Mek1 Phosphorylation Site Mutants Activate Raf-1 in NIH 3T3 Cells*

Alessandro Alessandrini†, Heidi Greulich, Weidong Huang, and Raymond L. Erikson‡

From the Department of Molecular and Cellular Biology, Harvard University, Cambridge, Massachusetts 02138

MAP (mitogen-activated protein) kinases are activated by a family of dual specificity kinases called Meks (MAP kinase/Erk kinase). Mek1 can be activated by Raf by phosphorylation on serine 218 and serine 222. Mutation of these sites to acidic residues leads to constitutively active Mek1 in some cases. When fibroblast lines were infected with high titer retroviral stocks carrying these Mek1 genes, the resultant transformation and morphological changes correlated with the kinase activity of the respective Mek1 enzymes. Although [Asp218] and [Asp218,Asp222]Mek immunoprecipitated from clonal cell lines could phosphorylate kinase-inactive Erk1 equally well in vitro, the endogenous MAP kinase activity was 5-7-fold greater in [Asp218]Mek1-infected clonal lines, and did not correlate with the degree of transformation. Analysis of the Erk1 pathway revealed Raf-1 activation, which correlated qualitatively with the MAP kinase activity seen in the [Asp218]- and [Asp218,Asp222]Mek1-infected clonal lines. Expression of dominant negative Ras did not affect the elevated Raf-1 activity observed in these cells, however. These data suggest that Mek1 phosphorylation site mutants activate Raf-1 and MAP kinase by a Ras-independent pathway and that the mechanism by which transformation occurs may utilize pathways that are MAP kinase-independent.

Cell growth and differentiation are controlled by a variety of extracellular signals. Many of these signals, including insulin, epidermal growth factor, nerve growth factor, and thrombin, activate a family of serine/threonine kinases named MAP1 (mitogen-activated protein) kinases or Erks (Extracellular signal-regulated kinases) (for reviews, see Refs. 1–4). The signal-transduction pathways that lead to the activation of MAP kinases have been intensively studied in recent years. Some components of these pathways, such as Gip2, Ras, and Raf cause oncogenic transformation in their constitutively active forms, suggesting that the activation of the MAP kinase pathway may be responsible for accelerated growth in NIH 3T3 and Rat 1a cells (5–7). Other studies indicate that the activation of MAP kinases is concurrent with the differentiation of PC12 cells induced by nerve growth factor (8, 9). These correlative observations, however, do not exclude the possibility that the activation of MAP kinases may be merely a secondary effect of the signaling processes.

Activation of MAP kinases requires the phosphorylation of both threonine and tyrosine residues in a conserved “TEY” region of the catalytic domain (10, 11). A family of dual specificity kinases called Meks (MAP kinase/Erk kinase) are responsible for the phosphorylation and activation of MAP kinases (10, 12). Mek1 is activated by phosphorylation on serine 218 and 222 by Raf (13, 14). Mutation of these two serine sites on Mek1 to acidic residues, particularly Asp218, Asp218/Asp222, and Glu219/Glu222, produces constitutively active forms of Mek1 that can activate MAP kinases both in vitro (13, 15) and when they are transiently expressed in COS cells (16, 17).

Stable expression of the constitutively active Mek1 mutants causes neuronal differentiation of PC12 cells (16) and oncogenic transformation of fibroblast cell lines (16, 18, 19). The transformed fibroblast lines exhibited increased AP-1 transcriptional activity and induced rapid tumor formation when they were injected into nude mice. We had previously generated constitutively active Mek1 mutants that have various specific activities (15, 17). To correlate Mek activity with transformation, a retroviral packaging system (20) was used to obtain high titer retroviruses carrying the constitutively active Mek1 mutants. Infection of NIH 3T3 and Swiss 3T3 cells with these retroviral constructs demonstrated that the transformation potential of the Mek1 mutants is closely related to their kinase activity, although growth in soft agar did not correlate with MAP kinase activity. Further analysis of [Asp218]- and [Asp218,Asp222]Mek1-infected clonal lines revealed that Raf1 activity is elevated.

** MATERIALS AND METHODS **

Production of Retroviruses—The HindIII-XhoI fragments containing Mek1 mutants from pG-MEK-C(B)lu (17) were subcloned into the HindIII-AvrII site of LC7A5X (a gift from Benjamin Neel, Beth Israel Hospital, Boston, MA). The LC7A5X constructs were transfected into BOSC23 cells to produce retroviruses following the method of Pear et al. (20). Briefly, BOSC23 cells were grown to subconfluence in selection medium (Dulbecco’s modified Eagle’s medium (DMEM), 10% dialyzed fetal bovine serum (FBS) (JRH Biosciences), 250 μg/ml xanthine, 2 mM glutamine, 1 × HAT supplement (Sigma), 25 μg/ml mycophenolic acid, 2 μg/ml aminopterin, 6 μg/ml thymidine, 100 units/ml penicillin, 100 μg/ml streptomycin) and plated on 6-cm dishes at a density of 2 × 104 cells/dish the day prior to transfection. Transfection was carried out by mixing equal volumes of HEPES-buffered saline (pH 7.05) and 250 mM calcium chloride solution containing 10 μg of DNA. This mixture was then added to the BOSC23 cells, and they were incubated for 10 h at 37 °C in DMEM, 10% FBS, 100 units/ml penicillin, 100 μg/ml streptomycin, 25 μg/ml chloroquine. The medium was then replaced with 4 ml of DMEM, 10% FBS, 100 units/ml penicillin, 100 μg/ml streptomycin.
Supernatants containing retroviruses were collected 48 and 72 h posttransduction in 2.5-ml volumes. Cell lines were then infected as described below.

**Infection and Characterization of Cell Lines**—NIH 3T3 and mouse embryo fibroblast (MEF) cells were grown in DMEM, 10% calf serum (CS), 100 units/ml penicillin, 100 µg/ml streptomycin. Swiss 3T3 cells were grown in DMEM, 10% FBS, 100 units/ml penicillin, 100 µg/ml streptomycin. Prior to infection, 1.5×10^6 cells were seeded in a 6-cm plate and incubated for 24 h. The cells were incubated at 37°C for 5 h with a mixture of 0.5 ml of retrovirus, 0.5 ml of medium, 8 µg/ml hexadimethrine bromide (Sigma) and then grown for 48 h before they were subcultured and characterized. MEF cells were seeded at 5×10^3 cells on 3-cm plates 24 h prior to infection. The cells were incubated at 37°C for 5 h with 2 ml of retrovirus in the presence of 4 µg/ml hexadimethrine bromide. MEFs were subcultured and characterized in the same manner as NIH 3T3 and Swiss 3T3 cells.

Growth in soft agar was measured following the method of Cowley et al. (16). In brief, 6-ml plates were coated with 5 ml soft agar (20% 2×DMEM, 10% serum, 50% DMEM, 20% 2.5% agar, 100 units/ml penicillin, 100 µg/ml streptomycin, melted and combined at 45°C). Duplicate of 1×10^3 cells were suspended in 0.5 ml of medium, mixed with 1 ml of soft agar and added to each plate. Cells were fed weekly with 2 ml of a mixture of 33% medium and 67% soft agar. After 14 days, the number of colonies (with more than 20 cells) from 10 randomly selected areas of each plate was counted to calculate the number of colonies per plate and the efficiency of colony formation.

To measure saturation density, 1×10^5 cells were seeded in a 6-cm plate in DMEM containing 10% FBS, 0.5% sodium azide and suspended in 50 ml of fixing medium (Hank’s balanced salt solution, 3% CS), 100 µg/ml streptomycin, and gradually separated on a 1% formaldehyde gel and transferred to Hybond-N (Amersham). Filters were prehybridized with BEPS (1% bovine serum albumin, 1% SDS, dried, and exposed to film. Filters were washed 3 times with 0.1×SSC (bed volume) of protein G-agarose (Santa Cruz Biotech, Inc.), and 400 µg of supernatant. Immunoprecipitates were washed three times with 1 ml of ice-cold potassium phosphate buffer and once with 1 ml of ice-cold ST (NaCl and Tris) buffer, then incubated with 40 µl kinase mixture for 30 min at 30°C. The Raf-1 associated with 25 µl of a monoclonal antibody against RasN17. Transfectants were selected in 2 µg/ml puromycin. Clonal lines were picked, expanded, and further analyzed.

**RESULTS**

**Stable Expression of Mek1 Phosphorylation Site Mutants**—Epitope-tagged Mek1 phosphorylation site mutants (17) were prepared under the control of an SV40 early promoter in a retroviral vector that also carries a human CD7 gene under the control of an LTR promoter. The retroviral constructs were transfected into BOSC23 cells (20) to produce high titer viruses. NIH 3T3, Swiss 3T3 and MEF cells were infected with the retroviruses. The percentage of infection was measured by FACS analysis using a monoclonal antibody against human CD7. For all Mek1 constructs, more than 90% of NIH 3T3 cells were infected, whereas Swiss 3T3 and MEF cells were infected 40–50% and 30–50%, respectively (Table I).

The expression of Mek1 in NIH 3T3 cells was examined by Western blotting with a monoclonal antibody against Mek1. The level of epitope-tagged Mek1 was 3–5-fold higher than that of endogenous Mek1 (Fig. 1A). To determine the specificity of the Mek1 mutants, a monoclonal antibody against the epitope tag was used for immunoprecipitation, and the kinase activity in the immunocomplex was assayed using kinase-inactive Erk1 as substrate (Fig. 1B). Consistent with our data from COS-7 cells (15), both [Asp^218,Asp^225]Mek1 and [Asp^218,Asp^225]Mek1 were highly activated, whereas [Glu^218,Glu^222]Mek1 was only marginally more active than wild-type Mek1.

**Characterization of Cell Lines Expressing Mek1 Mutants**—The high infection rate of NIH 3T3 cells allowed us to directly characterize the general populations without cloning. The NIH 3T3 cells expressing the Mek1 mutants exhibited growth rates similar to those of control cells (uninfected cells and cells expressing wild type Mek1) under both 10% serum and 0.1% serum conditions (data not shown). 48 h after infection, the Mek1 mutants were already expressed at the levels shown in Fig. 1A. At this point the cells still showed no evidence of morphological transformation. Three to 5 days after infection...
with one passage, however, cells expressing [Asp218,Asp222]Mek1 or [Asp218]Mek1 began to show distinct morphological changes (Fig. 2). Cells expressing [Asp218,Asp222]Mek1 were visibly smaller than control cells and contained a large proportion of round, refractile cells; cells expressing [Asp218]Mek1 showed a similar morphology, but to a lesser extent. In contrast, cells expressing wild type Mek1, [Asp222]Mek1, or [Glu218,Glu222]Mek1 were morphologically indistinguishable from control cells. Moreover, when cells were grown to confluence, the saturation density of [Asp218,Asp222]- and [Asp218]-infected cells was more than three times higher than that of control cells; and that of [Glu218,Glu222]-infected cells was 1.8 times higher (Fig. 3).

An important characteristic of transformed cells is anchorage-independent growth. NIH 3T3, Swiss 3T3, and MEF cells expressing the Mek1 mutants were tested for efficiency of colony formation in soft agar (Table I). Whereas MEF cells failed to form colonies, NIH 3T3 and Swiss 3T3 cell lines showed similar rates of growth in soft agar. [Asp218,Asp222]-infected cells exhibited the highest percentage of colony formation: 22.2% and 16.6% for NIH 3T3 and Swiss 3T3, respectively. [Asp218]-infected cells exhibited about 5% growth, and [Glu218,Glu222]-infected cells exhibited less than 1% growth. Cells expressing kinase-inactive Mek1 or [Asp222]Mek1 exhibited negligible growth; and control cells exhibited no growth in soft agar. Thus the capacity of anchorage-independent growth seems to correlate closely with the degree of Mek1 activation (Table I, Fig. 1B).

Isolation and Characterization of Clones Expressing Mek1 Mutants—It is possible that individual infected cells in the general population express Mek1 at significantly different levels, which would result in differential growth in soft agar. To address this concern, we isolated NIH 3T3 colonies that had grown in soft agar and examined their levels of Mek1 expression by Western blotting (Fig. 4). Without exception, the relative levels of epitope-tagged Mek1 to endogenous Mek1 in these clones were, for most parts, similar. Thus the ability of these clones to grow in soft agar was not due to an inordinately high level of Mek1.

The clonal cell lines showed growth rates similar to those of the general populations from which they had been isolated (data not shown). When these clonal lines were tested for efficiency of colony formation in soft agar, however, the results (Table II) differed from those of the general populations (Table I). There was a significant variation in efficiency of colony table:

| Mek1         | NIH 3T3 | Swiss 3T3 | MEF |
|--------------|---------|-----------|-----|
|              | Infection<sup>a</sup> | Colony<sup>b</sup> | Infection<sup>a</sup> | Colony<sup>b</sup> | Infection<sup>a</sup> | Colony<sup>b</sup> |
| Uninfected   | 0       | 0.0       | 0   | 0.0       | 0   | 0.0 |
| WT           | 92      | 0.0       | 43  | 0.0       | 32  | 0.0 |
| Asp218       | 96      | 5.0       | 42  | 5.8       | 52  | 0.0 |
| Asp222       | 92      | 0.1       | 50  | 0.0       | 33  | 0.0 |
| Asp218, Asp222 | 95    | 22.2      | 49  | 16.6      | 45  | 0.0 |
| Glu218, Glu222 | 98   | 0.2       | 55  | 1.0       | 51  | 0.0 |

<sup>a</sup> Percentage of cells infected with retrovirus, as represented by percentage of cells stained CD7+ in FACS analyses.

<sup>b</sup> Percentage of colony-forming cells after 14 days in soft agar, corrected with percentage of infection.
formation between clones expressing the same Mek1 mutant. On average, the clones exhibited a higher percentage of growth in soft agar than the general populations.

**Activity of MAP Kinases**—Since Mek1 is an activator of MAP kinases, it would be expected that the expression of constitutively active Mek1 mutants should lead to activation of MAP kinases. Because both [Asp218 Asp222]Mek1 and [Asp218]Mek1 phosphorylate GST-Erk-1(K63M) equally as well in vitro (Fig. 1B), we analyzed the endogenous MAP kinase activity in the clonal lines. We found that the [Asp218]Mek1-infected clonal lines (DS2 and DS4) exhibited 4–8-fold greater Erk-1 activity than the [Asp218 Asp222]Mek1-infected clonal lines (DD1 and DD3) (Fig. 5C) and this activity did not correlate with the efficiency of growth in soft agar (Table II). To see if the MAP kinase-specific phosphatase (MKP-1) (22) may be involved in this apparent difference, we analyzed the levels of MKP-1 RNA. Our data show that there was no significant increase in message levels of MKP-1 in [Asp218,Asp222]Mek1-infected clonal lines as compared to [Asp218]Mek1-infected clonal lines. Furthermore, MKP-1 levels in all of the Mek-transformed lines were lower than in NIH 3T3 cells (Fig. 6). Therefore, the difference in MAP kinase activity cannot be attributed to an increase in MKP-1 in these clonal lines, though other phosphatases (23–25) and/or inhibitors may be involved.

**Raf-1 Kinase Activity**—We analyzed signaling molecules upstream of Mek-1 in order to further investigate the activation of Erk-1 in the clonal lines, especially DS2 and DS4. Fig. 5A shows that Raf-1 activity is elevated in the clonal lines, and this activity qualitatively mirrors the Erk-1 kinase activity (Fig. 5C), in that the highest Raf-1 activity was observed in DS2 and DS4. Expression of dominant-negative RasN17 in these lines did not affect Raf-1 activity, suggesting that Raf-1 activation results from a Ras-independent pathway (Fig. 7B). Furthermore, these lines can tolerate high levels of RasN17 (Fig. 7A), suggesting that growth of these Mek1-transformed cell lines can be compensated by Ras-independent pathways.

**Dominant-negative Ras Expression**—As mentioned above, RasN17 was stably expressed in the Mek1-transformed lines using a puromycin-resistant vector. Both pooled transfected and clonal lines were characterized. The pooled puromycin-resistant NIH 3T3 cells expressed very low levels of RasN17 (2-fold greater than endogenous Ras), while pooled puromycin-resistant v-Src 3T3 did not express detectable levels of RasN17 (Fig. 8A). However, the pooled [Asp218]Mek- and [Asp218,Asp222] Mek-transformed 3T3 stably expressed high levels of dominant-negative Ras, more than 20-fold greater levels than endogenous Ras. No reversion of transformation was observed in these pooled Mek1-transformed lines (data not shown). Ras activity has been shown to be required for v-Src transformation (26, 27), but repeated attempts to stably express RasN17 in these pools failed. Clonal cell lines were therefore established to determine if a small percentage of the RasN17-transformed v-Src-transformed cells unable to be detected in a pooled

---

**Table II**  
Growth in soft agar of NIH 3T3 and Swiss 3T3 clonal cell lines expressing Mek1  

| NIH 3T3 | Swiss 3T3 |
|---------|-----------|
| Mek1 | Colony<sup>a</sup> | Mek1 | Colony<sup>b</sup> |
| Uninfected | 0.0 | Uninfected | 0.0 |
| DS2 | 2.2 | DS2 | 12.0 |
| DS4 | 7.8 | DS4 | 11.0 |
| DD1 | 43.0 | DD2 | 28.0 |
| DD3 | 25.0 | DD4 | 36.0 |
| EE1 | 29.0 | EE2 | 17.0 |
| EE3 | 28.0 | EE4 | 35.0 |

<sup>a</sup> Percentage of colony-forming cells after 14 days in soft agar.  
<sup>b</sup> Percentage of colony-forming cells after 10 days in soft agar.

---

**Fig. 4.** Western analysis of NIH 3T3 clonal lines. Lysates of NIH 3T3 cells were blotted with an anti-Mek1 monoclonal antibody (3D9). DS2 and DS4, clonal lines isolated from DS, expressing [Asp218]Mek1; DD1–4, clonal lines isolated from DD, expressing [Asp218,Asp222]Mek1; EE1–4, clonal lines isolated from EE, expressing [Glu218,Glu222]Mek1.  

**Fig. 5.** Activity of c-Raf1 and Erk1 in NIH 3T3 cells expressing Mek1 mutants. A, an anti-Raf1 polyclonal antibody (C-12) was used to immunoprecipitate c-Raf1 of Mek1 mutant clonal cell lines and uninfected NIH 3T3 cells. The c-Raf1 activity in the immunoprecipitates was assayed using Mek1(K97A) (kinase-inactive Mek1) as a substrate. Samples were run on a 10% SDS-PAGE gel. B, Western analysis of immunoprecipitated c-Raf1 using the anti-c-Raf1 polyclonal antibody (C-16). C, an anti-Erk1 polyclonal antibody (C-16) was used to immunoprecipitate Erk1 from lysates of Mek1 mutant clonal cell lines and uninfected NIH 3T3 cells. The activity of Erk1 in the immunoprecipitates was assayed using MBP as substrate. DS2 and DS4, clones of [Asp218]Mek1; DD1 and DD3, clones of [Asp218,Asp222]Mek1; N, asynchronous growing NIH 3T3 cells; N+TPA, asynchronous growing NIH 3T3 cells stimulated for 5 min with TPA.  

**Fig. 6.** Mek1 expression in Mek1 mutant clonal lines. 20 μg of total RNA was loaded per lane. Northern analysis was performed using Mek1 as a probe. The blot was reprobed with glyceraldehyde-3-phosphate dehydrogenase to check relative loading of total RNA. DS2 and DS4, clones of [Asp218]Mek1; DD1 and DD3, clones of [Asp218,Asp222]Mek1; N, asynchronous growing NIH 3T3 cells; N+serum, NIH 3T3 cells were starved for 24 h and then stimulated for 5 min with 10% serum.
assay did express RasN17 (Fig. 8B); such cells could then serve as a positive control for RasN17 function in the cell.

[Asp218]Mek, [Asp218,Asp222]Mek, and v-Src cells were therefore transfected with dominant negative RasN17, and puromycin-resistant colonies were isolated. Consistent with the RasN17 expression levels exhibited by the pooled transfecants, the number of drug-resistant colonies obtained from the [Asp218]Mek and [Asp218,Asp222]Mek was over an order of magnitude greater than the number of drug-resistant colonies obtained by transfection of the v-Src cells (only 6 out of $4 \times 10^5$ transfected). Furthermore, while greater than 50% of the drug resistant clones analyzed from transfection of the [Asp218]Mek and [Asp218,Asp222]Mek cells expressed the transfected cDNA, only 1 of the 6 v-Src-drug-resistant clones expressed RasN17.

Several clonal lines derived from RasN17-transfected [Asp218]Mek and [Asp218,Asp222]Mek were analyzed (Fig. 7A). Unlike the pooled cells, these lines did exhibit some slowed growth and a modest decrease in refractility, as well as partial inhibition of colony formation in soft agar (data not shown). However, the cells were still distinctly morphologically transformed, and were much more refractile than native NIH-3T3 cells (Fig. 9). Significantly, the single v-Src clone identified as expressing detectable levels of RasN17 was severely morphologically reverted, exhibiting slowed growth and complete flattening of the cells (Fig. 9).

**DISCUSSION**

In this report we show that activated Mek1 mutants lead to cellular transformation, but this process does not correlate with Erk activity (Table II, Fig. 5C). We also observe an increase in Raf1 activation in the [Asp218]Mek1-infected clonal lines that mirrors Erk activation (Fig. 5, A and C). Expression of dominant-negative Ras does not inhibit Raf1 activation (Fig. 7B), suggesting that this increase in activity occurs through a Ras-independent pathway. Although the [Asp218]Mek1-infected clonal lines expressing RasN17 show a slight reversion morphologically (Fig. 9), this does not correlate with Raf1 activity, which remains elevated. Furthermore, when we expressed RasN17 in v-Src- and [Asp218,Asp222]Mek1-transformed cells, we observed severe reversal of v-Src-expressing cells, and only slight reversal of [Asp218,Asp222]Mek1-expressing cells (Fig. 9). These data suggest that maintenance of transformation by Mek1 phosphorylation site mutants occurs through a Ras-independent pathway, and that the degree of transformation is independent of Raf1 and Erk1 activity.

We have expressed Mek1 phosphorylation site mutants in NIH 3T3, Swiss 3T3, and MEF cells using a retroviral vector. Because of the high infection rate, we were able to directly analyze and characterize the general populations of NIH 3T3 cells without selection of transformed foci. The Mek1 phosphorylation site mutants [Glu218,Glu222]Mek1, [Asp218]Mek1, and [Asp218,Asp222]Mek1 had been previously shown to be 7-, 50-, and 80-fold, respectively, more active than wild type Mek1 as measured by their capacity to phosphorylate Erk1 (16). In soft agar growth assays, cells infected with these mutants had colony forming efficiencies of 0.2%, 5%, and 22%, respectively. The [Asp218]- and [Asp218,Asp222]-infected cells were morphologically distinct from control cells and reached a saturation density 3-fold greater than that of control cells. The [Glu218,Glu222]-infected cells were morphologically similar to control cells and reached a saturation density 1.8 fold greater than that of control cells. Overall, the transformation potentials of the Mek1 phosphorylation site mutants appeared to correlate with their kinase activities.

There is, however, evidence that secondary events that promote transformation occur in these cells. We find for example significant variation in soft agar growth between the clones that were analyzed (Table II). There is also a striking increase in colony-forming efficiency of [Glu218,Glu222]Mek1 clones after they are picked from the initial population of infected cells (Table I and Table II). Since the level of mutant Mek1 expres-
More active in [Asp218]-infected cells than in [Asp218,Asp222] activation yet yielded the lowest percentage of soft agar colony formation and transformation (other factors appear to contribute to the observed cloning efficiency). The activation of MAP kinases only in [Asp218] Mek1-infected NIH Mek1 mutants caused transformation, we found moderate phosphorylation of Erk by activated Mek1. It is noteworthy that the Mek-transformed lines are able to stably express high levels of dominant-negative RasN17. Most published work involving RasN17 expression has been performed in transient or inducible systems because it is likely that Ras function is necessary for cell growth. This implies that the cells transformed by activated Mek1 at least partially bypass a requirement of Ras for cell growth. This is not surprising, as Mek1 has been shown to function downstream of Ras in growth factor signaling (31). However, the modest change in phenotype of the clonal RasN17-expressing Mek1-transformed cell lines and the lack of correlation of Erk activity with transformation suggests that the steady-state system for maintaining cell growth and transformation is more complex than simple phosphorylation of Erk by activated Mek1. Since MAP kinase activity does not correlate with the efficiency of growth in soft agar, component(s) other than MAP kinases may be responsible for transducing the mitogenic signal downstream from Mek1 in these transformed cells. No Mek1 substrate other than MAP kinases has been identified to date. Transformation by oncogenic Ras has been shown to involve Rac, a pathway independent of the MAP kinase cascade (32, 33). Furthermore, expression of MEKK1 in mammalian cells led to the constitutive activation of Mek1 and Mek2 but not of Erk2 (34). This suggests that these various Mek1 phosphorylation mutants may form complexes with distinct proteins and/or may localize to different parts of the cell where they would not normally be present. The elucidation of other pathway(s) involved in transformation by Mek1, together with the identification of new Mek1 substrates, will shed further light on our understanding of cell proliferation and differentiation.

Acknowledgments—We thank Benjamin Neel for pointing out the value of the BOSC23 packaging line and for a gift of the LC7ΔSX vector. We are grateful to Warren Pear for a gift of the BOSC23 cell line, Thomas Benjamin for a gift of the MEF cells, and Nick Tonks for the MKP-1 cDNA. We also thank Leslie Berg for the use of the FACScan and Steve Bunnell for help in FACS analysis.

REFERENCES

1. Ahn, N. G., Campbell, J. S., Seger, R., Jensen, A. L., Graves, L. M., and Krebs, E. G. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 5889–5892
2. Bennis, J. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 14553–14556
3. Crews, C. M., Alessandri, A., and Erikson, R. L. (1992) Cell Growth Diff. 3, 135–142
4. Davis, R. J. (1993) J. Biol. Chem. 268, 14553–14556
5. Gallego, C., Gupta, S. K., Heasley, L. E., Qian, N., and Johnson, G. L. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 7355–7359
6. Gupta, S. K., Gallego, C., Johnson, G. L., and Heasley, L. E. (1992) J. Biol. Chem. 267, 7987–7990
7. Leevers, S. J., and Marshall, C. J. (1992) EMBO J. 11, 569–574
8. Gotob, Y., Nishida, K., Yamashita, T., Hoshi, M., Kawakami, M., and Sakai, H.
Mek1 Transformation and the MAP Kinase Pathway

9. Miyasaka, T., Chao, M. V., Sherline, P., and Saltiel, A. R. (1990) J. Biol. Chem. 265, 4730–4735
10. Alessandri, A., Crewe, C. M., and Erikson, R. L. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 8200–8204
11. Payne, D. M., Rossomando, A. J., Martino, P., Erickson, A. K., Her, J., Shabanowitz, J., Hunt, D. F., Weber, M. J., and Sturgill, T. W. (1991) EMBO J. 10, 885–892
12. Rossomando, A., Wu, J., Weber, M. J., and Sturgill, T. W. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 5221–5225
13. Alessi, D. R., Saito, Y., Campbell, D. G., Cohen, P., Sithanandam, G., Rapp, U., Ashworth, A., Marshall, C. J., and Cowley, S. (1994) EMBO J. 13, 1610–1616
14. Zheng, C.-F., and Guan, K.-L. (1994) EMBO J. 13, 1123–1131
15. Huang, W., Kessler, D. S., and Erikson, R. L. (1995) Mol. Biol. Cell 6, 237–245
16. Cowley, S., Paterson, H., Kemp, P., and Marshall, C. J. (1994) Cell 77, 841–852
17. Huang, W., and Erikson, R. L. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 8960–8963
18. Brunet, A., Pages, G., and Pouyssegur, J. (1994) Oncogene 9, 3379–3387
19. Mansour, S. J., Matten, W. T., Hermann, A. S., Candida, J. M., Reng, S., Fukasawa, K., Vande Woude, G. F., and Ahn, N. G. (1994) Science 265, 966–970
20. Pear, W. S., Nolan, G. P., Scott, M. L., and Baltimore, D. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 8392–8396
21. Grussmeyer, T., Scheidtmann, K. H., Hutchinson, M. A., Ekkhart, W., and Walter, G. (1985) Proc. Natl. Acad. Sci. U. S. A. 82, 7953–7954
22. Sun, H., Charles, C. H., Lau, L. F., and Tonks, N. K. (1993) Cell 75, 487–493
23. Sarvevic, B., Erikson, E., and Maller, J. L. (1993) J. Biol. Chem. 268, 25075–25083
24. Ward, Y., Gupta, S., Jensen, P., Wartmann, M., Davis, R. J., and Kelly, K. (1994) Science 267, 651–654
25. Zheng, C.-F., and Guan, K.-L. (1993) J. Biol. Chem. 268, 16116–16119
26. DeClue, J. E., Zhang, K., Redford, P., Vass, W. C., and Lowy, D. R. (1991) Mol. Cell. Biol. 11, 2819–2825
27. Nori, M., Vogel, U. S., Gibbs, J. B., and Weber, M. (1991) Mol. Cell. Biol. 11, 2812–2818
28. Land, H., Parada, L. F., and Weinberg, R. A. (1983) Nature 304, 596–602
29. Ruley, H. E. (1983) Nature 304, 602–606
30. Bokemeyer, D., Sorokin, A., Yan, M., Ahn, N. G., Templeton, D. J., and Dunn, M. J. (1996) J. Biol. Chem. 271, 639–642
31. Roberts, T. M. (1992) Nature 360, 534–535
32. Qiu, R., Chen, J., Kirn, D., McCormick, F., and Symons, M. (1995) Nature 374, 457–459
33. Manser, E., Leung, T., Salihuddin, H., Zhao, Z., and Lim, L. (1994) Nature 367, 40–46
34. Xu, S., Robbins, D., Frost, J., Dang, A., Lange-Carter, C., and Cobb, M. H. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 6808–6812
