated in *S. cerevisiae* to suppress the pheromone response pathway [81]. The *MSG5* mRNA is induced by addition of mating pheromone. Furthermore, the *MSG5* gene product can dephosphorylate and inactivate the *FUS3* kinase. Both genetic data in yeast and biochemical data from mammalian cells show that the MAPK pathway is regulated by the balanced actions of specific kinases and phosphatases.

CONCLUDING REMARKS

The MAPK activation pathway is one of the central schemes in signal transduction. A linear pathway from the cell surface to cytoplasmic and nuclear events has just emerged (Fig. 1). Despite rapid progress in the last few years, numerous important questions remain to be clarified. It is unclear how Raf is activated although the association of Ras and Raf is likely to be an important event. MAPK is activated in mitogenic growth

factors as well as in factors which induce differentiation. For example, both EGF and NGF induce MAPK activation in PC12 cells, yet EGF causes proliferation while NGF causes differentiation. Therefore the role of MAPK activation in these apparent opposite effects in the cells is obscure. Several different MEK activators, including Raf, Mos and MEKK, have been discovered. The relationship between different MEK activators and different extracellular stimuli are not completely resolved. Furthermore, both MAPK and MEK are multiple enzyme families, and the physiological function of each isozyme in signal transduction is not understood. We know very little about the inactivation of this kinase cascade, especially in inactivation of MEK and Raf. Study to identify the negative regulators of the MAPK pathway will likely yield fruitful results in the regulation of this kinase cascade.

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