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Constitutive activation of Mek1 by mutation of serine phosphorylation sites

(signal transduction/mitogen-activated protein kinase/Raf-1)

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ABSTRACT **A variety of extracellular signals lead to the phosphorylation and activation of mitogen-activated protein kinases (MAP kinases). An activator of MAP kinases, Mek1, phosphorylates MAP kinases at threonine and tyrosine residues and is itself phosphorylated at serine-218 and -222 by the protooncogene product Raf-1. By introducing negatively charged residues that may mimic the effect of phosphorylation at positions 218 and 222, we have activated the capacity of Mek1 to phosphorylate MAP kinase by >100-fold. The most effective activation by a single substitution resulted from the introduction of aspartate at position 218, whereas the introduction of either aspartate or glutamate at position 222 was ineffective. Expression of the activated Mek1 phosphorylation-site mutants in COS-7 cells led to the activation of MAP kinase in the cells and resulted in an increase in the mass of the transfected COS-7 cell population, suggesting an important role of Mek1 in the transduction of mitogenic signals.**

Studies on the activation of serine/threonine-specific protein kinases in mitogen-treated animal cells have uncovered a highly conserved signal transduction pathway. The first enzyme in the pathway to be well-characterized, mitogen-activated protein kinase (MAP kinase, also known as Erk, for extracellular signal-regulated kinase) is related in sequence to Fus3p in *Saccharomyces cerevisiae* and Spk1p in *Schizosaccharomyces pombe* (for reviews, see refs. 1–4). The enzymes that activate MAP kinase have been characterized more recently. Matsuda *et al.* (5) purified a MAP kinase activator from mature *Xenopus* oocytes, using a recombinant MAP kinase expressed in bacteria as substrate. The *Xenopus* MAP kinase activator is a M_r 45,000 phosphoprotein that is inactivated by treatment with phosphatase 2A. Purification of a similar activator, denoted Mek (for MAP kinase/Erk kinase), from mammalian cells was carried out by using recombinant glutathione *S*-transferase (GST)–Erk1 fusion protein as substrate. Mek is a threonine/tyrosine-specific protein kinase that apparently phosphorylates and activates MAP kinase in animal cells (6, 7). Peptide sequencing revealed strong similarity to the *Sch. pombe byr1* gene product (8). Subsequently, the sequence of a Mek cDNA revealed it is 60% similar and 45% identical to Byr1p and closely related to Ste7p from *S. cerevisiae* (9–11). These three enzymes are also activated by conserved upstream protein kinases, Mek kinase, Ste11p, and Byr2p in animal cells, *S. cerevisiae* and *Sch. pombe*, respectively. The protooncogene product Raf-1 also serves as a Mek kinase in animal cells (12–14), but no protein kinase with a closely related sequence has been detected in yeast. In *S. cerevisiae*, members of this set of enzymes are utilized in three distinct cellular processes as diverse as mating-factor response, cell-wall construction, and response to osmotic changes in the environment (for review, see ref. 15). Evidently, unique features in enzymes with a similar sequence have evolved to limit their function to a single pathway.

In animal cells, protein kinases at each step in the pathway are often encoded by at least two distinct genes. For example, *Erk1* and *Erk2* encode $p44^{MAPK}$ and $p42^{MAPK}$, respectively (16), and *Mek1* and *Mek2* encode enzymes with distinct sequences (17, 18). Studies *in vitro* with purified enzymes have not revealed significant functional differences, nor have upstream extracellular signals been shown to selectively activate one of the pair at each step. The presence in *S. cerevisiae* of different signaling systems that utilize different versions of these enzymes suggests that *Mek1* and *-2* and *Erk1* and *-2* are not necessarily redundant but may, in fact, participate in different pathways. Assignment of these enzymes and others in this array to a specific signaling function requires, as it does in yeast, a combination of gene deletion, expression of the appropriate mutants of the enzymes, and biochemistry.

We have initiated studies to determine the influence of the introduction of activated enzymes on cell behavior. Recently, the serine residues in Mek1 that are required for phosphorylation and activation by Raf-1 have been identified (19, 20). These residues, Ser^{218} and Ser^{222}, lie between kinase subdomains VII and VIII. This region contains sites for activating phosphorylations in other protein kinases also, including Ste7p in *S. cerevisiae* (21). We show here that the double substitution of aspartate residues at positions 218 and 222 has a remarkable capacity to activate Mek1, whereas glutamate residues are usually less effective. The activity of Mek1 mutants *in vitro* correlates generally with their capacity to activate Erk1 in living cells.

MATERIALS AND METHODS

Mutagenesis and Protein Expression. pG·Mek·Cglu, a pGEM-7Zf(−) vector (Promega) carrying a mouse *Mek1* gene encoding a protein C-terminally tagged with the EE epitope (22), was a gift of S. G. Macdonald (Onyx Pharmaceuticals, Richmond, CA). Single mutations of Ser^{218} or Ser^{222} were generated in this vector by using Amersham's site-directed mutagenesis system (RPN1523) and were verified by dideoxynucleotide sequencing. Double mutations of Ser^{218} and Ser^{222} were generated by subcloning the *Aat* II–*Msc* I fragment of the Ser^{218} mutants into the *Aat* II–*Msc* I site of the Ser^{222} mutants. For expression in COS-7 cells, the *Sph* I–*Nhe* I fragment from pG·Mek·Cglu was subcloned into the *Sph* I–*Xba* I site of pCDNAI/Amp (Invitrogen). COS-7 cells were grown to 50–70% confluency in Dulbecco's modified Eagle's medium (DMEM)/10% heat-inactivated fetal bovine serum (FBS). COS-7 cells (10^7) were transfected with 10 μg of pCDNAI/Amp·Mek1 mutants by electroporation. After growth in DMEM/10% FBS for 48 hr, the cells were har-

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Abbreviations: MAP kinase, mitogen-activated protein kinase; MBP, myelin basic protein; GST, glutathione *S*-transferase; PMA, phorbol 12-myristate 13-acetate.

vested and frozen in liquid nitrogen. As a positive control, cells transfected with wild-type Mek1 construct were stimulated with phorbol 12-myristate 13-acetate (PMA, 100 ng/ml) for 5 min before they were harvested.

Antibodies and Western Analyses. The anti-EE monoclonal antibody, which reacts with the sequence used as an epitope tag, was a gift of G. Walter (23). The anti-Mek1 monoclonal antibody 3D9 was a gift of C. M. Crews (Harvard University). The anti-Erk1 polyclonal antibodies C-16 and K-23 were from Santa Cruz Biotechnology (Santa Cruz, CA). Proteins resolved by SDS/PAGE were transferred to poly(vinylidene difluoride) blotting membrane (Immobilon-P; Millipore) for 1.5 hr at 80 V in 25 mM Tris base/192 mM glycine/20% methanol/0.5 mM sodium vanadate at 4°C. Western blot analyses were performed at room temperature using the enhanced chemiluminescence (ECL) Western blot detection system (Amersham). Blots were blocked in 8% bovine serum albumin/phosphate-buffered saline (PBS)/0.1% Tween 20 for 1 hr and then washed with PBS/0.1% Tween 20 (PBST) for 5 min. After incubation with primary antibody at 0.1 μg/ml for 1 hr, blots were washed three times with PBST for 5 min each. Anti-mouse IgG or protein A 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.

Immunocomplex Kinase Assays. COS-7 cell pellets were Dounce-homogenized in lysis buffer A [10 mM potassium phosphate, pH 7.1/1 mM EDTA/5 mM EGTA/10 mM magnesium chloride/50 mM β-glycerophosphate/2 mM sodium vanadate/2 mM dithiothreitol/1 mM phenylmethanesulfonyl fluoride with leupeptin (10 μg/ml) and pepstatin A (10 μg/ml)]. The homogenates were centrifuged at 100,000 × *g* for 30 min. The protein concentration of the supernatants (cytosolic fractions) was determined by the Bradford assay (Bio-Rad, no. 5000-006). Immunoprecipitation was performed at 4°C in 1 ml of PBS/0.5% Triton X-100, using 1 μg of antibody, 10 μl (bed volume) of protein G agarose (Santa Cruz Biotechnology, Santa Cruz, CA) and 100 μg of cytosolic proteins. Immunoprecipitates were washed four times with 1 ml of ice-cold PBS/0.5% Triton X-100 and then incubated with 40 μl of kinase "cocktail" for 10 min at 30°C. The Erk1 kinase cocktail contained 50 mM Tris (pH 8.0), 5 mM dithiothreitol, 0.1 mg of ovalbumin per ml, 3 mM magnesium acetate, 50 μM ATP, 10 mM sodium fluoride, 1 mM sodium vanadate, 1 mM EGTA, 250 μCi of [γ-^{32}P]ATP per ml (1 μCi = 37 kBq), and 0.025 mg of GST–Erk1(K63M) [a kinase-inactive Erk1 protein fused to GST (6)] per ml. The myelin basic protein (MBP) kinase cocktail contained 25 mM Tris (pH 7.5), 10 mM magnesium chloride, 0.1 mg of bovine serum albumin per ml, 100 μM ATP, 0.125 mg of MBP per ml, and 25 μCi of [γ-^{32}P]ATP per ml. Kinase reactions were terminated by the addition of 10 μl of 5× SDS/PAGE sample buffer. The kinase reaction mixtures were resolved by SDS/PAGE in 10% (for Erk1 kinase assay) or 12.5% (for MBP kinase assay) polyacrylamide gels and the separated proteins were transferred to Immobilon-P membrane. After autoradiography, the quantity of phosphate incorporated into substrate was determined by liquid scintillation counting. The quantity of kinase in the immunocomplex was measured by Western blot analysis.

RESULTS

Construction and Expression of Mek1 Phosphorylation-Site Mutants. Ser^{218} and Ser^{222} of Mek1 are the major sites that c-Raf-1 phosphorylates *in vitro* (19, 20). To investigate the significance of these phosphorylation sites in living cells, we mutated Ser^{218} and Ser^{222} to acidic residues (aspartate and glutamate) in an attempt to mimic phosphoserine residues. Wild-type and mutant *Mek1* constructs, tagged with the EE epitope (22), were placed under the control of a cytomegalovirus immediate-early promoter and transfected into COS-7 cells by electroporation. The efficiency of transfection was 50–60%, based on 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside staining of cells that were transfected in parallel with the β-galactosidase gene. The expression levels of the EE-tagged proteins were examined by Western blot analysis using an anti-Mek1 monoclonal antibody, 3D9. For all mutant constructs, expression of the EE-tagged Mek1 proteins equaled or exceeded that of the endogenous Mek1 protein (Fig. 1).

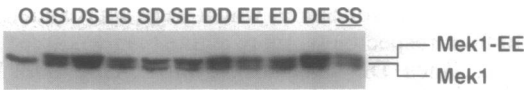

FIG. 1. Expression of Mek1 mutant proteins in COS-7 cells. Cytosolic proteins (20 μg per lane) from COS-7 cells that had been transfected with the epitope-tagged Mek1 constructs were resolved by SDS/10% PAGE and blotted with the anti-Mek1 antibody 3D9. Lanes: O, pCDNA/Amp vector alone; SS, wild-type Mek1; DS, [Asp^{218}]Mek1; ES, [Glu^{218}]Mek1; SD, [Asp^{222}]Mek1; SE, [Glu^{222}]-Mek1; DD, [Asp^{218},Asp^{222}]Mek1; EE, [Glu^{218},Glu^{222}]Mek1; ED, [Glu^{218},Asp^{222}]Mek1; DE, [Asp^{218},Glu^{222}]Mek1; SS, wild-type Mek1 stimulated with PMA.

Activation of Mek1 by Mutations of Ser^{218} and Ser^{222}. To assess the *in vitro* activity of the Mek1 phosphorylation-site mutants, we immunoprecipitated the EE-tagged Mek1 proteins from lysates of the COS-7 cells transfected with the mutant constructs and assayed their capacity to phosphorylate GST–Erk1(K63M), a kinase-inactive Erk1 protein fused to GST (Fig. 2*A*). These activities were compared with that of wild-type Mek1 from transfected COS-7 cells that were stimulated with PMA, a potent activator of the MAP kinase pathway. The quantities of Mek1 proteins in the immunocomplexes were measured by Western blot analysis and shown to be approximately equivalent to each other (Fig. 2*B*). There seemed to be no significant difference between the activity of wild-type Mek1 (Fig. 2*A*, lane SS) and that of [Asp^{222}]Mek1 or [Glu^{222}]Mek1 (Fig. 2*A*, lanes SD and SE). The [Asp^{218}]Mek1 and [Glu^{218}]Mek1 mutants were 50-fold (Fig. 2*A*, lane DS) and 2.5-fold (data not shown), respectively, more active than wild-type Mek1. It thus appeared that the mutation of Ser^{222} to acidic residues did not activate Mek1 but that the mutation of Ser^{218} to acidic residues did, with aspartate being a better substitute for phosphoserine than glutamate. These observations were corroborated by the activity of the Ser^{218}, Ser^{222} double mutants [Asp^{218},Asp^{222}]-Mek1 and [Asp^{218},Glu^{222}]Mek1, both containing the substi-

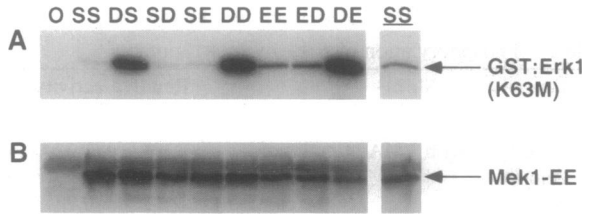

FIG. 2. Erk1 kinase activity of Mek1 mutant proteins. The Mek1 mutant proteins expressed in COS-7 cells were immunoprecipitated with the anti-EE antibody. (*A*) The Erk1 kinase activity of the immunocomplex was assayed using GST–Erk1 (K63M) as substrate. (*B*) The quantity of Mek1 protein in the immunocomplex was examined by Western blot analysis using the anti-Mek1 antibody 3D9. Lanes: O, pCDNA/Amp vector alone; SS, wild-type Mek1; DS, [Asp^{218}]Mek1; SD, [Asp^{222}]Mek1; SE, [Glu^{222}]Mek1; DD, [Asp^{218},Asp^{222}]Mek1; EE, [Glu^{218},Glu^{222}]Mek1; ED, [Glu^{218}, Asp^{222}]Mek1; DE, [Asp^{218},Glu^{222}]Mek1; SS, wild-type Mek1 stimulated with PMA.

tution of aspartate for Ser^{218}, which were approximately 80- and 110-fold, respectively, more active than wild-type Mek1 (Fig. 2*A*, lanes DD and DE). [Glu^{218},Asp^{222}]Mek1 and [Glu^{218},Glu^{222}]Mek1, both containing the substitution of glutamate for Ser^{218}, were about 6-fold more active than wild-type Mek1 (Fig. 2*A*, lanes ED and EE). These results are summarized in Table 1.

Activation of Erk1 by Mek1 Phosphorylation-Site Mutants. Erk1 is a downstream target of Mek1 in the MAP kinase pathway (8, 24). If the phosphorylation of Ser^{218} and Ser^{222} in Mek1 is important for the signal transduction from Mek1 to Erk1, it is expected that the expression of active Mek1 mutants should activate Erk1 in living cells. To test this hypothesis, we immunoprecipitated Erk1 from lysates of the COS-7 cells transfected with the Mek1 mutant constructs and assayed its MBP kinase activity (Fig. 3*A*). The quantity of Erk1 protein in each immunocomplex as measured by Western blot analysis was approximately equivalent (Fig. 3*B*). Only the three Mek1 mutants that were most active in phosphorylating GST-Erk1(K63M)—namely, [Asp^{218}]-Mek1, [Asp^{218},Asp^{222}]Mek1, and [Asp^{218},Glu^{222}]Mek1—were able to activate Erk1 in the cells (Fig. 3*A*, lanes DS, DD, and DE). Although the double mutations Glu^{218}, Asp^{222} and Glu^{218}, Glu^{222} activated Mek1 to the extent achieved by PMA stimulation (Fig. 2*A*, lanes EE, ED, and SS), they did not activate Erk1 to the extent observed with PMA stimulation (Fig. 3*A*, lanes EE, ED, and SS). This suggests that Mek family members other than Mek1 may also contribute to the activation of Erk1 during PMA stimulation.

Effects of Mek1 Activation on Growth. The MAP kinase pathway is activated by various mitogenic signals. If Mek1 plays a role in transducing mitogenic signals, its activation may result in accelerated growth. As an approximate index of growth, we measured the quantity of cytosolic protein of the COS-7 cell population 48 hr after transfection with the Mek1 phosphorylation-site mutants. The results (Fig. 4) correlated, in general, with the activity of the mutants; the cell populations transfected with the highly activated Mek1 mutants—namely, [Asp^{218}]Mek1, [Asp^{218},Asp^{222}]Mek1, and [Asp^{218}, Glu^{222}]Mek1—yielded about 2-fold more cytosolic protein than those transfected with wild-type Mek1. The cell populations transfected with the inactive [Asp^{222}]Mek1 and [Glu^{222}]Mek1 mutants did not yield significantly more cytosolic protein than those transfected with wild-type Mek1. The exceptions, however, are the [Glu^{218},Asp^{222}]Mek1 and [Glu^{218},Glu^{222}]Mek1 mutants. Although these mutants were only 6-fold more active than wild-type Mek1 (Table 1), the cell populations transfected with these two mutants also yielded twice as much cytosolic protein as those transfected with wild-type Mek1.

Table 1. Phosphorylation of Erk1 by Mek1 mutants

Mek1	Relative activity
Wild-type	1
Wild-type with PMA stimulation	8.7 ± 1.3
Asp^{218}	49 ± 3
Asp^{222}	1.0 ± 0.2
Glu^{222}	1.5 ± 0.5
Asp^{218},Asp^{222}	78 ± 11
Glu^{218},Glu^{222}	6.6 ± 2.3
Glu^{218},Asp^{222}	6.4 ± 2.3
Asp^{218},Glu^{222}	112 ± 4

Mek1 mutant proteins expressed in COS-7 cells were immunoprecipitated with the anti-EE antibody. The Erk1 kinase activity of the immunocomplex was assayed with GST-Erk1(K63M) as substrate. The activity of Mek1 mutants is presented relative to that of wild-type Mek1. The data for [Glu^{218}]Mek1 are not shown because of an insufficient number of experiments.

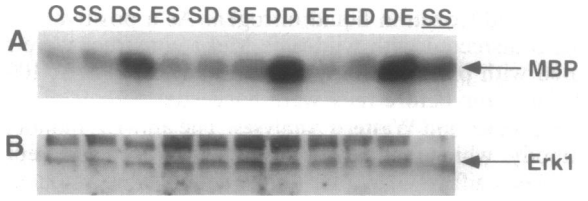

FIG. 3. Erk1-specific MBP kinase activity in COS-7 cells expressing the Mek1 mutant proteins. The Erk1 protein in the cytoplasm of transfected COS-7 cells was immunoprecipitated with the anti-Erk1 antibody C-16. (*A*) The MBP kinase activity of the immunocomplex was assayed. (*B*) The quantity of Erk1 protein in the immunocomplex was examined by Western blot analysis using the anti-Erk1 antibody K-23. Lanes: O, pCDNA/Amp vector alone; SS, wild-type Mek1; DS, [Asp^{218}]Mek1; ES, [Glu^{218}]Mek1; SD, [Asp^{222}]Mek1; SE, [Glu^{222}]Mek1; DD, [Asp^{218},Asp^{222}]Mek1; EE, [Glu^{218},Glu^{222}]Mek1; ED, [Glu^{218},Asp^{222}]Mek1; DE, [Asp^{218}, Glu^{222}]Mek1; SS, wild-type Mek1 stimulated with PMA.

DISCUSSION

In this communication we have demonstrated that replacement of the serine sites of phosphorylation in Mek1 with the acidic residues aspartate and/or glutamate enhanced the protein kinase activity up to 100-fold. This degree of activation is >10-fold greater than that achieved by PMA stimulation. Among the single replacements analyzed, aspartate at 218 had the most dramatic effect, whereas aspartate at 222 caused an insignificant change. In contrast, glutamate alone had only modest influence at either position 218 or position 222. For double replacements, the substitution of aspartate at 218 and 222 or aspartate at 218 and glutamate at 222 resulted in significant activation. When glutamate was substituted at 218 together with aspartate or glutamate at 222, the activity of Mek1 was increased by <10-fold. Others have reported that mutation of Ser^{222} to glutamate alone activated Mek1 by 30-fold, however (20). This discrepancy may be due to the fact that the wild-type Mek1 protein in our study had a higher basal level of activity. The wild-type Mek1 that Alessi *et al.* (20) expressed in *Escherichia coli* is unlikely to be phosphorylated, whereas the wild-type Mek1 studied for this report was expressed in mammalian cells and may be phosphorylated at a basal level even in the absence of mitogenic signals.

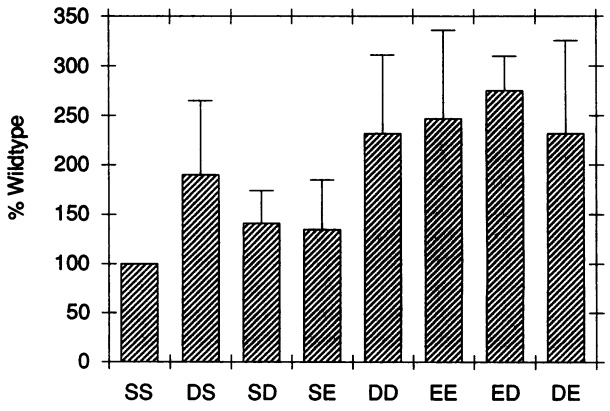

FIG. 4. Quantity of cytosolic protein in COS-7 cells expressing the Mek1 mutant proteins. Cytosolic protein in cells transfected with the Mek1 mutant constructs was quantitated by the Bradford assay and is presented as a percentage of that in cells transfected with the wild-type Mek1 construct. The graph represents data from five independent experiments. The data for [Glu^{218}]Mek1 are not shown because of an insufficient number of experiments. SS, wild-type Mek1; DS, [Asp^{218}]Mek1; SD, [Asp^{222}]Mek1; SE, [Glu^{222}]Mek1; DD, [Asp^{218},Asp^{222}]Mek1; EE, [Glu^{218},Glu^{222}]Mek1; ED, [Glu^{218}, Asp^{222}]Mek1; DE, [Asp^{218},Glu^{222}]Mek1.

The dual phosphorylation sites of Mek1 are strikingly parallel to those of MAP kinase, which are also located between subdomains VII and VIII. The mechanisms of activation for these two enzymes may differ, however. The activation of MAP kinase requires the phosphorylation of both the threonine and the tyrosine site (6), and the dephosphorylation of either site inactivates the enzyme (25, 26). Our success in the activation of Mek1 by the Asp^{218} mutation suggests that the phosphorylation of Ser^{218} is sufficient for at least the partial activation of Mek1. Moreover, the inactivation of Mek1 by phosphatases appears to require the dephosphorylation of both position 218 and position 222 (20). Thus, it is possible that the phosphorylation of either Ser^{218} or Ser^{222} partially activates Mek1 and that the phosphorylation of both sites fully activates it. Nonetheless, this notion has to be reconciled with the observation that the single substitution of alanine for either Ser^{218} or Ser^{222} in Mek1 abolishes its capacity to be activated by Raf-1 (19, 20). It is possible, however, that the single alanine substitution at either of these two sites alters the kinase structure to such an extent that the mutant Mek1 is no longer active even when the other site is phosphorylated. Similarly, our failure to activate Mek1 by Ser^{222} mutation may be due to a structural alteration introduced by the mutation that prevents aspartate from mimicking phosphoserine at position 222 as adequately as it does at position 218. Clarification of these questions requires the production of mutant proteins in sufficient quantity to test their capacity to be phosphorylated and activated by Raf-1 in soluble assays. These mutant enzymes will be necessary in order to obtain more quantitative data than is possible with immunocomplex assays.

We also showed that expression of the highly activated Mek1 phosphorylation-site mutants led to the activation of Erk1 in cultured cells. This result supports the proposal that the phosphorylation of Ser^{218} and Ser^{222} in Mek1 is one of the key events responsible for passing the mitogenic signal from the cell surface to Erk1. The degree of Erk1 activation in the cells, however, was only 3- to 4-fold, whereas we observed up to 100-fold activation of the Mek1 mutants *in vitro*. This apparent attenuation of the phosphorylation-induced signal may result from a high basal level of Erk1 activity even in the absence of activated Mek1 or mitogenic signals. Feedback regulatory mechanisms that employ Erk-specific phosphatases (27–30) may also significantly offset the effect of the active Mek1 mutants on Erk1.

MAP kinase family members are the only known physiological substrates of Mek1. The availability of Mek1 mutants in a constitutively active conformation should facilitate the search for other downstream targets of Mek1 through either affinity chromatography or the interaction-trap assay (31). The constitutively active Mek1 mutants may also be a useful tool for studying the role of the Mek family kinases in growth regulation. As a preliminary approach to address the question of whether these Mek1 mutants can stimulate cell growth or contribute to oncogenic transformation of cells, we estimated the rate of growth of the COS-7 cells transfected with the Mek1 mutants by measuring the protein content of the cell population (Fig. 4). We found that the quantity of cytosolic protein was significantly increased by the expression of the activated Mek1 mutants, suggesting a growth-stimulating effect. Surprisingly, the expression of the [Glu^{218}, Asp^{222}]Mek1 or [Glu^{218},Glu^{222}]Mek1 mutant, which did not activate Erk1 in COS-7 cells, also significantly increased the protein content of the cell population. It is possible that these two mutants exerted their effects through downstream targets other than Erk1. The increased protein content of the cell population could result, however, from one or more factors, such as an increase in individual cell size or an acceleration of protein synthesis and cell division of cells specifically expressing a high *Mek1* copy number. A thorough investigation of the *in vivo* function of the Mek family calls for the establishment and characterization of cell lines that stably express activated Mek1 and Mek2 mutants.

Note Added in Proof. After this manuscript was submitted, Crowley *et al.* (32) reported that [Glu^{218},Glu^{222}]Mek1 transforms NIH 3T3 cells in culture and induces differentiation in PC12 cells.

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