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Biochemical and Biological Analysis of Mek1 Phosphorylation Site Mutants

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Recently, we described the constitutive activation of Mek1 by mutation of its two serine phosphorylation sites. We have now characterized the biochemical properties of these Mek1 mutants and performed microinjection experiments to investigate the effect of an activated Mek on oocyte maturation. Single acidic substitution of either serine 218 or 222 activated Mek1 by 10–50 fold. The double acidic substitutions, [Asp^{218}, Asp^{222}] and [Asp^{218}, Glu^{222}], activated Mek1 over 6000-fold. The specific activity of the [Asp^{218}, Asp^{222}] and [Asp^{218}, Glu^{222}] Mek1 mutants, 29 nanomole phosphate per minute per milligram, is similar to that of wild-type Mek1 activated by Raf-1 in vitro. Although the mutants with double acidic substitutions could not be further activated by Raf-1, three of those with single acidic substitution were activated by Raf-1 to the specific activity of activated wild-type Mek1. Injection of the [Asp^{218}, Asp^{222}] Mek1 mutant into *Xenopus* oocytes activated both MAP kinase and histone H1 kinase and induced germinal vesicle breakdown, an effect that was only partially blocked by inhibition of protein synthesis. These data provide a measure of Mek's potential to influence cell functions and a quantitative basis to assess the biological effects of Mek1 mutants in a variety of circumstances.

INTRODUCTION

Protein phosphorylation is one of the major cellular mechanisms for regulating protein functions. The enzymes that catalyze protein phosphorylation, protein kinases, are often regulated by protein phosphorylation themselves. Thus, a series of protein kinases that phosphorylate and regulate others would comprise a pathway that is capable of transmitting and amplifying regulatory signals and that is potentially the subject of multiple controls. The mitogen-activated protein (MAP)[1] kinase pathway is such a cascade, which eukaryotic cells employ to transmit a variety of extracellular signals to regulatory proteins in the cytoplasm and nucleus (for reviews, see Crews *et al.*, 1992; Ahn *et al.*, 1993; Blenis, 1993; Davis, 1993). The first mammalian enzyme discovered in this pathway, MAP kinase (also known as Erk) is a serine/threonine-specific kinase that is related to Fus3p and Kss1p in *Saccharomyces cerevisiae* and Spk1p in *Schizosaccharomyces pombe* (Boulton *et al.*, 1991). The kinase that regulates MAP kinase, Mek, activates MAP kinase by phosphorylating it on threonine and tyrosine residues (Alessandrini *et al.*, 1992; Rossomando *et al.*, 1992). Mek is homologous to the *S. cerevisiae STE7* gene product and the *S. pombe byr1* gene product (Crews *et al.*, 1992b; Seger *et al.*, 1992; Wu *et al.*, 1993). Genetic analyses place two other protein kinases, Ste11p and Byr2p, directly upstream of Ste7p and Byr1p in *S. cerevisiae* and *S. pombe*, respectively (Wang *et al.*, 1991; Cairns *et al.*, 1992). A mammalian counterpart of Ste11p and Byr2p, termed Mek kinase (MEKK), was identified by homology screening and shown to be an activator of Mek (Lange-Carter *et al.*, 1993). Another mammalian kinase, the proto-oncogene product Raf-1, can also phosphorylate and activate Mek (Dent *et al.*, 1992; Howe *et al.*, 1992; Kyriakis *et al.*, 1992). Raf-1 is not closely related in sequence to MEKK and no yeast homologue of Raf-1 has been discovered to date. The upstream regulator of MEKK or Raf-1 is still unknown. Although the proto-oncogene product Ras physically associates

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[1] Abbreviations used: GVBD, germinal vesicle breakdown; MAP kinase, mitogen-activated protein kinase; Mek, MAP kinase/Erk kinase; MPF, maturation-promoting factor.

with Raf-1 and is required for the activation of the MAP kinase pathway, it appears that Ras alone is insufficient to activate Raf-1 (Vojtek *et al.*, 1993; Warne *et al.*, 1993; Zhang *et al.*, 1993).

The MAP kinase pathway has been implicated in multiple cellular processes. In mammalian cells, the pathway is activated in response to growth factors (Crews *et al.*, 1992a), UV irradiation (Derijard *et al.*, 1994), hyperosmolarity, and endotoxin stimulation (Galcheva-Gargova *et al.*, 1994; Han *et al.*, 1994). In *S. cerevisiae*, it is involved in pheromone response, cell wall construction, and osmosensing (for review, see Errede and Levin, 1993). In *Xenopus laevis*, the MAP kinase pathway participates in meiotic maturation of oocytes. Immature *Xenopus* oocytes are arrested at prophase of meiosis I. Upon exposure to progesterone released from the surrounding follicle cells, they undergo a series of metabolic alterations, known as meiotic maturation, to complete meiosis I and progress to metaphase of meiosis II. Several cytoplasmic kinases are activated concurrently, including maturation-promoting factor (MPF), Raf-1 (Fabian *et al.*, 1993; Muslin *et al.*, 1993), Mek (Matsuda *et al.*, 1992), and MAP kinase (Gotoh *et al.*, 1991; Barrett *et al.*, 1992). The kinase activity of Raf-1 is required for the maturation process (Fabian *et al.*, 1993b; Muslin *et al.*, 1993), and injection of an activated mutant of Raf-1 induces maturation (Fabian *et al.*, 1993b). Mek is also required for oocyte maturation (Kosako *et al.*, 1994). However, it is not yet clear whether the activation of Mek alone can promote maturation. Moreover, stimulation by progesterone induces the synthesis of Mos, a germ-line specific serine/threonine kinase (Sagata *et al.*, 1988). The synthesis of Mos is necessary for progesterone-induced oocyte maturation (Sagata *et al.*, 1988), and a recombinant Mos protein can promote maturation in the absence of protein synthesis (Yew *et al.*, 1992). Interestingly, Mos can directly phosphorylate and activate Mek in vitro (Posada *et al.*, 1993; Shibuya and Ruderman, 1993). Thus Mek may be regulated simultaneously by Raf-1 and Mos during meiotic maturation.

It is likely that different versions of the MAP kinase pathway are responsible for its multiple functions in each organism. In *S. cerevisiae*, for example, the pheromone response, cell wall construction, and osmosensing processes utilize three different versions of the MAP kinase pathway, each consisting of different homologues of MEKK, Mek, and MAP kinase (Errede and Levin, 1993). In mammalian cells, the MAP kinase family currently consists of six members: Erk1, Erk2, Erk3 (Boulton *et al.*, 1991), Jnk1, Jnk2 (Derijard *et al.*, 1994), and p38 (Galcheva-Gargova *et al.*, 1994); the Mek family consists of two: Mek1 and Mek2 (Brott *et al.*, 1993; Zheng and Guan, 1993). In the absence of convenient genetic tools, the assignment of these mammalian enzymes to specific signaling functions may be aided by biochemical approaches, which entail determining the mechanism of activation of each enzyme, its substrate specificity, and the in vivo effects of mutant proteins.

The residues in Mek1 that are required for phosphorylation and activation by Raf-1 were identified as serine 218 and 222 (Alessi *et al.*, 1994; Zheng and Guan, 1994). By introducing acidic residues to these two phosphorylation sites, we recently succeeded in activating Mek1 over 100-fold in a transient expression system and demonstrated that the activated Mek1 mutants can activate Erk1 in vivo (Huang and Erikson, 1994). Others have reported that activated Mek mutants transform NIH 3T3 cells in culture (Cowley *et al.*, 1994; Mansour *et al.*, 1994). To quantitatively study the significance of each phosphorylation site in the activation of Mek1, we have now expressed these mutant proteins in insect cells using recombinant baculoviruses and characterized their biochemical properties. The results support a model in which phosphorylation of either serine 218 or 222 partially activates Mek1, and phosphorylation of both fully activates Mek1. The availability of the mutant proteins permits us to correlate in a more quantitative fashion the activity of Mek1 with a biological response. We have initiated studies of the biological response of *Xenopus* oocytes to Mek activation and found that injection of an activated Mek1 mutant activates histone H1 kinase and promotes maturation.

MATERIALS AND METHODS

Mutagenesis and Protein Expression

Mutations of serine 218 and serine 222 were generated as previously described (Huang and Erikson, 1994) in *pG·MEK·Cglu*, a *pGEM-7Zf(minus)* vector (Promega, Madison, WI) carrying a mouse *MEK1* gene C-terminally tagged with the EE epitope (Rubinfeld *et al.*, 1991). The *Xba*I-*Bam*HI fragments containing *MEK1* mutants from *pG·MEK·Cglu* were subcloned into the *Xba*I-*Bam*HI site of *pVL1392* (Invitrogen, San Diego, CA). *pVL1392·MEK1* mutants were co-transfected into Sf9 cells with Baculogold (PharMingen, San Diego, CA). Baculoviruses were harvested 10 days later and then amplified once. Mek1 mutant proteins were expressed in Sf9 cells and purified by anti-EE affinity chromatography as described (Huang *et al.*, 1993). The baculovirus vector that expresses Y340D/Raf-1, an activated Raf-1 mutant (Fabian *et al.*, 1993a) tagged with the FLAG epitope (Hopp *et al.*, 1988), was a gift of D.K. Morrison (National Cancer Institute-Frederick Cancer Research and Development Center, Frederick, MD). The Y340D/Raf-1 protein was expressed in Sf9 cells and purified with an anti-FLAG M2 affinity column (International Biotechnologies, New Haven, CT) following the manufacturer's recommended procedure.

Kinase Assays

To assay Mek1 activity, we used GST-Erk1 (K63M), a kinase-inactive mutant of Erk1 fused to glutathione-*S*-transferase (GST) (Alessandrini *et al.*, 1992) as substrate. Affinity-purified Mek1 mutant protein (0.1 μg) was incubated at 30°C for 5 min in 40 μl Erk1 kinase cocktail (50 mM Tris (pH 8.0)/5 mM dithiothreitol/0.1 mg ovalbumin per ml/3 mM $MgAc_2$/50 μM ATP/10 mM NaF/1 mM Na_3VO_4/1 mM EGTA/250 μCi [γ-^{32}P]ATP per ml) containing 8 μg

GST-Erk1(K63M). To assay histone H1 kinase activity, 2 μl oocyte extract was incubated at 22°C for 20 min in 40 μl H1 kinase cocktail (80 mM sodium β-glycerophosphate (pH 7.0)/20 mM EGTA/15 mM $MgCl_2$/50 μM ATP/250 μCi [γ-^{32}P]ATP per ml) containing 5 μg histone H1 (Boehringer Mannheim, Indianapolis, IN). Kinase reactions were terminated by the addition of 10 μl 5× Laemmli sample buffer (Laemmli, 1970). The reaction mixtures were resolved by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). After autoradiography, the gel pieces containing substrate were excised and phosphate incorporation was determined by liquid scintillation counting. To assay MAP kinase activity in oocyte extracts, we used a synthetic peptide derived from epidermal growth factor receptor (Gonzalez *et al.*, 1991) as substrate. Two microliters of oocyte extract was incubated at 22°C for 20 min in 40 μl MAP kinase cocktail (25 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethane-sulfonic acid (HEPES) (pH 7.4)/10 mM $MgCl_2$/50 μM ATP/250 μCi [γ-^{32}P]ATP per ml) containing 20 μg of the peptide. Kinase reactions were terminated by the addition of 10 μl 45% formic acid/125 mM ATP. Twenty-five microliters of the reaction mixture was applied onto phosphocellulose paper (P81, Whatman, Maidstone, UK). The filters were washed twice with 1 M acetic acid/4 mM sodium pyrophosphate and phosphate incorporation was determined by liquid scintillation counting. The incubation time of all kinase assays was within the linear range, as determined by using the most active enzyme under similar conditions.

In Vitro Activation of Mek1 by Raf-1

Affinity-purified Y304D/Raf-1 protein (0.3 μg) was incubated with 0.1 μg affinity-purified Mek1 at 30°C for 10 min in 40 μl Erk1 kinase cocktail. Then 8 μg GST-Erk1 (K63 M) was added and the reaction mixtures were incubated at 30°C for 5 min. The reactions were terminated by the addition of 10 μl 5× Laemmli sample buffer. The reaction mixtures were resolved by 10% SDS-PAGE. After autoradiography, the gel pieces containing Mek1 and GST-Erk1 (K63 M) were excised and the amount of phosphate incorporation was determined by liquid scintillation counting. Under these conditions, wild-type Mek1 was maximally phosphorylated and the ERK1 kinase assay was within the linear range.

Oocyte Microinjection

Xenopus laevis females (Xenopus I) were primed with 35 units of pregnant mare's serum (Sigma Chemical, St. Louis, MO). After 2–3 days, a portion of ovary was surgically removed from anesthetized females and digested with 2 mg per ml collagenase (Sigma Chemical, type IA) in OR2 (5 mM HEPES (pH 7.8)/82.5 mM NaCl/2.5 mM KCl/1 mM Na_2HPO_4) for 2 h. After extensive washing, oocytes were cultured overnight at 19°C in OR2 supplemented with 1 mM $CaCl_2$, 1 mM $MgCl_2$, and 0.5 mg bovine serum albumin per ml. Stage VI oocytes were selected and incubated in MBS (88 mM NaCl/1 mM KCl/0.7 mM $CaCl_2$/1 mM $MgSO_4$/5 mM HEPES (pH 7.8)/2.5 mM $NaHCO_3$) with or without 10 μg per ml cycloheximide. Oocytes were injected with 40 nl of Mek1 protein at 0.15, 0.3, or 0.5 mg per ml in 88 mM NaCl/20 mM HEPES (pH 6.8). Some uninjected oocytes were treated with 10 μg per ml progesterone. After injection (8–10 h), pools of 50 oocytes were scored for GVBD by the presence of a white spot on the animal pole. To confirm GVBD, some oocytes were fixed in 10% trichloroacetic acid/MBS and then dissected to determine the integrity of the nucleus. For kinase assays, groups of five oocytes were homogenized by pipetting in 50 μl lysis buffer A (10 mM potassium phosphate (pH 7.1)/1 mM EDTA/5 mM EGTA/10 mM $MgCl_2$/50 mM β-glycerophosphate/2 mM sodium vanadate/2 mM dithiothreitol/1 mM phenylmethylsulfonyl fluoride/10 μg leupeptin per ml/10 μg pepstatin A per ml). The homogenates were centrifuged for 5 min at 14,000 × *g* and the supernatants were frozen in liquid nitrogen and stored at −70°C.

RESULTS

Activity of Mek1 Phosphorylation Site Mutants

The activation of Mek1 by Raf-1 requires the phosphorylation of serine 218 and 222, two residues that are conserved among the Mek family members (Figure 1). To evaluate the role that each serine site may play in activation, we constructed a series of Mek1 mutants in which acidic residues (aspartate or glutamate) were introduced into the two phosphorylation sites to mimic phosphoserine residues (Figure 1). When these mutants were expressed in COS-7 cells, they exhibited various degrees of activation compared with wild-type Mek1. The [Asp^{218}, Asp^{222}] and [Asp^{218}, Glu^{222}] mutants were most active (~100-fold greater than wild type) and could activate Erk1 in COS-7 cells (Huang and Erikson, 1994). To further characterize the biochemical properties of these mutant proteins, we have incorporated the Mek1 mutant genes into a baculovirus vector and expressed them in Sf9 cells. The Mek1 mutant proteins, C-terminally tagged with the EE epitope (Rubinfeld *et al.*, 1991), were purified from Sf9 cell lysate by anti-EE affinity chromatography.

The activity of the Mek1 mutant proteins was assayed using GST-Erk1 (K63M), a kinase-inactive mutant of Erk1 fused to glutathione-*S*-transferase as substrate (Alessandrini *et al.*, 1992) (Table 1). The Mek1 mutants with neutral substitutions, namely [Val^{218}], [Val^{222}], and [Val^{218}, Val^{222}], displayed activity levels equivalent to that of wild-type Mek1. This result indicates that the wild-type Mek1 protein purified from Sf9 cells was probably unphosphorylated on both serines and thus displayed basal level activity. Single acidic substitutions, namely [Asp^{218}], [Asp^{222}], [Glu^{218}], and [Glu^{222}], activated Mek1 10- to 50-fold. The highest degree of activation was achieved by substituting aspartate for serine 218 and aspartate or glutamate for serine 222. The [Asp^{218}, Asp^{222}] and [Asp^{218}, Glu^{222}] mutants were over 6000-fold more active than wild-type Mek1. If the acidic substitutions altered the structure of Mek1 in a fashion similar to that of phosphorylation, these results would suggest that the phosphorylation of either serine 218 or 222

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STE7  GVSKKLINSIADTFVGTSTYMSPER
BYR1  GVSGELVNSVAQTFVGTSTYMSPER
PBS2  GVSGNLVASLAKTNIGCQSYMAPER
MEK1  GVSGQLIDSMANSFVGTRSYMSPER
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Figure 1. Conserved phosphorylation sites of the MEK family. Sequences of subdomain VIII of the catalytic domain of four members of the MEK family are aligned: mouse Mek1 (Crews *et al.*, 1992), *S. pombe* Byr1 (Nadin-Davis and Nasim, 1988), *S. cerevisiae* STE7 (Teague *et al.*, 1986), and PBS2 (Brewster *et al.*, 1993). The conserved serine/threonine phosphorylation sites are underlined.

Table 1. Activation of Mek1 by mutation of serine phosphorylation sites[a]

Mek1 mutant	Fold activation[b]
Wildtype	1
Val^{218}	1
Val^{222}	1
Val^{218}, Val^{222}	1
Asp^{218}	10
Asp^{222}	40
Glu^{218}	50
Glu^{222}	50
Asp^{218}, Asp^{222}	6000
Asp^{218}, Glu^{222}	6000

[a] Each value is the mean of four independent determinations.
[b] Activity of each mutant is represented as fold activation over unphosphorylated wildtype Mek1.

alone partially activates Mek1, but when combined, the two phosphorylation events have a synergistic effect in activating Mek1.

In Vitro Activation of Mek1 by Raf-1

It is not clear whether phosphorylation sites other than serine 218 and 222 also contribute to the activation of Mek1. The fact that an Mek1 mutant with alanine substitution at both serine 218 and 222 is not phosphorylated and activated by Raf-1 (Zheng and Guan, 1994; Alessi *et al.*, 1994) may suggest that such phosphorylation sites are unlikely to exist. It is still possible, however, that there exist phosphorylation site(s) whose phosphorylation depends on the phosphorylation of serine 218 and 222. To investigate such a possibility, we used an activated mutant of Raf-1 (Y340D/Raf-1) to phosphorylate the Mek1 phosphorylation site mutants in vitro. After phosphorylation, we measured the activity of the Mek1 mutants and the quantity of phosphate incorporated.

The results of these in vitro phosphorylation experiments (Table 2) show that none of the three Mek1 mutants with double substitutions at 218 and 222 were significantly phosphorylated by Raf-1. The [Asp^{218}, Asp^{222}] and [Asp^{218}, Glu^{222}] mutants were not further activated by Raf-1. Thus, it is unlikely that there exists a third phosphorylation site that contributes to the activation by Raf-1. The specific activity of wild-type Mek1 phosphorylated by Raf-1 equalled that of [Asp^{218}, Asp^{222}] Mek1 and [Asp^{218}, Glu^{222}] Mek1. Because the stoichiometry of phosphorylation achieved here is half the expected 2 mol of phosphate per mol of Mek1 for both serines to be phosphorylated, we estimate that the [Asp^{218}, Asp^{222}] and [Asp^{218}, Glu^{222}] Mek1 mutants are about 50% as active as the fully phosphorylated wild-type Mek1. The Mek1 mutants with single substitutions, either neutral or acidic, were all phosphorylated by Raf-1 to a ratio of 0.4–0.7 mol of phosphate per mol of Mek1. This suggests that the phosphorylation of serine 218 or 222 does not depend on the phosphorylation of the other site. Although the quantity of phosphate incorporated into the four mutants with single acidic substitution was approximately half of that incorporated into the wild-type Mek1, three of the mutants were activated by Raf-1 and yielded approximately the same specific activity as the wild-type Mek1. These data further support the suggestion that the acidic residues sufficiently mimic phosphoserines and that the phosphorylation of serine 218 and 222 activates Mek1 synergistically.

The Activation of Mek1 Promotes Xenopus Oocyte Maturation

The MAP kinase pathway is activated during meiotic maturation of *Xenopus* oocytes. It has been demonstrated that the kinase activity of Raf-1 is required for the maturation process (Muslin *et al.*, 1993; Fabian *et al.*, 1993) and that the activation of Raf-1 induces maturation (Fabian *et al.*, 1993). Because Mek is a downstream target of Raf-1, it is likely that Mek is required for oocyte maturation. This has been recently demonstrated by the injection of oocytes with an inhibitory antibody against Mek (Kosako *et al.*, 1994). However, Mek may not be the only downstream target of Raf-1. It remains to be determined whether the activation of Mek is sufficient to promote maturation.

The availability of activated Mek1 mutant proteins allowed us to address this question by microinjection experiments. We found that the injection of *Xenopus* oocytes with the affinity-purified [Asp^{218}, Asp^{222}]

Table 2. In vitro activation of Mek1 by Raf-1[a]

Mek1 mutant	Phosphorylation[b]	Specific activity Before[c]	Specific activity After[c]	Fold[d]
Wildtype	1.0	5×10^{-3}	31	6000
Val^{218}	0.4	6×10^{-3}	1.6	250
Val^{222}	0.7	1×10^{-2}	1.3	130
Val^{218}, Val^{222}	0.03	7×10^{-3}	3×10^{-1}	40
Asp^{218}	0.5	4×10^{-2}	2.2	50
Asp^{222}	0.4	2×10^{-1}	14	70
Glu^{218}	0.7	2×10^{-1}	36	180
Glu^{222}	0.5	3×10^{-1}	20	70
Asp^{218}, Asp^{222}	0.07	29	22	N/A
Asp^{218}, Asp^{222}	0.1	29	29	N/A

[a] Each value is the mean of four independent determinations.
[b] Moles of phosphate incorporated into per mole of Mek1 by Raf-1.
[c] Nanomoles of phosphate incorporated into GST:Erk1(K63M) per minute per milligram of Mek1. Before, specific activity of Mek1 before phosphorylation; After, specific activity of Mek1 after phosphorylation.
[d] Fold activation by phosphorylation.

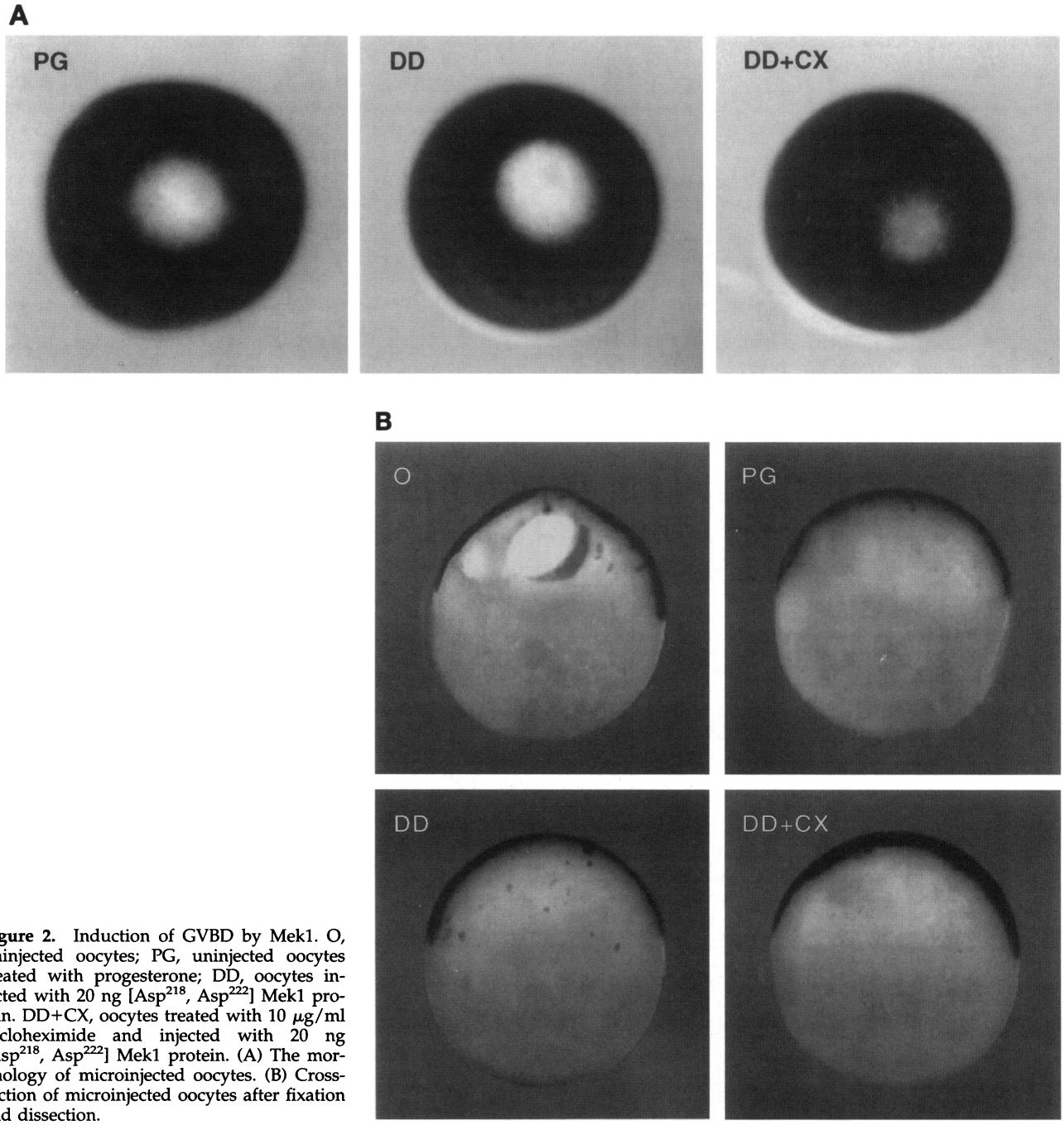

Figure 2. Induction of GVBD by Mek1. O, uninjected oocytes; PG, uninjected oocytes treated with progesterone; DD, oocytes injected with 20 ng [Asp^{218}, Asp^{222}] Mek1 protein. DD+CX, oocytes treated with 10 μg/ml cycloheximide and injected with 20 ng [Asp^{218}, Asp^{222}] Mek1 protein. (A) The morphology of microinjected oocytes. (B) Cross-section of microinjected oocytes after fixation and dissection.

Mek1 protein was sufficient to induce meiotic maturation. Although the injection of wild-type Mek1 protein did not induce GVBD in oocytes, GVBD was observed in more than 50% of the oocytes injected with 20 ng [Asp^{218}, Asp^{222}] Mek1 protein. The morphology of GVBD observed in oocytes injected with [Asp^{218}, Asp^{222}] Mek1 was indistinguishable from that in oocytes treated with progesterone (Figure 2). To correlate the activity of Mek1 with the response of oocytes, we injected varying quantities of [Asp^{218}, Asp^{222}] Mek1 (Figure 3A). Although the percentage of oocytes undergoing GVBD increased with dosage, there appeared to be a dose threshold of Mek1 for its effectiveness. Little GVBD was observed in oocytes injected with less than 12 ng [Asp^{218}, Asp^{222}] Mek1.

To confirm that the oocytes injected with [Asp^{218}, Asp^{222}] Mek1 indeed underwent maturation, we measured the histone H1 kinase activity in extracts of

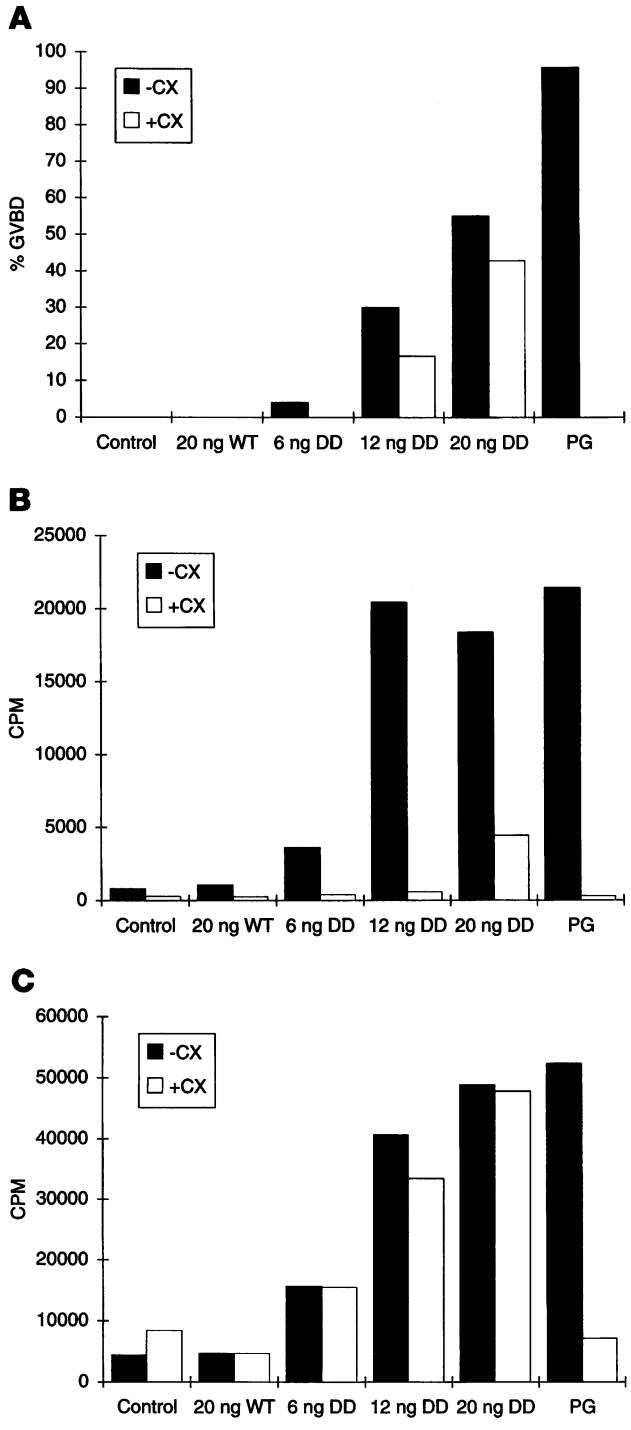

Figure 3. Activation of histone H1 kinase and MAP kinase. Control, uninjected oocytes; SS, oocytes injected with wild-type Mek1; DD, oocytes injected with [Asp^{218}, Asp^{222}] Mek1 protein; PG, uninjected oocytes treated with 10 μg/ml progesterone; −CX, in the absence of cycloheximide; +CX, in the presence of 10 μg/ml cycloheximide. (A) The percentage of oocytes showing GVBD. (B) Histone H1 kinase activity in oocyte extracts. (C) MAP kinase activity in oocyte extracts.

these oocytes. Active MPF phosphorylates histone H1 in vitro and this property can be employed to measure MPF activity (Lohka *et al.*, 1988). We found that in oocytes injected with 12 ng or more [Asp^{218}, Asp^{222}] Mek1, histone H1 kinase was activated to a level comparable to that in oocytes treated with progesterone (Figure 3B). Similarly, MAP kinase was activated to approximately the same degree in oocytes injected with 12 ng or more [Asp^{218}, Asp^{222}] Mek1 and in those treated with progesterone (Figure 3C).

Although protein synthesis is required for the induction of maturation by progesterone (Schorderet-Slatkine, 1972), it appears that [Asp^{218}, Asp^{222}] Mek1 was able to induce oocyte maturation in the absence of protein synthesis (Figure 2). Cycloheximide, an inhibitor of protein synthesis, only partially inhibited the GVBD induced by the injection of [Asp^{218}, Asp^{222}] Mek1 (Figure 3A). Although the inhibitory effect of cycloheximide was more drastic on the activation of histone H1 kinase, the injection of 20 ng [Asp^{218}, Asp^{222}] Mek1 could still activate histone H1 kinase by more than 15-fold in the presence of cycloheximide (Figure 3B).

DISCUSSION

The constitutive activation of a protein kinase by replacement of phosphorylation site residues with acidic residues has not been frequently observed. To our knowledge, the only precedent before the studies of Mek1 is the 30-fold activation of Raf-1 by replacement of phosphotyrosine (Tyr^{340}) with aspartate (Fabian *et al.*, 1993a). By substituting aspartate for serine 218 and aspartate or glutamate for serine 222, we have achieved the constitutive activation of Mek1 by over 6000-fold. The degree of activation that we report here was determined in vitro with recombinant proteins produced in insect cells. It is greater than the ~100-fold activation that we previously determined with proteins transiently expressed in COS-7 cells (Huang and Erikson, 1994). This discrepancy may be due to the fact that the wild-type Mek1 protein produced in insect cells has a lower basal level of phosphorylation and thus is less active than that expressed in COS-7 cells. The specific activity of the [Asp^{218}, Asp^{222}] and [Asp^{218}, Glu^{222}] Mek1 mutants, 29 nmol phosphate per min per mg, is at the same level as that of a v-Src/v-Ras–activated Raf-1 protein (Force *et al.*, 1994). More importantly, it is similar to that of the wild-type Mek1 activated by Raf-1 in vitro. Therefore, the mutations [Asp^{218}, Asp^{222}] and [Asp^{218}, Glu^{222}] activated Mek1 to a degree near that achieved by stoichiometric phosphorylation. The effectiveness of these mutations argues that acidic residues can sufficiently mimic phosphoserines in the structure of Mek1. Others have reported that bacterially expressed [Glu^{218}, Glu^{222}] and [Glu^{218}, Asp^{222}] Mek1 mutants are 40- and 80-fold,

respectively, more active than wild-type Mek1 (Alessi *et al.*, 1994; Mansour *et al.*, 1994). These reports are consistent with our previous finding that the [Glu^{218}, Glu^{222}] and [Glu^{218}, Asp^{222}] mutants are less active than [Asp^{218}, Asp^{222}] and [Asp^{218}, Glu^{222}] when they are expressed in COS-7 cells (Huang and Erikson, 1994). It is possible that due to spatial hindrance, the more bulky glutamate residue is less effective in mimicking phosphoserine than aspartate at 218. Consistent with this notion is the observation that among Mek family members, the serine at 218 is conserved whereas the serine at 222 is frequently replaced by the more bulky threonine (Figure 1).

The dual phosphorylation sites of Mek1 are strikingly parallel to those of MAP kinase, suggesting that they may have a similar mechanism of activation. The activation of Erk1 requires the phosphorylation of both threonine 183 and tyrosine 185 (Alessandrini *et al.*, 1992) and the dephosphorylation of either site inactivates the enzyme (Anderson *et al.*, 1990; Gomez and Cohen, 1991). Previous studies have shown that alanine substitution of either serine 218 or 222 substantially reduces the capacity of Mek1 to be activated by Raf-1 (Alessi *et al.*, 1994; Zheng and Guan, 1994). This observation is corroborated by the results of our in vitro phosphorylation experiments. The [Val^{218}] and [Val^{222}] Mek1 mutants were activated by Raf-1 to a specific activity less than 5% that of wild-type Mek1 activated by Raf-1 (Table 2). Moreover, although the double acidic substitutions can activate Mek1 by over 6000-fold, the single acidic substitutions activate Mek1 by only 10- to 50-fold (Table 1). The apparent synergistic effect of the acidic substitutions suggests that the two phosphorylation events on serine 218 and 222 play cooperative roles in the activation of Mek1. Unlike Erk1, in which the preferred order of phosphorylation is tyrosine 185 first and threonine 183 second (Haystead *et al.*, 1992), the two phosphorylation events on serine 218 and 222 of Mek1 do not appear to have a particular order. The Mek1 mutants with a single acidic substitution on either site were all phosphorylated by Raf-1 to approximately the same extent as those mutants with a single neutral substitution (Table 2). The Mek1 mutants with acidic substitutions at both 218 and 222 were not further activated by Raf-1, suggesting that it is unlikely that phosphorylation sites other than serine 218 and 222 contribute to the activation of Mek1. It should be noted that although the [Val^{218}, Val^{222}] Mek1 mutant was minimally phosphorylated by Raf-1, it was activated about 40-fold (Table 2). We speculate that a stable association of Mek1 and Raf-1 (Huang *et al.*, 1993) may partially activate Mek1 by altering its structure.

In the oocyte maturation pathway, Mos acts upstream of Mek (Dent *et al.*, 1992; Howe *et al.*, 1992; Kyriakis *et al.*, 1992; Posada *et al.*, 1993; Shibuya and Ruderman, 1993). The activation of Mos is sufficient to induce GVBD and activate histone H1 kinase (Yew *et al.*, 1992). Our microinjection experiments show that the activation of Mek is also sufficient to induce GVBD and activate histone H1 kinase. Like Mos, the induction of maturation by Mek can only be partially inhibited by cycloheximide. The similar fashion in which Mos and Mek promote maturation strongly suggests that Mek is the major downstream effector of Mos in the initiation of meiotic maturation.

The downstream target of Mek, MAP kinase, is activated during oocyte maturation. Consistent with our results, experiments using a thiophosphorylated form of MAP kinase have demonstrated that the activation of MAP kinase is sufficient to induce meiotic maturation and activate histone H1 kinase (O. Haccard and J. Maller, unpublished data). Together, these data suggest that the MAP kinase pathway plays a key role in transmitting the maturation signal from Mos to MPF. It is of immediate importance to elucidate the signaling process between the MAP kinase pathway and MPF.

Two approaches may be taken to identify signaling components between the MAP kinase pathway and MPF. The first approach would involve identifying components that directly regulate MPF. For example, a protein kinase has been identified that activates MPF through phosphorylation of $p34^{cdc2}$, the catalytic subunit of MPF (Frequet *et al.*, 1993; Poon *et al.*, 1993; Solomon *et al.*, 1993). The kinase, termed CAK, is composed of $p40^{MO15}$ (a $p34^{cdc2}$-related protein) and cyclin H (Fisher and Morgan, 1994; Makela *et al.*, 1994). Our unpublished results indicate that Mek and MAP kinase do not phosphorylate $p40^{MO15}$ in vitro (W. Huang and R.L. Erikson, unpublished data). Indeed, because the activity of $p40^{MO15}$ remains constant throughout meiotic maturation (Brown *et al.*, 1994), it seems unlikely that Mek may act as a $p40^{MO15}$ kinase. The second approach is to search for downstream targets of Mek (and MAP kinase) using tools such as affinity chromatography and the interaction-trap screen (Fields and Song, 1989). The constitutively active Mek1 mutants that we describe here should facilitate this latter approach.

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