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The MAP kinase cascade. Discovery of a new signal transduction pathway

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Abstract

Using biochemical techniques similar to those used by Krebs and Fischer in elucidating the cAMP kinase cascade, a protein kinase cascade has been found that represents a new pathway for signal transduction. This pathway is activated in almost all cells that have been examined by many different growth and differentiation factors, suggesting control of different cell responses. At this writing, four tiers of growth factor regulated kinases, each tier represented by more than one enzyme, have been reconstituted *in vitro* to form the MAP kinase cascade. Preliminary findings suggesting multiple feedback or feedforward regulation of several components in the cascade predict higher complexity than a simple linear pathway. (Mol Cell Biochem **127/128:** 201–209, 1993)

Key words: mitogen activated protein kinase, kinase cascade, growth factor, protein phosphorylation, signal transduction

Introduction and background

It is well established that the response of cells to hormones and growth factors usually involves protein phosphorylation at one or more stages in the signal transduction pathway. The prototypical example of this was shown by the work of Krebs and Fischer who demonstrated the role of cAMP-dependent protein kinase and phosphorylase kinase in the regulation of glycogen phosphorylase. This was the first of many cases illustrating how protein phosphorylation follows the release of second messengers within the cell, which activate messenger-dependent protein kinases. Among these are cAMP-dependent protein kinase, cGMP-dependent protein kinase, protein kinase C, and several Ca^{+2}, calmodulin-dependent protein kinases (reviewed in [1]). Another major route of protein phosphorylation involves protein kinases that are second messenger-independent, but somehow still activated by extracellular stimuli. In recent years, a new signal transduction pathway has been identified, in which several second messenger-independent protein kinases form a protein kinase cascade, in which each enzyme is activated by protein phosphorylation during the response of cells to growth and differentiation factors. This pathway, which will be referred to as the 'MAP kinase cascade', is the subject of this review.

Early evidence for growth factor regulated messenger independent kinases emerged from studies on insulin signalling. Changes in activity of several insulin-regulated metabolic enzymes could be accounted for by alterations in their state of serine/threonine phosphorylation (reviewed in [2]). Thus, following insulin binding, regulation of serine/threonine kinases or phosphatases by the insulin receptor tyrosine kinase was indicated, suggesting a mechanism of communication between protein tyrosine and protein serine/threonine phosphorylation.

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Ribosomal protein S6, a constituent of 40S ribosomes, is a prominent intracellular target for phosphorylation. Serine phosphorylation of S6 increases in response to insulin as well as a number of other growth factors and mitogens [3–6]. This observation led to the characterization of ribosomal S6 kinase, the first kinase activity to be described that was growth factor regulated, and second messenger independent, suggesting a mode of regulation involving covalent modification [7–10]. Importantly, sensitivity of S6 kinase to dephosphorylation was suggested by the stabilizing effect of protein phosphatase inhibitors added to extraction buffers [7]. Direct deactivation of S6 kinase by protein phosphatases was then confirmed [11], thus it was expected that S6 kinase would be regulated by a kinase cascade mechanism, in analogy to the cAMP-dependent activation of phosphorylase kinase.

Since the studies on S6 kinase were initiated, a dozen or more growth factor regulated kinase activities have been reported. Most, if not all, have been characterized by using techniques developed for analysis of S6 kinase, in which cultured cells treated with growth factors, or tissues of live animals injected with insulin or hepatectomized to induce liver regeneration are extracted in the presence of phosphatase inhibitors. Kinase activity is then assayed by measuring the phosphorylation of an exogenous protein or peptide substrate added to the extract. Like S6 kinase, many of these enzymes are sensitive to inactivation by protein phosphatases.

Cumulative evidence demonstrates that three kinases characterized in this manner, namely pp90 ribosomal S6 kinase, MAP kinase and MAP kinase kinase, are ubiquitously found in cells, and are stimulated in response to wide variety of reagents, including growth factors whose receptors are tyrosine kinases, mitogens whose receptors couple to heterotrimeric G proteins, tumor promoters, interleukins, and agents that induce oocyte maturation. To date, the pathway comprised of these enzymes provides one of the most thoroughly investigated examples of a mitogen-activated protein kinase cascade. Our understanding of this pathway has mostly been obtained using a biochemical approach of identifying a downstream target of phosphorylation, and then performing *in vitro* reconstitution experiments to identify each successive upstream component. This approach of working backwards has been modeled on that first used by Krebs and Fischer in elucidating the kinase cascade involved in the regulation of glycogen phosphorylase.

pp90 and pp70 ribosomal S6 kinases

Two types of mitogen-stimulated ribosomal S6 kinase have been identified, which are both found in most cell types, and are activated by many of the same stimuli (reviewed in [12]). A 70kDa form ("pp70^{rsk}") has been purified from rat liver [13] and from 3T3 cells [14], while two isoforms of a 90kDa enzyme ('S6 kinase I and II', also named 'pp90^{rsk}'), have been purified from unfertilized eggs of *Xenopus laevis* [9, 15]. A mammalian form of pp90^{rsk} has been purified from rabbit liver [16]. Although both forms recognize all five of the physiological serine phosphorylation sites at the C terminus of ribosomal protein S6 [14, 17], they are distinguishable based on their chromatographic properties and antibody crossreactivity. Molecular cloning has confirmed that pp90^{rsk} and pp70^{rsk} are unrelated, although they share ~60% sequence identity within their catalytic domains [18–20]. Interestingly, the sequence of pp90^{rsk} predicts a tandem arrangement of two catalytic domains, one which shows strong similarity to protein kinase C, cGMP dependent protein kinase, and the catalytic subunit of cAMP-dependent protein kinase, whereas the other is more closely related to the catalytic subunit of phosphorylase kinase [18]. The contribution of each catalytic domain to kinase activity is currently unknown.

The activities of the different forms of S6 kinase can be distinguished by selective immunoprecipitation from extracts, followed by phosphorylation of 40S ribosomes [21], or by the use of peptide substrates. The latter assay is based on the recognition by pp70^{rsk} of a block of four basic amino acids (KRRR) preceding the phosphorylatable residue, so that an octapeptide (RRLSSLRA), based on the first two of the five phosphorylation sites in S6, serves as a good substrate for the pp90^{rsk} but is not as effective in the detection of pp70^{rsk} [22, 23]. Using these assays, pp90^{rsk} activation was found to be transient, peaking at about five minutes and decaying within one hour, while activation of pp70^{rsk} was sustained over 1–2 hours [21, 24].

Both pp70^{rsk} and pp90^{rsk} can be inactivated by protein phosphatases, including serine/threonine protein phosphatases 1 and 2A, suggesting that they are activated by serine/threonine phosphorylation [11, 15, 25]. This has been confirmed by metabolic ^{32}P-labelling of the enzymes following growth factor treatment of cells [26, 27]. However, the enzymes appear to be regulated by separate pathways, as discussed below.

MAP kinase

MAP kinase was first reported in insulin-stimulated 3T3-L1 adipocytes and in EGF-stimulated fibroblasts as an activity that phosphorylated microtubule-associated protein-2 [28, 29]. Other laboratories reported a growth factor-stimulated activity that phosphorylated myelin basic protein (MBP kinase) [24, 30, 31]; this activity was found to be identical to MAP kinase. MAP kinase activity is activated in response to a wide range of stimuli, including insulin, epidermal growth factor, platelet-derived growth factor, nerve growth factor, serum, phorbol esters, nicotine, okadaic acid, growth hormone, hormones that induce oocyte maturation, and T cell activation (reviewed in [32]).

Cloning of MAP kinase revealed several homologous forms of the MAP kinase, which have been named ERKs, for 'extracellular signal regulated kinases' [33, 34]. Two isoforms, ERK1 and ERK2 (90% identical in sequence; ref. [34]), are identical to two MAP-2 or MBP kinase activities that have similar enzymatic properties but resolve chromatographically [35]. The ERKs are related in sequence to the cdc2 family of protein kinases, which, in addition to the cell cycle dependent kinases, includes the yeast protein kinases FUS3 and KSS1 from *S. cerevisiae,* and Spk1 from *S. pombe,* involved in yeast signal tranduction pathways leading to cell cycle arrest and mating. A third enzyme that has been cloned, ERK3, is 50% identical to ERK1 and ERK2, but shows more restricted tissue expression and substrate specificity [34]. Other isoforms may also exist, based on evidence from antibody crossreactivity [32].

The first evidence for a growth factor-stimulated kinase cascade was the finding that the insulin-stimulated MAP kinase could phosphorylate and activate S6 kinase II (a *Xenopus laevis* form of $pp90^{rsk}$), which had been previously inactivated by protein phosphatase 2A [25]. The activation of $pp90^{rsk}$ by MAP kinases has also been demonstrated by the reactivation of phosphatase-inactivated rabbit liver $pp90^{rsk}$ by MAP kinase purified from fibroblasts [16], and by the activation of several forms of $pp90^{rsk}$ by ERK1 and ERK2, derived from a single source, Swiss 3T3 cells [36]. Importantly, activation of $pp70^{rsk}$ by MAP kinase could not be demonstrated [37], indicating that the $pp90^{rsk}$ and $pp70^{rsk}$ forms of S6 kinase appear to be regulated by divergent pathways after growth factor binding. In support of this model, the immunosuppressant, rapamycin, which inhibits the activation of $pp70^{rsk}$ by growth factors and by interleukin-2, had no effect on MAP kinase or $pp90^{rsk}$ activation [38–40]. Recently, an enzyme named MAPKAP kinase-2, which has a substrate specificity distinct from $pp90^{rsk}$, was shown to be activated *in vitro* by ERK1 or ERK2. This interesting observation suggests that the MAP kinases may represent a branch point in the kinase cascade [41].

That the MAP kinases themselves might be targets for phosphorylation in a kinase cascade was suggested initially by their phosphorylation on threonine and tyrosine residues in intact cells treated with growth factors [42], and by their inactivation by protein phosphatases [24, 25, 29]. In fact, ERK1 and ERK2 were originally observed as phosphoproteins, named pp44 and pp42, respectively, which were phosphorylated on tyrosine residues upon serum, phorbol ester, or growth factor stimulation of intact cultured cells [43, 44]. A key finding was made by Anderson *et al.* [45], who showed that MAP kinase undergoes dephosphorylation on phosphothreonine when treated with the serine/threonine specific protein phosphatase 2A, whereas treatment with the tyrosine-specific phosphatase, CD45, led to specific dephosphorylation of phosphotyrosine residues. In both cases, complete inactivation of MAP kinase activity was observed, leading to the conclusion that both threonine and tyrosine residues must be phosphorylated for the enzyme to be active.

The sites of threonine and tyrosine phosphorylation have both been identified in ERK2, and are located, one residue apart, within subdomain 8 of the consensus kinase sequence [46]. These sites are conserved in ERK1 [34]. Comparison of this sequence with that of the catalytic subunit of cAMP-dependent protein kinase, for which an X-ray structure is known, suggests that these sites are located on a loop that is involved in substrate binding, located near the catalytic cleft [47, 48]. Site directed mutagenesis studies substituting the phosphorylated threonine and tyrosine residues with nonphosphorylatable residues have been initiated in order to understand how conformational changes caused by phosphorylation of residues in this region contribute to catalytic activation [49].

MAP kinase kinase

When the regulation of MAP kinases by tyrosine phosphorylation was first discovered, it was reasonable to predict that these enzymes would be direct substrates for regulation by receptor tyrosine kinases. As it often

happens, the predicted results were supplanted by observed results that turned out to be more interesting. Instead, a growth factor-regulated dual specificity kinase has been identified, which can phosphorylate both of the physiologically relevant residues on MAP kinase, accounting for its complete activation. The enzyme was discovered using inactive MAP kinase derived from unstimulated cells or phosphatase-inactivated MAP kinase as substrates to probe growth factor-treated cells for a factor that could phosphorylate and activate MAP kinase [50, 51]. The factor, variously named MAP kinase activator, MAP kinase kinase, or MAP/ERK kinase (MEK), is also able to activate recombinant forms of MAP kinase [52–58]. Evidence for at least two forms of MAP kinase kinase has been reported [50, 52, 56].

In all cases examined so far, activation of MAP kinase kinase correlates with the activation with MAP kinase. Thus the MAP kinase kinase has been shown to be a target for activation in G protein-coupled and phorbol ester coupled pathways as well as those regulated by receptor tyrosine kinases (reviewed in [59]).

Identification of MAP kinase kinase as a kinase was difficult to establish for two reasons. First, both ERK1 and ERK2 have properties of dual specificity kinases in being able to autophosphorylate on threonine and tyrosine residues [60–62]. Thus the possibility that the activating factor induced an autophosphorylation and autoactivation of MAP kinase could not be eliminated. Second, MAP kinase kinase is very selective for ERK1 and ERK2, so phosphorylation of nonkinase substrates could not be established [50, 52]. Current evidence suggests that MAP kinase kinase is highly selective for its substrate, in contrast to MAPK and $pp90^{rsk}$, which both recognize a larger spectrum of substrates *in vitro*. Heat inactivated MAP kinase or peptides based on the sequence surrounding the MAP kinase phosphorylation sites are not recognized by MAP kinase kinase, suggesting that MAP kinase kinase recognizes higher order structures on its substrate [52]. The issue was resolved in several laboratories by using mutants of ERK2 that were unable to autophosphorylate. These mutants, containing a single amino acid substitution of arginine for lysine-52, were phosphorylated on both threonine and tyrosine residues by MAP kinase kinase, demonstrating that the MAP kinase kinase is indeed a protein kinase [52, 54, 55, 57, 58]. It is thus an example of a dual specificity kinase, unusual among the other dual specificity kinases that have been identified so far, in its ability to phosphorylate *exogenous* substrates on serine/threonine and tyrosine residues [63].

Primary sequence of MAP kinase kinase has been obtained in several laboratories [64–68], and an interesting homology has been noted with several yeast kinases, including STE7 and PBS2 from *S. cerevisiae* and Byr1 and Wis1 from *S. pombe* [69–72]. Although the resemblance in sequence is variable (30–40% identity overall, 60% identity within subdomains VI–X of the consensus kinase catalytic sequence [73]), the match is striking because of the homology noted between the MAP kinases and the yeast enzymes FUS3 and KSS1 from *S. cerevisiae* and Spk1 from *S. pombe*. Recent studies have demonstrated the functional homology between the MAP kinases and the yeast enzymes, by complementing *S. pombe* spk1 mutants using mammalian ERK1 [74].

Like MAP kinase and pp90rsk, MAP kinase kinase is inactivated upon treatment with protein phosphatases, suggesting that its activity is also regulated by protein phosphorylation. Complete inactivation occurred with the serine/threonine phosphatases 1 and 2A [51, 53, 55, 75]. Metabolic labeling studies performed in progesterone treated *Xenopus laevis* oocytes and in growth factor stimulated mammalian cells show that MAP kinase kinase is phosphorylated on serine and threonine residues [75, 76]. Inactivation of mammalian enzyme by phosphatase 2A led to loss of phosphoserine, indicating that serine phosphorylation at least is important for activity [76]. Tyrosine phosphorylation was not found *in situ* and tyrosine phosphatases had no effect on enzyme activity [51], in spite of the fact that MAP kinase kinase autophosphorylates weakly on tyrosine residues *in vitro* [52, 62, 75]. Thus a role for upstream serine/threonine kinases in regulating MAP kinase kinase is strongly suggested. Several enzymes that are candidates for these upstream kinases are discussed below.

Raf-1

The proto-oncogene, Raf-1, which encodes a serine/threonine kinase, has been proposed to function as a direct regulator of MAP kinase kinase [77–79]. In NIH3T3 cells transformed with the v-raf oncogene, MAP kinase and MAP kinase kinase were found to be constitutively activated, suggesting a role for Raf-1 upstream of MAP kinase kinase. Upon incubation in the presence of MgATP, Raf-1 or v-raf, partially purified by immunoprecipitation from cell extracts, were able to reactivate MAP kinase kinase that had been previously inactivated with protein phosphatase 2A.

Raf-1 itself appears to be activated by growth factors,

and mitogenic stimulation or transformation of cells leads to an increased phosphorylation of this kinase on serine and threonine, and in some examples, tyrosine residues (reviewed in [80]). This finding, together with the sensitivity of Raf-1 to inactivation by protein phosphatases [81, 82], suggests that another kinase further upstream may function in this kinase cascade. Several growth factor-stimulated kinase activities have been found that phosphorylate Raf-1, including ERK1 and ERK2 [83–85]. In these studies, peptide mapping indicated that Raf-1 was phosphorylated by MAP kinase *in vitro* on a subset of the sites seen in ^{32}P-labelled cells, suggesting that Raf-1 may be a downstream target of MAP kinase. Although phosphorylation by MAP kinase has not yet been shown to affect Raf-1 activity *in vitro*, the result raises the possibility that Raf-1 might be regulated through complex feedback mechanisms.

MEK kinase

The sequence similarity of MAP kinase to FUS3, KSS1 and Spk1, and the similarity of MAP kinase kinase to STE7 and Byr2 suggested a role for a STE11/Byr2-like kinase upstream of MAP kinase kinase (reviewed in [86]). In *S. cerevisiae,* the protein kinase STE11 is required for the activation of STE7, which in turn regulates FUS3 [87]. In *S. pombe,* a homolog of STE11, named Byr2, lies upstream of Byr1 and Spk1 [88]. Recently, a novel kinase with sequence similarity to STE11 and Byr2 has been identified in mouse cDNA libraries using degenerate oligonucleotides that correspond to regions of sequence identity between the STE11 and Byr2 genes [89]. Upon expression in mammalian cells, this enzyme, named MEK kinase, phosphorylates and activates MAP kinase kinase in a manner that is independent of Raf-1 activation. MEK kinase may thus function as an upstream regulator of MAP kinase kinase. An attractive hypothesis is that MEK kinase may be stimulated upon ligand binding to receptors coupled to heterotrimeric G protein-coupled pathways, as it appears to be in *S. cerevisiae.*

Mos

Several protein kinases are activated following progesterone-stimulated induction of maturation in Xenopus laevis oocytes. These include Xenopus homologs of the cdc2, cell cycle kinase, pp90^{rsk}, MAP kinase, MAP kinase kinase, and a serine/threonine kinase proto-oncogene, c-mos [53, 90, 91]. The activities of all of these kinases remain elevated in M phase arrest following the two meiotic cell divisions, then decrease after fertilization. Cyclic activation of cdc2 then leads to mitotic cycling.

Microinjection of a recombinant maltose binding protein (MBP)-mos fusion protein into immature oocytes stimulated MAP kinase and MAP kinase kinase, indicating a role for mos as an upstream activator of MAP kinase kinase [92]. *In vitro,* immunoprecipitated *Xenopus* MBP-mos was able to phosphorylate and activate a mammalian form of MAP kinase kinase that had been inactivated by phosphatase 2A treatment. Reactivation was ineffective using immunoprecipitates of kinase-dead mos mutants, suggesting that mos activity is required to activate MAP kinase kinase. As with Raf-1, however, further studies are needed to establish that mos directly regulates MAP kinase kinase by phosphorylation.

Microinjection of MBP-mos or v-mos into oocytes induced germinal vesicle breakdown [93], and also led to activation of cdc2 [92]. In these studies, the activation of MAP kinase preceded the activation of cdc2, suggesting a role of MAP kinase upstream or parallel to cdc2 activation [92]. Microinjection of p21^{ras} into Xenopus oocytes (see below) also led to the activation of MAP kinase, prior to the activation of cdc2 and germinal vesicle breakdown [94]. Paradoxically, microinjection of cdc2 into oocytes or addition of cdc2 to oocyte extracts led to the activation of MAP kinase kinase and MAP kinase, suggesting a role of MAP kinase downstream of cdc2 [53]. In addition, a *Xenopus* protein factor of high molecular mass (~440kDa, by sizing gel filtration) has been reported to activate and phosphorylate MAP kinase kinase]95]. Thus, the available data suggests multiple regulatory pathways for MAP kinase kinase activation in this system, perhaps involving feedback interactions between MAP kinase kinase and cdc2.

p21^{ras}

An upstream role for p21^{ras} in the MAP kinase cascade has been indicated by several studies showing that the expression of a dominant negative N-ras mutant (Ser17Asn) inhibits the activation of pp90^{rsk}, MAP kinase, MAP kinase kinase, and Raf-1 [96–101]. Kinase activation was blocked in response to phorbol esters, NGF, insulin, and PDGF. On the other hand, kinase activation

by aluminum fluoride was not affected, suggesting that $p21^{ras}$ is an intermediate between growth factor tyrosine kinase receptors and Raf-1, but is not involved in G protein coupled pathways leading to activation of the MAP kinase cascade. Such data suggest that MAP kinase kinase may be a convergence point for multiple signalling pathways involving receptor tyrosine kinase activation (*via* $p21^{ras}$ and Raf-1) *vs* pathways involving receptor-G protein interactions (perhaps *via* MEK kinase). However, this is probably an oversimplification, as further evidence for crosstalk between these pathways comes to light.

Microinjection of normal or transforming $p21^{ras}$ into *Xenopus* oocytes, or addition of $p21^{ras}$ to cytosolic egg extracts resulted in the stimulation of endogeneous MAP kinase kinase and MAP kinase [94, 101–104]. These observations have led to the development of *in vitro* assays for dissecting the function of $p21^{ras}$ upstream of MAP kinase kinase. Using inactive MAP kinase kinase as a probe, a 150–200kDa component has been identified that activates MAP kinase kinase in a $p21^{ras}$ dependent manner [104]. Assays such as these aimed at identifying and characterizing the upstream regulators of MAP kinase kinase are expected to lead to the discovery of cellular effectors of $p21^{ras}$, a long elusive goal in tumor cell research.

Summary

The MAP kinase kinase can be activated by several distinct kinases, each presumably regulated by different receptor systems, and seems to represent a point of convergence of different signalling pathways. The MAP kinase can phosphorylate and activate at least two classes of downstream protein kinases, and seems to represent a downstream divergence point in the pathway. Of particular interest is the finding that MAP kinase and pp90rsk phosphorylate and activate several nuclear transcription factors [105–108]. Reports of nuclear localization of MAP kinase and pp90rsk, and evidence for their translocation from the cytoplasm to nuclei in response to cell activation further support the role for these kinases in phosphorylation nuclear targets [109, 110]. The accumulating evidence suggests that the MAP kinase cascade will be an important mechanism by which activation of cell surface receptors ultimately control cell responses through the regulation of gene transcription.

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