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Biochemistry

Raf-1 forms a stable complex with Mek1 and activates Mek1 by serine phosphorylation

(mitogen-activated protein kinase/protein kinases/signal transduction)

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ABSTRACT **Recombinant Mek1 and Raf-1 proteins produced in Sf9 cells undergo a tight association both *in vivo* and *in vitro*, which apparently does not depend on additional factors or the kinase activity of Mek1 or Raf-1. The complex can be disrupted by two polyclonal antibodies raised against Raf-1 peptides. Coinfection with Raf-1 activates Mek1 >150-fold, and coinfection with Raf-1 and Mek1 activates Erk1 ≈90-fold. The activation of Mek1 by Raf-1 involves only serine phosphorylation, which is directly proportional to the extent of Mek1 activation. Phosphopeptide maps suggest a single Raf-1 phosphorylation site on Mek1.**

A variety of mitogenic signals, including phorbol ester, insulin, epidermal growth factor, platelet-derived growth factor, and nerve growth factor, activate a family of serine/threonine kinases named MAP (mitogen-activated protein)/MBP (myelin basic protein) kinases or Erks (extracellular signal-regulated kinases). Erks are believed to play important roles in transducing extracellular signals from receptor tyrosine kinases to downstream serine/threonine kinases (1–4). Activation of Erk1 involves sequential phosphorylation of its Tyr-185 and Thr-183 residues (5–7). The upstream activator responsible for these phosphorylation events, the dual-specificity kinase Mek1 (MAP kinase/Erk kinase; also called MAP kinase kinase), has been purified and cloned from mouse (8), rat (9), human (10), *Xenopus* (11), and *Drosophila* (12). Mek1 appears to be regulated by reversible serine/threonine phosphorylation but not by tyrosine phosphorylation. It can be inactivated by treatment with the serine/threonine-specific protein phosphatase 2A (13, 14).

Several recent studies suggested that Raf-1, the protein kinase encoded by the protooncogene c-*raf-1*, is an activator of Mek1 (15–17). It has been reported that the *in vitro* activation of partially purified murine Mek1 by Raf-1 correlates with phosphorylation of Mek1 on serine and threonine residues (15). *In vivo*, activated Mek1 is also phosphorylated on serine and threonine residues in mammalian cells (18) and on threonine residues in *Xenopus* (19). In this report, we describe the tight association of Mek1 and Raf-1 and the activation of Mek1 by serine phosphorylation.

MATERIALS AND METHODS

Expression of Proteins in Insect Cells. The *ERK1* baculovirus construct, pBacERK1, was made by cloning a *Bam*HI/*Aat* II (blunt end) *ERK1* fragment from pGEX3X-ERK1 (20) into the *Bam*HI/*Kpn* I (blunt end) site of pAc702 (Invitrogen). pBacERK1 was then cotransfected into Sf9 cells with Baculogold (Pharmigen). BacERK1 baculovirus was harvested 10 days later and then underwent two further rounds of amplification. Baculovirus constructs of epitope-tagged *MEK1* and *MEK1* (K97A) were gifts of S. G. Macdonald (Onyx Pharmaceuticals, Richmond, CA). Baculovirus constructs of *RAF-1* and *RAF301* were gifts of T. M. Roberts (21). Sf9 cells were cultured in suspension at 27°C in IPL-41 (JRH)/10% heat-inactivated fetal bovine serum/Yeastolate (VWR Scientific)/lactalbumin hydrolysate (VWR Scientific)/0.1% pluronic acid F-68 (GIBCO). After baculoviral infection proceeded for 48 h, Sf9 cells were harvested by centrifugation and stored at −70°C.

Antibodies and Western Blot Analyses. The anti-EE monoclonal antibody, which reacts with the sequence used as an epitope tag, was a gift of G. Walter (22). The C12 and K153 anti-Raf-1 polyclonal antibodies and the 956/837 anti-Erk1 polyclonal antibody were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The raf1-III anti-Raf-1 polyclonal antibody was purchased from Upstate Biotechnology (Lake Placid, NY). Proteins resolved by SDS/PAGE were transferred to poly(vinylidene difluoride) blotting membrane (Immobilon-P; Millipore) for 1.5 h at 80 V in 25 mM Tris·HCl/192 mM glycine/20% methanol/0.5 mM Na_3VO_4 at 4°C. Western blot analyses were performed at room temperature using the enhanced chemiluminescence (ECL) Western detection system (Amersham). Blots were blocked in 8% bovine serum albumin/phosphate-buffered saline (PBS)/0.1% Tween 20 for 1 h and then washed with PBS/0.1% Tween 20 (PBST) for 5 min. After incubation with 0.1 μg of primary antibody per ml for 1 h, blots were washed three times with PBST for 5 min each. Anti-mouse or anti-rabbit IgG conjugated to horseradish peroxidase (Amersham) was diluted 1:2000 in PBST and incubated with blots for 20 min. Finally, blots were washed with PBST four times for 5 min each, incubated with the ECL detection reagent (Amersham) for 1 min, and exposed to Fuji RX film.

Affinity Chromatography. Anti-EE antibody (20 mg) was conjugated to 1 ml (bed volume) of protein G plus agarose (Santa Cruz Biotechnology) as described (23). Sf9 cells were Dounce-homogenized in lysis buffer A: 10 mM potassium phosphate, pH 7.1/1 mM EDTA/5 mM EGTA/10 mM $MgCl_2$/50 mM β-glycerol phosphate/2 mM Na_3VO_4/2 mM dithiothreitol (DTT)/1 mM phenylmethylsulfonyl fluoride/10 μg of leupeptin per ml/10 μg of pepstatin per ml. The homogenate was cleared by centrifugation at 12,000 × *g* for 10 min. NaCl and *n*-octyl glucoside were added to the supernatant to a concentration of 80 mM and 0.1%, respectively, before it was applied to the anti-EE affinity column. The column was washed with 10 column vol of PBS/0.1% Nonidet P-40 and 10 column vol of lysis buffer A/100 mM NaCl/0.1% *n*-octyl glucoside and eluted with 1 column vol of EE peptide at 50 μg/ml/20 mM Tris·HCl, pH 8.2/100 mM NaCl/2 mM 2-mercaptoethanol.

Kinase Assays. MBP kinase assays and Mek assays were performed as described (7, 14). Mek1 kinase activity was

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Abbreviations: MAP kinase, mitogen-activated protein kinase; MBP, myelin basic protein; Erk, extracellular signal-regulated kinase; Mek, MAP kinase/Erk kinase; GST, glutathione *S*-transferase.

assayed in 40 μl of 100 μM ATP/10 mM DTT/20 mM $MgCl_2$/100 mM Tris·HCl, pH 7.5/0.2 mg of ovalbumin per ml, using 2 μg of Mek1 (K97A) and 20 μCi of [γ-^{32}P]ATP (1 Ci = 37 GBq) per reaction. The kinase reaction mixtures were incubated at 30°C for 10 min and analyzed by SDS/PAGE. Incorporated phosphate was determined by liquid scintillation counting of dried SDS/PAGE gel bands.

Immunoprecipitation and Binding Assays. Immunoprecipitation was performed at 4°C in 1 ml of PBS/0.5% Triton X-100, using 1 μg of antibody and 10 μl (bed vol) of protein G plus agarose. Immunoprecipitates were washed four times with 1 ml of ice-cold PBS/0.5% Triton X-100 before they were used for binding assays or kinase assays. In Mek1 binding assays, 5 μg of affinity-purified Mek1 was incubated with immunoprecipitates in 100 μl of lysis buffer A/100 mM NaCl/0.1% *n*-octyl glucoside at 37°C for 30 min. Then immunoprecipitates were washed twice with 1 ml of lysis buffer A/100 mM NaCl/0.1% *n*-octyl glucoside and twice with 1 ml of PBS/0.5% Triton X-100.

Phosphoamino Acid Analyses and Phosphopeptide Mapping. Phosphoamino acid analyses and phosphopeptide mapping were performed as described (20).

RESULTS

***In Vivo* Association of Mek1 and Raf-1.** Sf9 cells were coinfected with recombinant baculoviruses expressing mouse Mek1, which had been N-terminally tagged with the EE epitope (24), and with baculoviruses expressing human Raf-1. Anti-EE affinity chromatography of the lysate of infected cells resulted in the copurification of two proteins with apparent molecular masses of ≈70 and ≈47 kDa upon SDS/PAGE (Fig. 1*A*, lane 2). Western blot analyses using an anti-Raf-1 antibody (raf1-III) and an anti-EE antibody (22) identified the ≈70-kDa protein as Raf-1 (Fig. 1*B*, lane 2) and the ≈47-kDa protein as the epitope-tagged Mek1 (Fig. 1*C*, lane 2). Because the anti-EE antibody did not recognize the Raf-1 protein (Fig. 1*C*, lane 2), we concluded that Raf-1 was purified by the anti-EE affinity chromatography because of its association with Mek1. Sf9 cells were also infected with baculovirus constructs expressing Mek1 and Raf301, a kinase-inactive mutant of Raf-1 (Lys-375 to Trp). From the lysate of the coinfected cells, Raf301 also copurified with Mek1 by anti-EE affinity chromatography (data not shown). This result indicates that the kinase activity of Raf-1 is not required for its association with Mek1 *in vivo*.

The association of Mek1 and Raf-1 persisted in 3.0 M NaCl, 70% ethylene glycol, 0.1% Nonidet P-40, or 3% *n*-octyl glucoside. The complex did not seem to depend on disulfide bonds, as it was dissociated by SDS/PAGE in the absence of 2-mercaptoethanol (data not shown). To examine the effects of anti-Raf-1 antibodies on the association of Mek1 and Raf-1, we used three different anti-Raf-1 polyclonal antibodies (C12, K153, and raf1-III) to immunoprecipitate the affinity-purified Mek1/Raf-1 complex. Although the K153 and raf1-III antibodies immunoprecipitated about the same amount of Raf-1 (Fig. 2*A*, lanes 6 and 8) as the C12 antibody (Fig. 2*A*, lane 4), the Mek1 coimmunoprecipitated with K153 and raf1-III (Fig. 2*B*, lanes 6 and 8) was significantly less than that coimmunoprecipitated with C12 (Fig. 2*B*, lane 4). Since the Mek1/Raf-1 complex was previously purified by anti-EE affinity chromatography, all the Raf-1 was associated with Mek1 before interaction with the antibody. It appeared that the K153 and raf1-III antibodies (raised against residues 37–49 and 449–473 of Raf-1, respectively) disrupted the association of Mek1 and Raf-1.

***In Vivo* Activation of Mek1 and Erk1 by Raf-1.** Using a kinase-inactive recombinant Erk1 protein, glutathione *S*-transferase (GST):Erk1 (K63M) (7) as substrate, we compared the specific activities of Mek1 affinity-purified from Sf9 cells singly infected, or coinfected with Raf-1 or Raf301. We found that coinfection with Raf-1, but not with Raf301, increased the ability of Mek1 to phosphorylate GST:Erk1 (K63M) >150-fold (data not shown). To test whether the phosphorylation of Erk1 by Raf-1-activated Mek1 results in activation of Erk1, we coinfected Sf9 cells with baculovirus constructs of *ERK1*, *RAF-1*, and *MEK1* or *MEK1* (K97A), a kinase-inactive mutant of MEK1 (Lys-97 to Ala). From the lysates of infected cells, we immunoprecipitated Erk1 (Fig. 3*B*) and measured its MBP kinase activity (Fig. 3*A*). The coinfection with Mek1 did not increase the capacity of Erk1 to phosphorylate MBP (Fig. 3*A*, lane 2). The coinfection with Mek1 and Raf-1, however, activated Erk1 ≈90-fold (Fig. 3*A*, lane 3). This activation apparently requires the kinase activity of Mek1, since the coinfection with Mek1 (K97A) and Raf-1 increased Erk1 activity <3-fold (Fig. 3*A*, lane 4).

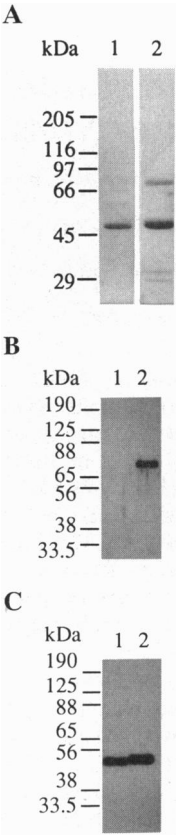

FIG. 1. Raf-1 is copurified with Mek1 upon anti-EE affinity chromatography. Lysates of 1.5 × 10^7 Sf9 cells infected either with *MEK1* or with *MEK1* and *RAF-1* baculovirus constructs were applied to 0.2-ml anti-EE affinity columns. The columns were washed and eluted with the EE peptide as described. The eluted proteins (1/100th) were resolved by SDS/10% PAGE. Lanes: 1, *MEK1* single infection; 2, *MEK1* and *RAF-1* coinfection. (*A*) Gel stained with Coomassie blue. (*B*) Anti-Raf-1 (raf1-III) Western blot. (*C*) Anti-EE Western blot.

***In Vitro* Association of Mek1 and Raf-1.** It is possible that factors other than Mek1 and Raf-1 are required for their association. To investigate this possibility, we incubated immunoprecipitates of Raf-1 or Raf301 with affinity-purified Mek1 in the presence or absence of ATP and/or GTP. The proteins bound to the immunoprecipitates were analyzed by Western blot analyses. As shown in Fig. 4, Mek1 associated with the immunoprecipitates only when Raf-1 was present. This association apparently did not require any other factor and was not influenced by the presence of ATP or GTP (Fig. 4*B*, lanes 2–5). The kinase activity of Raf-1 was not required, as Mek1 associated with the immunoprecipitates of Raf301 (Fig. 4*B*, lane 6). In a separate similar experiment, we found

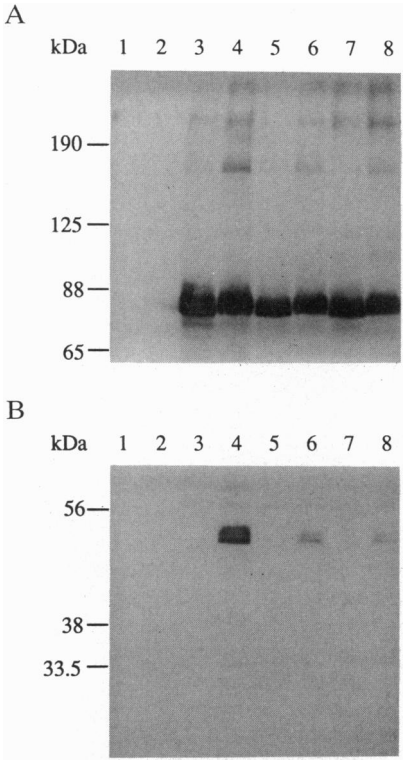

FIG. 2. Two anti-Raf-1 polyclonal antibodies reduce the association of Mek1 and Raf-1. Three polyclonal antibodies were used to immunoprecipitate Raf-1 from either a lysate of *RAF-1*-infected Sf9 cells or the affinity-purified Mek1/Raf-1 complex. The immunoprecipitates were resolved by SDS/7.5% PAGE and subjected to Western blot analyses. Lanes: 1, 3, 5, and 7, immunoprecipitates of *RAF-1*-infected Sf9 cell lysate; 2, 4, 6, and 8, immunoprecipitates of affinity-purified Mek1/Raf-1 complex; 1 and 2, no antibody; 3 and 4, 1 μg of C12; 5 and 6, 1 μg of K153; 7 and 8, 3.5 μg of raf1-III. (*A*) Anti-Raf-1 (C12) Western blot. (*B*) Anti-EE Western blot.

that affinity-purified Mek1 (K97A) also associated with the immunoprecipitate of Raf-1 (data not shown). Thus, the kinase activity of Mek1 is not required for the interaction of Raf-1 and Mek1.

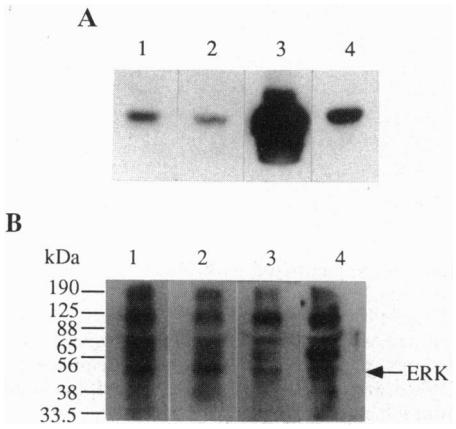

FIG. 3. Raf-1 activates Erk1 through Mek1. An anti-Erk1 polyclonal antibody (956/837) was used to immunoprecipitate Erk1 from lysates of Sf9 cells infected with various combinations of *ERK1*, *MEK1*, *MEK1* (K97A), and *RAF-1* baculovirus constructs. The activity of Erk1 in the immunoprecipitates was assayed with MBP as substrate and the amount of Erk1 in the immunoprecipitates was compared by Western blot analysis. Lanes: 1, *ERK1*; 2, *ERK1* plus *MEK1*; 3, *ERK1* plus *MEK1* plus *RAF-1*; 4, *ERK1* plus *MEK1* (K97A) plus *RAF-1*. (*A*) MBP kinase activity. (*B*) Anti-Erk1 (956/837) Western blot.

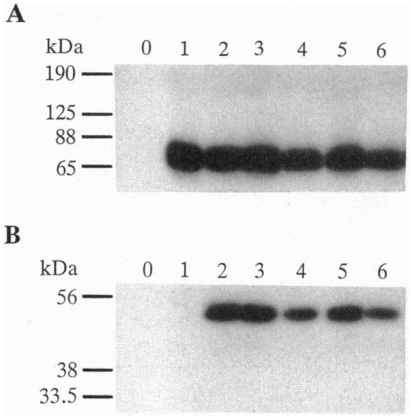

FIG. 4. Mek1 specifically associates with immunoprecipitated Raf-1. Anti-Raf-1 (2 μg of C12) immunoprecipitates from lysates of 1.5×10^6 *RAF-1*- or *RAF301*-infected Sf9 cells were incubated with 5 μg of affinity-purified Mek1 at 37°C for 30 min in the presence or absence of ATP or GTP. After incubation, the immunoprecipitates were washed, resolved by SDS/10% PAGE and subjected to Western blot analyses. Lanes: 0, mock immunoprecipitate (without antibody) of Raf-1 plus Mek1; 1, Raf-1 immunoprecipitate; 2, Raf-1 immunoprecipitate plus Mek1; 3, Raf-1 immunoprecipitate plus Mek1 plus 0.2 mM ATP; 4, Raf-1 immunoprecipitate plus Mek1 plus 0.2 mM GTP; 5, Raf-1 immunoprecipitate plus Mek1 plus 0.2 mM ATP plus 0.2 mM GTP; 6, Raf301 immunoprecipitate plus Mek1 plus 0.2 mM ATP plus 0.2 mM GTP. (*A*) Anti-Raf-1 (C12) Western blot. (*B*) Anti-EE Western blot.

***In Vitro* Activation of Mek1 by Raf-1.** When affinity-purified Mek1 was incubated with immunoprecipitates of Raf-1 in the presence of ATP, we observed that both the Mek1 associated with the immunoprecipitate and the Mek1 present in the supernatant became phosphorylated and activated. Both phosphorylation and activation required ATP as well as the kinase activity of Raf-1 as the immunoprecipitate of Raf301 did not phosphorylate or activate Mek1 (data not shown). The amount of phosphate incorporated into Mek1 during the course of incubation was directly proportional to the extent of Mek1 activation (Fig. 5*A*). The autophosphorylation of Mek1 appears to be insignificant, since Mek1 (K97A) showed a time course of phosphorylation similar to that of Mek1 (Fig. 5*B*). Phosphoamino acid analyses showed that Mek1 (K97A) was phosphorylated exclusively on serine (Fig. 5*D*) and that Mek1 was phosphorylated on serine with trace amounts of threonine and tyrosine phosphorylation (Fig. 5*C*). The two-dimensional tryptic phosphopeptide maps of Mek1 and Mek1 (K97A) both revealed one major phosphopeptide (Fig. 5 *E* and *F*). The tryptic phosphopeptide of Mek1 appears to be identical to that of Mek1 (K97A), as they comigrated upon two-dimensional fractionation of the tryptic peptides (data not shown).

DISCUSSION

We have demonstrated the association of Mek1 and Raf-1 both *in vivo* and *in vitro*. The formation of the complex does not appear to require any additional factors or the kinase activity of either protein. Moreover, the interaction between the two proteins survived common chemical treatments but was dissociated under strong denaturing conditions. Therefore, it was surprising to observe that the binding of two anti-Raf-1 antibodies could also promote dissociation. The K153 and raf1-III anti-Raf-1 antibodies may disrupt the Mek1/Raf-1 complex either by competitive binding of the Raf-1 domain that interacts with Mek1 or by changing the global conformation of Raf-1 protein. The former case seems unlikely, however, because small amounts of Mek1 were coimmunoprecipitated with Raf-1 by K153 and raf1-III (Fig. 2*B*). It is generally accepted that the physical interaction between kinases and

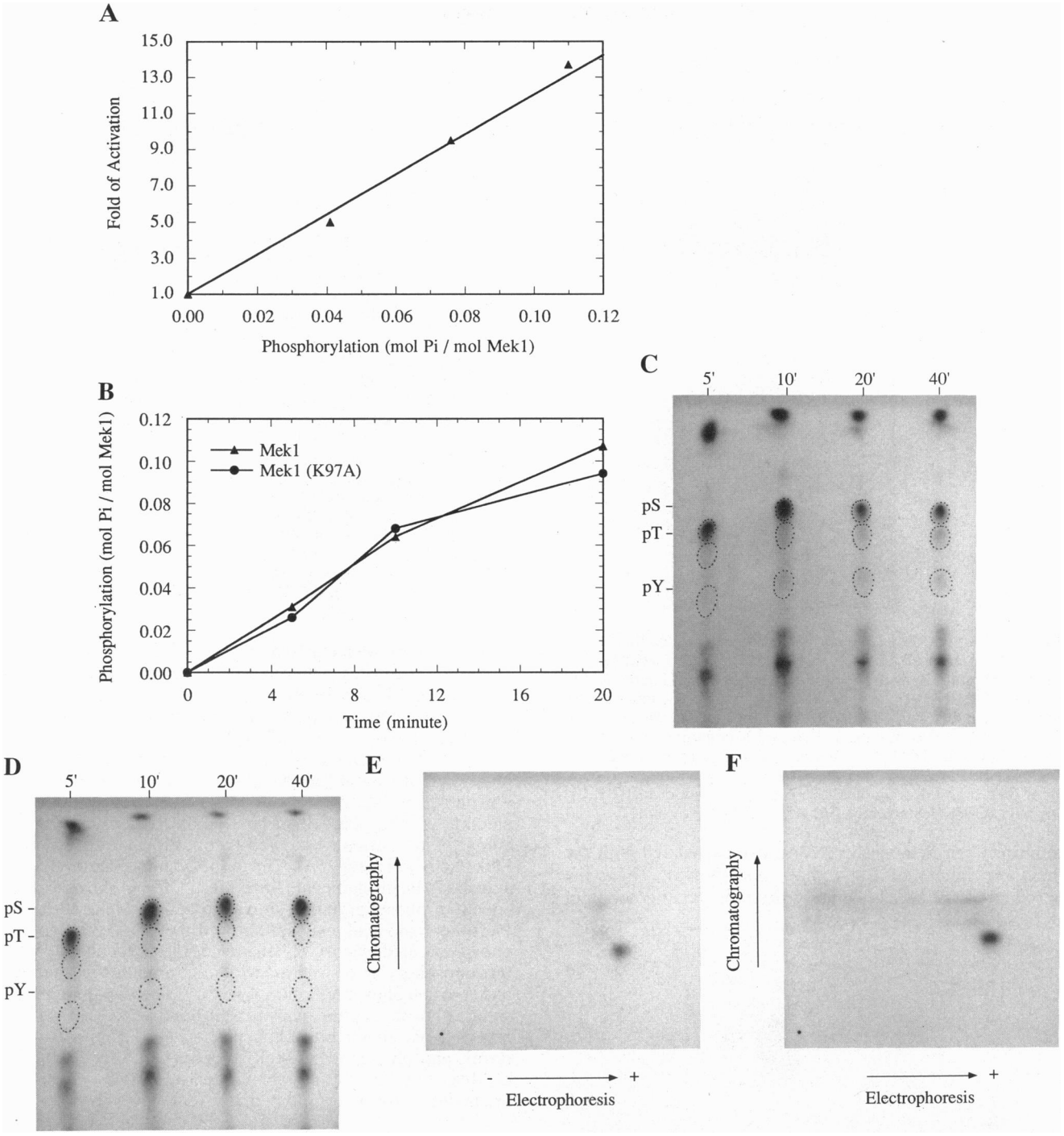

FIG. 5. Raf-1 activates Mek1 by serine phosphorylation. Immunoprecipitates of Raf-1 were incubated with Mek1 or Mek1 (K97A) in the presence of 100 μM ATP and 1 μCi of [γ-^{32}P]ATP per μl at 30°C. At various times, Mek1 and Mek1 (K97A) supernatants were separated from Raf-1 immunoprecipitates and the amount of phosphate incorporation was measured. The ability of Mek1 to phosphorylate GST:Erk1 (K63M) was also measured to determine the extent of activation. (*A*) Activation of Mek1 vs. phosphorylation of Mek1. (*B*) Time course of Mek1 and Mek1 (K97A) phosphorylation. (*C*) Phosphoamino acid analyses of Mek1 at various times. pS, phosphoserine; pT, phosphothreonine; pY, phosphotyrosine. (*D*) Phosphoamino acid analyses of Mek1 (K97A) at various times. (*E*) Two-dimensional tryptic phosphopeptide map of Mek1. (*F*) Two-dimensional tryptic phosphopeptide map of Mek1 (K97A). Black dot at bottom left marks sample origin.

their substrates is transient and may only be detected by sensitive methods such as the "interaction trap" system (25). The Raf-1/Mek1 association appears to be an exception to this paradigm. It seems unlikely that the strong association of Mek1 and Raf-1 is necessary as an intermediate step of the phosphorylation process. A significant fraction of Mek1 is known to be present in the membrane fraction of mitogen-stimulated cells (14). One possible role of this association could be to localize Raf-1 to the cell membrane. Others have recently used the interaction trap system to demonstrate interactions between Ras and Raf-1 and between Mek1 and Raf-1 (26). Thus, it is possible that Raf-1 may be localized to the membrane by association with Mek1 and Ras and the localization promotes downstream effects.

In the studies described here, we have obtained activation of Mek1 and Erk1 in insect cells by expression of Raf-1 alone. Others (21) have described the activation of Raf-1 that requires upstream components such as Src and Ras. We have

not compared the degree of activation of Mek1 that is possible by coinfection with Src or Ras, but it appears from these data that overexpression of Raf-1 alone is sufficient for at least partial activation of Mek1.

We have also shown that Raf-1 activates Mek1 by serine phosphorylation. Our phosphopeptide maps suggest a single phosphorylation site of Mek1 by Raf-1 (Fig. 5 *E* and *F*). Although a small fraction of threonine and tyrosine phosphorylation was observed in Mek1 (Fig. 5*C*), it probably resulted from autophosphorylation because Mek1 (K97A) was phosphorylated only on serine (Fig. 5*D*). It has been reported that the *Xenopus* Mek1 is activated by a high molecular weight kinase, which phosphorylates it mainly on serine and is also phosphorylated by *Xenopus* MAP kinase on threonine without activation (27). We have also found that MAP kinase can phosphorylate Mek1 on threonine (unpublished data). It is likely that previous reports describing Mek1 phosphorylation on threonine as well as serine are complicated by the fact that kinases other than Raf-1 may phosphorylate Mek1. Recently, a Mek1 kinase homologous to *Saccharomyces cerevisiae* STE11 protein and *Schizosaccharomyces pombe* Byr2 protein has been cloned and shown to activate Mek1 independently of Raf-1 (28). Thus, it seems plausible that the reported serine and threonine phosphorylations of Mek1 may have resulted from phosphorylation by multiple kinases.

The significance of the protein interaction reported here is unclear. It will be necessary to define the domains of Raf-1 and Mek1 necessary for complex formation. Our observation that Raf-1 can phosphorylate the Mek1 protein with which it associates suggests that the catalytic domain of Raf-1 and the phosphorylation sites of Mek1 are involved in their interaction. To delineate the Mek1/Raf-1 interaction more precisely, mutagenesis studies of each protein will be required in conjunction with the determination of Mek1 phosphorylation sites. A test of limited domains of each protein may reveal regions that are important for the formation of the complex. The use of specific peptides to inhibit complex formation should serve to elucidate additional details of Mek1/Raf-1 interaction.

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