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Regulation and function of the MAP kinase cascade in *Xenopus* oocytes

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SUMMARY

In *Xenopus* oocytes, activation of MAP kinase occurs during meiotic maturation through a protein kinase cascade (the MAP kinase cascade), which is utilized commonly in various intracellular signaling pathways in eukaryotes. Studies with a neutralizing antibody against *Xenopus* MAP kinase kinase (MAPKK), a direct upstream activator for MAP kinase, have shown that the MAP kinase cascade plays a crucial role in both initiating oocyte maturation and inducing metaphase arrest.

Key words: MAP kinase, *Xenopus*, kinase cascade, MPF, oocyte maturation

INTRODUCTION

Mitogen-activated protein (MAP) kinases are serine/threonine kinases highly conserved throughout evolution and are activated commonly by various extracellular stimuli inducing mitogenesis or differentiation (reviewed by Cobb et al., 1991; Nishida and Gotoh, 1992; Pelech and Sanghera, 1992; Ruderman, 1993; Thomas, 1992). They are supposed to play a central role in intracellular signal transduction pathways. Full activation of MAP kinases requires phosphorylation of both tyrosine and threonine residues (Anderson et al., 1990). These phosphorylation sites have been determined to be located in the TEY sequence between kinase subdomains VII and VIII (Payne et al., 1991). A 45 kDa protein factor that can induce phosphorylation and activation of inactive MAP kinases in vitro was purified first from *Xenopus* unfertilized eggs (Matsuda et al., 1992) and subsequently from mammalian cells (Crews and Erikson, 1992; Nakielny et al., 1992b; Seger et al., 1992a; Shirakabe et al., 1992). This MAP kinase activating factor can undergo autophosphorylation on serine, threonine and tyrosine residues (Kosako et al., 1992; Nakielny et al., 1992b) and phosphorylate the kinase-deficient mutant of MAP kinase on tyrosine and threonine residues (Crews and Erikson, 1992; Kosako et al., 1993; Nakielny et al., 1992a; Seger et al, 1992a). Therefore, this factor is a dual specificity kinase and has been named MAP kinase kinase (MAPKK). cDNA cloning of MAPKK (Ashworth et al., 1992; Crews et al; 1992; Kosako et al., 1993; Seger et al., 1992b; Wu et al., 1993) revealed that MAPKK shows high similarities to several yeast protein kinases functioning in various signal transduction pathways such as the mating process and osmotic regulation. This suggests that the MAPKK/MAP kinase cascade functions universally in eukaryotic systems (reviewed by Errede and Levin, 1993; Nishida and Gotoh, 1993).

It has been shown that the activation of MAPKK and MAP kinase occurs during *Xenopus* oocyte maturation (Ferrell et al., 1991; Gotoh et al., 1991a,b; Matsuda et al., 1992; Posada et al., 1991). Fully grown *Xenopus* oocytes (immature oocytes) are arrested at the first meiotic prophase. Exposure to progesterone induces the resumption of the meiotic process, leading to the production of the unfertilized egg, which is arrested at the second meiotic metaphase (metaphase II). The key event in this oocyte maturation process is thought to be the activation of maturation promoting factor (MPF), a complex of $p34^{cdc2}$ kinase and cyclin B, which is stored in immature oocytes as an inactive complex called pre-MPF (reviewed by Lohka, 1989; Maller, 1991; Nurse, 1990). MPF activity rises before germinal vesicle breakdown (GVBD), falls after metaphase I, and rises again and remains high during metaphase II. A cytostatic factor (CSF) is responsible for the metaphase II arrest with high MPF activity, and the product of the c-*mos* proto-oncogene, a 39 kDa serine/threonine protein kinase, is thought to be a component of CSF (Sagata et al., 1989). Translation of Mos is induced by progesterone and is necessary for meiosis I as well as for meiosis II and CSF arrest (Sagata et al., 1988). Moreover, bacterially expressed Mos protein can promote oocyte maturation when injected into immature oocytes without any hormonal stimulation and induce CSF arrest when injected into a two-cell embryo (Yew et al., 1992). Recent studies shed light on the roles of these four protein kinases (MPF, Mos, MAPKK and MAP kinase) during oocyte maturation process.

REGULATORY MECHANISM OF THE MAP KINASE CASCADE IN OOCYTE MATURATION

Activities of MAPKK and MAP kinase are elevated at about the same time as MPF during the course of oocyte maturation, remain high in unfertilized eggs and decrease to a basal level after fertilization (Ferrell et al., 1991; Gotoh et al., 1991a,b; Matsuda et al., 1992; Posada et al., 1991). Activation of MAPKK during this process is accompanied by its phosphorylation on threonine and serine residues (Kosako et al., 1992). Since MAPKK is deactivated by protein phosphatase 2A treatment in vitro (Gomez and

Cohen, 1991; Matsuda et al., 1992), MAPKK itself is thought to be activated by phosphorylation catalyzed by an upstream serine/threonine kinase(s), MAPKK kinase. Recent work has shown that MAPKK is phosphorylated on serine residues by MAPKK kinase and on threonine residues by its target kinase, MAP kinase (Matsuda et al., 1993; see Fig. 1). In the *Xenopus* MAPKK sequence there are two serine residues, S^{222} and S^{218}, located 9 and 13 amino acid residues upstream of the S(A)PE kinase motif, respectively. These two serine residues are conserved as serine or threonine residues among all the MAPKK homologs in vertebrates, *Drosophila* and yeasts. Site-directed mutagenesis studies have revealed that both or either of these serine residues may be important for activation of MAPKK by a variety of MAPKK kinases including Raf-1 (Gotoh et al., 1994). *Xenopus* MAPKK contains a single consensus sequence for phosphorylation by MAP kinase ($PST^{388}P$), and this sequence is conserved in mammalian and *Drosophila* MAPKK (Tsuda et al., 1993). A mutant MAPKK having $threonine^{388}$ changed to alanine was not phosphorylated by MAP kinase purified from unfertilized eggs (Gotoh et al., 1994). This phosphorylation might have some regulatory role.

Recently, it has been revealed that Mos can work as a MAPKK kinase (Nebreda et al., 1993; Posada et al. 1993). Posada et al. (1993) showed that bacterially expressed Mos protein rapidly activates MAPKK and MAP kinase when injected into immature oocytes, and Nebreda and Hunt (1993) showed the activation of MAPKK and MAP kinase by adding recombinant Mos to cell-free extracts prepared from *Xenopus* immature oocytes. Both groups reported further that the recombinant Mos when expressed in *Escherichia coli* has no MAPKK kinase activity but it acquires the kinase activity after incubation with rabbit reticulocyte lysate (Posada et al., 1993) or with *Xenopus* egg extracts (Nebreda et al., 1993). Synthesis of Mos in response to progesterone may be responsible, at least in part, for activation of the MAPKK/MAP kinase cascade in oocyte maturation (Fig. 1).

On the other hand, it has been reported that the product of the c-*raf-1* proto-oncogene, a 74-76 kDa serine/threonine protein kinase, lies upstream of the MAPKK/MAP kinase cascade and functions as a MAPKK kinase in various signal transduction systems of mammals and *Drosophila* (Dent et al., 1992; Kyriakis et al., 1992; Howe et al., 1992; Tsuda et al., 1993). Since expression of dominant-negative Raf-1 inhibits progesterone-induced activation of MAP kinase in *Xenopus* oocytes (Fabian et al., 1993; Muslin et al., 1993), Raf-1 has been suggested to lie upstream of the MAP kinase cascade in the oocyte maturation process. However, activation of Raf-1 kinase as MAPKK kinase has not been demonstrated during oocyte maturation, and Ras, a putative direct upstream factor of Raf-1 (Moodie et al., 1993; Van Aelst et al., 1993; Vojtek et al., 1993; Zhang et al., 1993), is not supposed to be involved in progesterone-induced oocyte maturation (Deshpande and Kung, 1987). Thus, participation of Raf-1 in activation of the MAP kinase cascade during this process remains unclear (Fig. 1).

In yeasts, STE11, BCK1 and Byr2 are homologous serine/threonine protein kinases functioning upstream of each MAPKK homolog (STE7, MKK1/MKK2 and Byr1, respectively; reviewed by Errede and Levin, 1993; Nishida and Gotoh, 1993). A mammalian homolog of these putative yeast MAPKK kinases, termed MEKK, was shown to phosphorylate and activate MAPKK independently of Raf-1 (Lange-Carter et al., 1993). *Xenopus* MEKK has not been isolated yet, but bacterially expressed STE11 protein can activate the MAP kinase cascade in cell-free extracts prepared from *Xenopus* immature oocytes (K. Takenaka et al., unpublished). Thus, MAPKK kinases other than Mos and Raf-1 could also function during oocyte maturation (Fig. 1).

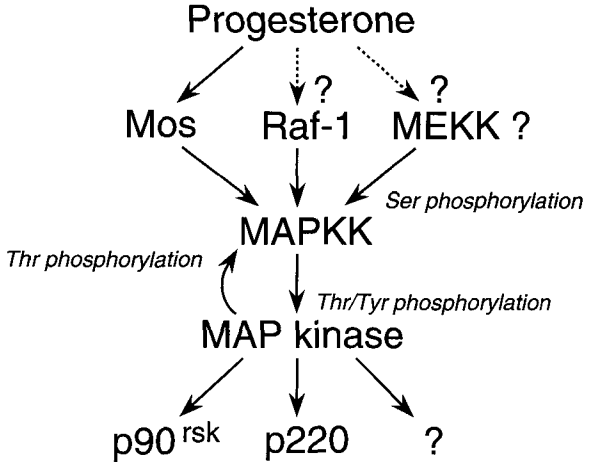

Fig. 1. Kinase cascade pathways resulting in MAP kinase activation during progesterone-induced oocyte maturation. Progesterone treatment induces synthesis of Mos protein, one of the MAPKK kinases. Whether other MAPKK kinases (Raf-1 and putative *Xenopus* MEKK) are activated by progesterone is unknown. These MAPKK kinases activate 45 kDa MAPKK by its serine phosphorylation (Gotoh et al., 1994). Then MAPKK (a dual specificity kinase) activates MAP kinase by phosphorylation on threonine and tyrosine residues. Activated MAP kinase phosphorylates several proteins, including MAPKK (an upstream kinase; Matsuda et al., 1993), $p90^{rsk}$ (a downstream kinase; Sturgill et al., 1988) and p220 (a microtubule-associated protein; Shiina et al., 1992). It is likely that MAP kinase has other physiological substrates in maturing oocytes.

Several groups have identified a family of mammalian dual specificity phosphatases that can specifically dephosphorylate and inactivate MAP kinase in vitro (reviewed by Nebreda, 1994). One of these phosphatases (3CH134 or CL100) is an immediate early gene product and is shown to be a physiological MAP kinase phosphatase by transient transfection studies (thus named MKP-1), suggesting a shut-off mechanism for the transient activation of MAP kinase in mitogenic stimulation (Sun et al., 1993). In *Xenopus* oocytes, the MAP kinase activity which is fully active during metaphase II arrest drops upon fertilization, but no gene expression occurs during early embryogenesis. Therefore, inactivation of MAP kinase after fertilization may occur by a different mechanism. Interestingly, a 47 kDa phosphatase purified from *Xenopus* eggs showed absolute specificity toward phosphotyrosine but not phosphothreonine of MAP kinase in vitro (Sarcevic et al., 1993). Regulation of this tyrosine phosphatase may provide an alternative mechanism for inactivation of MAP kinase.

FUNCTION OF THE MAP KINASE CASCADE IN *XENOPUS* OOCYTES

Requirement of the MAP kinase cascade for initiation of oocyte maturation

Recently, we prepared many polyclonal and monoclonal anti-

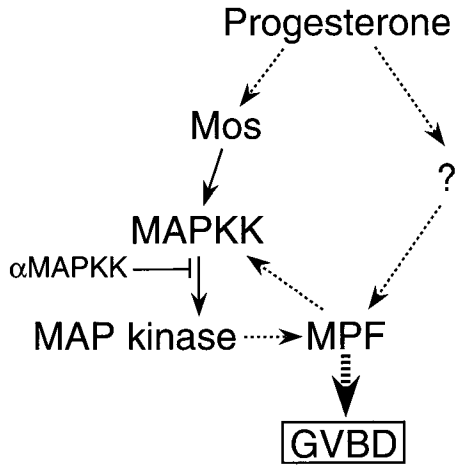

Fig. 2. A model for signal transduction pathways in oocyte maturation. This model proposes that progesterone-induced Mos, an essential protein for oocyte maturation, exerts its function through the MAPKK/MAP kinase cascade, resulting in MPF activation and GVBD. It is quite possible that other signaling pathways are required for progesterone-induced MPF activation.

bodies against bacterially expressed *Xenopus* 45 kDa MAPKK, one of which was found to be a neutralizing antibody that can specifically and efficiently inhibit *Xenopus* MAPKK activity in vitro (Kosako et al., 1994). This neutralizing antibody inhibited Mos- or okadaic acid-induced activation of MAP kinase when added to cell-free extracts prepared from *Xenopus* immature oocytes, suggesting that these agents activate MAP kinase through the 45 kDa MAPKK in a cell-free system. Furthermore, microinjection of this antibody into immature oocytes prevented progesterone- or Mos-induced activation of MAP kinase (Kosako et al., 1994). Our previous report showed that microinjection of the purified *Xenopus* MAPKK into immature oocytes resulted in rapid activation of endogenous MAP kinase (Matsuda et al., 1992). Thus, it is suggested that MAPKK, originally identified by its ability to activate MAP kinase in vitro, is the only direct activator of MAP kinase in *Xenopus* oocytes in vivo. Since there exist several putative MAPKK kinases (Raf-1, Mos and MEKK), MAPKK may function at a convergent point in various signaling pathways resulting in activation of MAP kinase (see Fig. 1).

The inhibition of activation of the MAP kinase cascade by microinjecting immature oocytes with the neutralizing antibody against MAPKK blocked the progesterone- or Mos-induced activation of MPF, as judged by inhibition of both GVBD and histone H1 kinase activation (Kosako et al., 1994). This suggests that the MAP kinase cascade plays a critical role in MPF activation during oocyte maturation and that there exists a signal transduction pathway consisting of Mos, MAPKK, MAP kinase and MPF (Fig. 2). The activated MAP kinase in this pathway may directly or indirectly regulate some proteins controlling MPF activity, such as cdc25, wee1 and CAK (reviewed by Solomon, 1993). However, whether MAP kinase activation is sufficient for MPF activation is unknown. It is possible that the MAP kinase cascade-independent pathways are also required for progesterone-induced MPF activation. It has been reported that $p70^{s6k}$, which is shown to be activated by mitogenic stimulation independently of the MAP kinase cascade in mammalian cultured cells (Ballou et al., 1991), is rapidly activated by progesterone treatment in *Xenopus* oocytes (Lane et al., 1992). Other signaling pathways, such as inactivation of cAMP-dependent protein kinase, may also be necessary for progesterone-induced MPF activation (Daar et al., 1993).

We showed previously that purified MPF can activate MAPKK and MAP kinase when microinjected into immature oocytes or added to cell-free extracts prepared from interphase eggs (Gotoh et al., 1991b; Matsuda et al., 1992). Therefore, it is supposed that the MAPKK/MAP kinase cascade and MPF form a positive feedback loop (Fig. 2). This might explain the synchronous activation of MAP kinase and MPF during progesterone-induced oocyte maturation (Nebreda and Hunt, 1993).

Function of the MAP kinase cascade in CSF arrest

Mos, one of the MAPKK kinases, functions not only as an initiator of oocyte maturation but also as a component of cytostatic factor (CSF) that causes the natural arrest of unfertilized eggs in second meiotic metaphase (metaphase II arrest; Sagata et al., 1988, 1989). Recently, Haccard et al. (1993) reported that microinjection of thiophosphorylated MAP kinase (thiophosphorylated proteins are generally resistant to dephosphorylation by protein phosphatases) into one blastomere of a two-cell embryo induced metaphase arrest similar to that induced by Mos. This assay is the only index of CSF activity, and their result reveals that active MAP kinase is sufficient for metaphase arrest in *Xenopus* fertilized eggs. It is suggested that active MAP kinase in mature oocytes (unfertilized eggs) functions, downstream of Mos, to induce metaphase II arrest (Fig. 3). Interestingly, MAP kinase is deactivated before GVBD in clam oocytes that are not arrested at metaphase II (Shibuya et al., 1992a). We have shown by using the neutralizing antibody against MAPKK that the CSF activity of Mos is mediated by the MAP kinase cascade (H. Kosako, Y. Gotoh and E. Nishida, unpublished). Microinjection of bacterially expressed Mos protein into one blastomere of a two-cell embryo induced metaphase arrest as had been reported by Yew et al. (1992), but coinjection of Mos and the neutralizing antibody prevented the Mos-induced metaphase arrest. The previous report that Ras has CSF activity (Daar et al., 1991) may also be explained by Ras-induced activation of the MAP kinase cascade (Hattori et al., 1992; Itoh et al., 1993; Leevers and Marshall, 1992; Shibuya et al., 1992b; Fig. 3). Thus, the MAP kinase cascade is thought to play a pivotal role in both initiating oocyte maturation by hormonal stimulation and maintaining metaphase arrest in mature oocytes (Figs 2 and 3). The mechanism by which the same kinase cascade induces apparently different cellular responses is unclear, but MAP kinase may regulate, directly or indirectly, a factor(s) involved in both activation and stabilization of MPF.

PROSPECTS

In vertebrates, the MAP kinase cascade is activated downstream of several proto-oncogene products in various intracellular signal transduction pathways, but its significance for cellular function was unclear. The study utilizing the neutralizing antibody against *Xenopus* MAPKK has shown the physiological significance of the MAP kinase cascade in *Xenopus* oocyte maturation. However, target proteins of MAP kinase

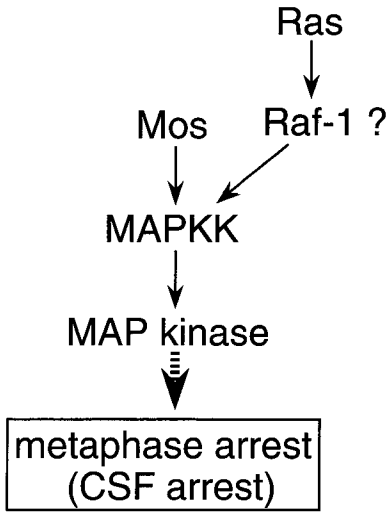

Fig. 3. A model for signal transduction pathways inducing metaphase arrest. This model proposes that the CSF activity of Mos is mediated by the MAP kinase cascade. The CSF activity of Ras may also be mediated by this kinase cascade probably through Raf-1, another MAPKK kinase.

during the oocyte maturation process have not been identified fully. It has been reported that MAP kinase phosphorylates a downstream kinase ($p90^{rsk}$ or S6 kinase II; Sturgill et al., 1988) and a microtubule-associated protein (p220; Shiina et al., 1992) present in *Xenopus* oocytes. Elucidation of a catalog of MAP kinase substrates and their function will increase our understanding of the function of the MAP kinase cascade not only in oocyte maturation but also in other cellular processes.

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