Cytoplasmic Localization of the Mitogen-activated Protein Kinase Activator MEK*

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The mitogen-activated protein kinase (MAPK) or extracellular signal-regulated kinase (ERK) is phosphorylated and activated by an upstream activator kinase, MEK (MAPK or ERK kinase), in response to mitogenic growth factors. ERKs translocate into the nucleus upon mitogen stimulation, suggesting that the subcellular redistribution of ERK may play a critical role in signal transfer from cytoplasm to the nucleus. We demonstrated in this report that MEK was exclusively localized in cytoplasm in several cell lines, including Swiss 3T3, HeLa, COS, and PC12. Immunofluorescence analysis of both native and transiently expressed MEK with a MEK-specific antibody revealed that both MEK1 and MEK2 were localized only in the cytoplasm. The cytoplasmic localization of MEK was further supported by subcellular fractionations as well as detergent permeabilization experiments. In contrast to ERK, mitogen stimulation did not cause any nuclear accumulation of MEK. These data suggest that ERK is phosphorylated and activated in the cytoplasm. The activated ERK could subsequently translocate into the nucleus and phosphorylate its nuclear substrates.

The mitogen-activated protein kinase (MAPK),1 also known as the extracellular signal-regulated protein kinase (ERK), is a family of serine/threonine kinases which are activated by various extracellular signals including mitogenic growth factors (1–6). Growing evidence suggests that ERK plays a central role in signal transduction. ERK phosphorylates cellular proteins, including protein kinases (7), transcription factors (8, 9), cytoskeletal proteins (10, 11), and proteins known to be important for cell cycle regulation (12, 13). Activation of ERK requires phosphorylation of both threonine and tyrosine by MEK (14, 15).

Biochemical purification and molecular cloning have demonstrated that MEK is a dual-specific protein kinase which activates ERK by phosphorylating threonine 183 and tyrosine 185 (14–24). MEK, whose activity is also rapidly stimulated by mitogens, displays an extremely high substrate specificity toward ERK (17). Although autophosphorylation (mainly on serine and threonine) partially stimulates MEK activity (25), phosphorylation by an upstream activating kinase is required for full activation of MEK (26–30). MEK activation is accompanied by serine and threonine phosphorylation (31–33). Several serine/threonine kinases, including c-Raf, c-Mos, and MEKK, directly phosphorylate and activate MEK (26–30). Activation of MEK1 by c-Raf requires the phosphorylation of both serine residues 218 and 222 (34).

Substrates of ERK include not only cytoplasmic proteins but also nuclear proteins such as the transcription factors of c-Jun and c-Myc (8, 9). Immunofluorescence studies have demonstrated that ERK is localized in both cytoplasm and nucleus (35–39). Mitogen stimulation causes a significant subcellular redistribution of ERK (35–38). The ERK localization may be cell type-dependent, because ERK was constitutively found in the nucleus of chick hepatoma DU249 cells and embryo fibroblasts (39), whereas in other cells the nuclear localization of ERK is stimulated by mitogens (35–38). Amino acid sequence analysis of ERK reveals no typical nuclear localization signal. These observations raise questions such as: is ERK activated in the nucleus?, what is the relationship between ERK activation and nuclear translocation?, and is MEK a nuclear protein?

In this report, we demonstrate that both MEK1 and MEK2 are localized exclusively in the cytoplasm as measured by indirect immunofluorescence, subcellular fractionation, and detergent permeabilization techniques. In contrast to ERK, MEK was not translocated into the nucleus when cells were treated with mitogens, indicating that ERK was phosphorylated and activated by MEK in the cytoplasm.

EXPERIMENTAL PROCEDURES

Cell Culture—Swiss 3T3 cells were obtained from ATCC and maintained in DMEM supplemented with 10% calf serum (Life Technologies, Inc.). For mitogen stimulation experiments, cells were starved with the same medium containing 0.1% serum for 24 h and then treated with either EGF (100 ng/ml, Sigma), PMA (190 nm) (Sigma), or fetal bovine serum (10%) (Life Technologies, Inc.) for various times. HeLa cells were cultured in DMEM supplemented with 10% fetal calf serum. COS cells were cultured in DMEM supplemented with 10% calf serum. PC12 cells were cultured in DMEM supplemented with 7.5% fetal calf serum and 7.5% horse serum. Chinese hamster ovary (CHO) cells were cultured in F-12 medium (Sigma) supplemented with 10% fetal calf serum.

Transient Transfection—To express MEK1 or MEK2 in mammalian cells, the BamHI fragments of pGEX-2T/MEK1 or pGEX-2T/MEK2 (25) were subcloned into the BamHI site of the eukaryotic expression vector pCMV5 (40) to construct pCMV-MEK1 and pCMV-MEE2. Approximately 106 cells were plated into each well of a 24-well plate for 1 day. The cells were washed twice with phosphate-buffered saline (PBS, 150 mM NaCl, 20 mM phosphate, and 0.2% KCl) then transfected with 2.5 μg of pCMV-MEK1 or pCMV-MEE2 in 1 ml of OptiMEM containing 2.5 μg of lipofectin (Life Technologies, Inc.). The transfection was terminated with 1 ml of F-12 medium supplemented with 20% fetal calf serum and incubated for 2 days. Immunofluorescence of transfected cells was performed as described below.

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1 The abbreviations used are: MAPK, mitogen-activated protein kinase; ERK, extracellular signal-regulated kinase; MEK, MAPK or ERK kinase; PMA, phorbol 12-myristate 13-acetate; EGF, epidermal growth factor; NGF, nerve growth factor; DMEM, Dulbecco's modified Eagle's medium; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; DAPI, 4',6-diamidino-2-phenylindole; PC12, pheochromocytoma-12; CHO, Chinese hamster ovary.
Expression and Purification of Histidine-tagged MEK2—An N-terminal primer (AAAAAGATCTCAGTCTGCGCCGAGGAA) and a C-terminal primer (AAAAAGATCTCAGGCGGTCGCGGCTG) were used for polymerase chain reaction amplification of MEK2 coding sequence using one of the original full-length clones (20). The polymerase chain reaction product was digested with BamHI and BglII and ligated to pQE-16 (Qiagen) digested with the same enzymes. The resulting plasmid (HT-MEK2) was used to express histidine-tagged MEK2.

Cells from a liter of HT-MEK2 expressing culture were resuspended in 20 ml of buffer A (100 mM phosphate and 10 mM Tris, pH 8.0) containing 6.0 M guanidine hydrochloride (Sigma) and stirred for 40 min at room temperature. The lysate was centrifuged at 15,000 rpm for 15 min, and the supernatant was slowly loaded on a nitrilotriacetic acid-nickel-agarose column (2 ml, Qiagen) equilibrated with buffer A. The column was washed sequentially with 15 ml of buffer A plus 6.0 M guanidine, 15 ml of buffer A containing 8.0 M urea, and 15 ml of buffer A, pH 6.1, with 8.0 M urea. Histidine-tagged MEK2 was then eluted by 10 ml of 250 mM imidazole in buffer A, pH 8.0.

Antibody Preparation and Purification—Human MEK2 and ERK1 were expressed as glutathione S-transferase fusion proteins and purified as described (20, 25) and used to immunize rabbits for antibody production (Cocalico Biologicals, Inc., Reamstown, PA). Expression plasmids for ERK2 and ERK3 were generous gifts of Dr. M. Cobb. Purified histidine-tagged MEK2 (2 mg) and ERK1 (1.5 mg) were used for affinity purification of antibodies as described (41).

Immunofluorescence—Cells cultured on coverslips in 24-well culture plates were analyzed by immunofluorescence essentially as described by Wang et al. (42). Cells on the coverslip were washed twice with 1 ml of ice-cold PBS. Cells were fixed with 1 ml of 3% formaldehyde in PBS for 10 min at room temperature followed by 1 ml of 1:1 mixture of methanol and acetone for 5 min with gentle shaking. After being washed three times with 1 ml of PBS, the coverslips were incubated for 45 min with the affinity-puriﬁed anti-MEK2 (1:100 dilution) or ERK1 antibodies (1:50 dilution) in 0.4 ml of PBS supplemented with 1% BSA, 1 mg/ml thyglobulin, and 0.1% saponin (Sigma), then washed four times with PBS plus 0.1% saponin. Anti-rabbit IgG horseradish conjugate (1:100, Boehringer Mannheim) was then incubated with the coverslips for 45 min in the dark in 0.4 ml of PBS containing 0.1% saponin and 1% BSA. The coverslips were washed for 4 x 2 min with 1 ml of PBS plus 0.1% saponin followed by 2 x 2 min with PBS. DAPI (1 pm/ml, Sigma) was added in the second wash to stain nuclei. Finally, the coverslips were mounted with 4 μl of buffered glycerol (10% PBS, 0.1% p-phenylenediamine and 90% glycerol) on a glass slide, sealed with nail polish, and examined by fluorescence microscopy (Zeiss).

Subcellular Fractionation—Swiss 3T3 Cells were fractionated into cytosolic, nuclear, and postnuclear membrane fractions according to Czenkowski et al. (43). The activity of MEK and ERK was determined in each fraction. For Western blotting experiments, proteins from each fraction were precipitated with 10% trichloroacetic acid. Equal portions of each fraction (39 pg of cytosolic protein, 20 pg of nuclear protein, and 6 pg of membrane protein) were subjected to SDS-PAGE followed by immunoblotting with anti-MEK or anti-ERK antibody. Lactate dehydrogenase was determined as a cytosolic marker (43). Detergent permeabilization of cells was done following previously described procedures (46).

Western Blotting—The cell lysate was resolved by 10% SDS-PAGE and transferred to nitrocellulose filters (HybondTM-ECL, Amersham). The filters were blocked overnight in Tris-buffered saline containing 0.2% Tween 20, 5% Carnation dry milk, and 0.5% BSA, and incubated with anti-MEK or anti-ERK antibodies (1:1000 for antisera or 1:500 for affinity-puriﬁed antibodies) for 1 h. Filters were washed for 3 x 5 min with Tris-buffered saline plus 0.3% Triton X-100. The second antibody, a goat anti-rabbit IgG conjugated to either horseradish peroxidase or alkaline phosphatase (Life Technologies, Inc., 1:5000), was incubated and washed in the same buffers as for the primary antibody. ECL detection was performed using reagents from Amersham, as recommended by the manufacturer.

Immunoprecipitation—Swiss 3T3 cells were harvested in RIPA buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 1% Triton X-100, 0.5% deoxycholate, 1 mM EDTA, 1 mM phenylmethylsulfonyl Fluoride, 1 mM dithiothreitol, 0.2 mM sodium vanadate, 25 mM NaF, 10 mM sodium pyrophosphate, 25 mM glycerophosphate). The cell lysate was briefly sonicated and centrifuged in an Eppendorf microcentrifuge for 5 min. The supernatant (0.4 mg of protein) was incubated with 5 μl of preimmune or specific antibody for 1 h on ice. Protein A-agarose (10 μl, Pierce Chemical Co.) was added and incubated for 30 min on ice with shaking.

The protein A-agarose was recovered by centrifugation and washed three times with RIPA buffer and once with kinase buffer minus ATP.

**RESULTS**

**Multiple Forms of MEK Detected by Specific Antibodies**—To study the subcellular localization of MEK and ERK, we prepared anti-ERK and anti-MEK sera from rabbits. The anti-ERK serum was raised against recombinant human ERK1 which was expressed and purified from Escherichia coli. Fig. 1 shows that the anti-ERK serum recognized both ERK1 and ERK2 (Fig. 1, lanes 4 and 5) which share 90% amino acid sequence identity (45). The anti-ERK serum did not detect the recombinant ERK3 (Fig. 1, lane 6) which shares approximately 55% sequence identity with ERK1 and ERK2 (45). The anti-MEK serum, which was prepared against purified recombinant human MEK2 (20), reacted with recombinant MEK1, MEK2, and MEK3 (Fig. 1, lanes 1–3). The predicted amino acid sequence of MEK2 shares approximately 82% identity to MEK1 (20). MEK3 is an alternative splicing form of MEK1. The anti-ERK serum did not recognize MEKs nor did the anti-MEK serum recognize ERKs (not shown).

Affinity-purified antibodies were used to detect ERK and MEK in cultured Swiss 3T3 and PC12 cells. In Swiss 3T3 cells, the anti-ERK antibody recognized two major bands corresponding to ERK2 and ERK1, respectively (Fig. 2, panel B, lane 3). A slight mobility shift was observed when cells were stimulated with EGF (Fig. 2, panel B, lane 4). Proteins of similar molecular weights were detected in PC12 cells (Fig. 2, panel B, lane 5). A mobility shift of the 42- and 44-kDa bands was also observed in NGF-stimulated PC12 cells (Fig. 2, panel B, lane 5). Preimmune antibody did not detect any band in similar experiments (Fig. 2, panel B, lane 1). The reactivity to 42- and 44-kDa bands can be completely eliminated if the antibody was pretreated with purified ERK1 (Fig. 2, panel B, lane 2), suggesting that the 42- and 44-kDa proteins are ERK2 and ERK1, respectively.

It is worth noting that the ratio of the 42- to 44-kDa bands can be completely eliminated if the antibody was pretreated with purified ERK1 (Fig. 2, panel B, lane 2), suggesting that the 42- and 44-kDa proteins are ERK2 and ERK1, respectively.

Affinity-purified anti-MEK antibody detected two major proteins (44 and 45 kDa) in Swiss 3T3 and PC12 cells (Fig. 2, panel
that the staining was specific. We also examined the subcellular localization of ERK in Swiss 3T3 cells with affinity-purified anti-ERK antibody. ERK was found to be distributed in the cytoplasm as well as in the nucleus, consistent with previous observations (Fig. 3, panel 3A) (35-38). The specificity of the anti-ERK antibody was confirmed by competition with purified ERK1 protein (not shown).

In addition to Swiss 3T3 cells, several different cell lines, including PC12, HeLa, and COS, were examined for the subcellular localization. Immunofluorescence staining of MEK was found exclusively in the cytoplasm of these cells (not shown).

Both MEK1 and MEK2 Are Cytoplasmic Proteins—Because the anti-MEK antibody recognized both MEK1 and MEK2, it is possible that the cytoplasmic immunofluorescence staining may be due to one of the more abundant MEK, whereas the other may localize into different compartments of the cell. To study the subcellular localization of individual MEK1 and MEK2, CHO cells were transfected with pCMV-MEK1 or pCMV-MEK2 followed by immunofluorescence staining. The level of expression of MEK1 and MEK2 in transfected cells was significantly higher than that of the endogenous proteins, therefore the immunofluorescence staining observed was mainly due to protein expressed from the transfected plasmids. Both MEK1 and MEK2 were found exclusively in the cytoplasm of the transfected cells (Fig. 4). Similar results have been observed in MEK1- or MEK2-transfected Swiss 3T3 cells (not shown).

Effect of Mitogens on the Subcellular Localization of MEK and ERK—To study the effect of mitogens on the subcellular distribution of MEK, Swiss 3T3 cells stimulated with serum, EGF, or phorbol ester were examined by indirect immunofluorescence. The activities of ERK and MEK were first determined to demonstrate their activation by mitogens. EGF induced a 10-fold activation of MEK and 4-5-fold activation of ERK. Immunofluorescence staining showed that MEK remained in the cytoplasm following treatment with EGF (Fig. 5, panels 1-3). Similarly, neither PMA (Fig. 5, panels 4 and 5) nor serum (not shown) induced a nuclear translocation of MEK. Thus, activation of MEK by mitogens had no effect on its subcellular distribution. In contrast, ERK was found to be redistributed into the nucleus when cells were stimulated with mitogens (Fig. 5, panels 6 and 7), consistent with observations made in other cell lines (35-39). Because the ERK antibody reacted with both ERK1 and ERK2, we were unable to distinguish whether both ERK1 and ERK2 or only one of the ERKs was translocated into the nucleus after mitogen stimulation.

To exclude the possibility that our anti-MEK antibody may not recognize the activated MEK which might be localized to the nucleus, the anti-MEK antibody was used in immunoprecipitation. MEK activity was determined in both the anti-MEK immunoprecipitate and the post-anti-MEK supernatant. The majority of MEK activity (approximately 70%) was found in the anti-MEK immunoprecipitate (not shown). Furthermore, this anti-MEK precipitable activity was greatly stimulated by EGF. These data demonstrate that the anti-MEK antibody used in this study can recognize the activated MEK.

Cytoplasmic Localization of MEK by Subcellular Fractionation—Careful examination of the immunofluorescence data indicated that MEK may not be uniformly distributed in the cytosol (Fig. 3, panel 1A), we tested the possibility that MEK might be associated with cellular structures such as endoplasmic reticulum. Swiss 3T3 cells were fractionated into nuclear, cytosol, and membrane fractions by differential centrifugation (35). Immunoblotting with the anti-MEK antibody was used to detect MEK in those fractions. No MEK protein was detected in nuclear or membrane fractions even after a
Fig. 3. **Immunofluorescence localization of ERK and MEK in Swiss 3T3 cells.** Cells stained with anti-MEK antibody (panel IA, 1:100 dilution) or DAPI (panel IB); panel IC, cells viewed by phase contrast. Controls with preimmune antibody (panels 2A, 2B, and 2C). Immunofluorescence with anti-ERK antibody (panel 3A, 1:50 dilution) or DAPI (panel 3B); panel 3C, cells viewed by phase contrast. Controls with preimmune antibody (panels 4A, 4B, and 4C). I.F. denotes immunofluorescence. Nuclei were visualized by DAPI staining for DNA.

I.F. Nucleus Cell

A-MEK

P.I.

A-ERK

P.I.

I.F. denotes anti-MEK immunofluorescence (top row). The middle row is nuclear staining by DAPI. The bottom row is phase contrast of the cells.

Fig. 4. **Immunofluorescence of transiently expressed MEK1 and MEK2.** Both MEK1 and MEK2 cDNAs were transiently transfected into CHO cells and followed by immunofluorescence with anti-MEK antibody. Left column, cells transfected with pCMV-MEK1; right column, cells transfected with pCMV-MEK2. I.F. denotes anti-MEK immunofluorescence (top row). The middle row is nuclear staining by DAPI. The bottom row is phase contrast of the cells.

MEK1  MEK2

I. F.

Nucleus

Cell

longer exposure in which the two minor bands of 48 and 34 kDa were evident (Fig. 6, panel A). Both MEK1 and MEK2 were detected exclusively in the cytosolic fraction (Fig. 6). The same subcellular fractions were also blotted with the anti-ERK antibody, revealing a small but significant fraction of ERK protein in the nuclear fraction (Fig. 6, panel A). These data are consistent with the immunofluorescence data and similar to previously published observations (35). In addition, enzymatic activities of ERK and MEK in each fraction were determined as was the activity of lactate dehydrogenase as a marker for subcellular fractionation (Fig. 6, panel B), and the results were consistent with immunoblotting in Fig. 6, panel A. Although a small amount of MEK activity was detected in the nuclear fraction, this could be contributed by a small percent of cyto-
plasmic contamination.

The cytosolic localization of MEK was further confirmed by detergent release experiments. Digitonin was used to permeabilize monolayer Swiss 3T3 cells. Western blotting with anti-MEK serum of digitonin-released samples showed that MEK protein was quantitatively released from the cells under these conditions (not shown). Data from subcellular fractionation and detergent permeabilization support the immunofluorescence results, demonstrating that MEK1 and MEK2 are soluble cytosolic proteins.

DISCUSSION

Rapid activation of ERK is common to signal transduction by various mitogenic growth factors (1-6). The complete pathway of mitogenic signal transduction is just beginning to emerge (47, 48). ERK is activated exclusively by MEK, which phosphorylates ERK on threonine and tyrosine residues. MEK can be activated by several different upstream activators, including c-Raf, MEKK, and c-Mos (26-30). We have shown that both MEK1 and MEK2 are exclusively localized in the cytoplasm of cells, including Swiss 3T3, PC12, CHO, COS, and HeLa. Neither MEK protein nor MEK activity was found in the nucleus. Immunofluorescence results can be affected by many factors such as cell morphology, fixation methods, and antibody accessibility. It is important to confirm the immunofluorescence data by different techniques. We performed subcellular fractionation experiments to support our immunofluorescence data. However, we cannot exclude the possibility that a very small fraction of MEK1 and/or MEK2, not detected by the anti-MEK antibody, exists in the cell nucleus. Furthermore, it is possible that the anti-MEK antibody may selectively recognize one isoform of the endogenous MEK by immunofluorescence even though the antibody was able to immunoblot and immunoprecipitate both MEKs.

The amount of ERK protein detected in the nuclear fraction appears to be less than expected from the immunofluorescence data (Fig. 5). One possibility for the low recovery of ERK in the nuclear fraction may be leakage of ERK from the nucleus into cytosol during fractionation. The leakage of soluble nuclear
proteins into the cytosol during subcellular fraction has been observed for several proteins. For example, CTP:phosphocholine cytidylyltransferase fractions as a cytosolic protein, but appears nuclear by immunofluorescence with specific antibody (42). Likewise, DNA polymerase (50) and DNA ligase (51) appear cytosolic upon fractionation, but nuclear upon immunocytochemical localization. Similar phenomena have been observed in casein kinase II (52) and unliganded progesterone receptor (53).

Growth factor signals cross the cytoplasmic membrane barrier via transmembrane tyrosine kinase receptors. Signals are integrated and amplified in the cytoplasm via a kinase cascade which includes Raf, MEK, ERK, and rsk (48). How the cytoplasmic phosphorylation signals cross the nuclear envelope barrier is not yet well understood. Recently, Chen et al. (35) reported that ERK is localized in both and in unstimulated HeLa cells. When cells were stimulated with growth factors or serum, significant redistribution of ERK from cytoplasm to nucleus occurred. Similar results have also been observed in other cells stimulated by different mitogens (36-39). We showed in this report that ERK was translocated into the nucleus when Swiss 3T3 cells were stimulated by mitogens. The cytoplasmic localization of MEK indicates that ERK is phosphorylated and activated in the cytosol. The phosphorylated active ERK may be subsequently translocated into the nucleus where phosphorylation of nuclear substrates occurs, suggesting that translocation of ERK may play a pivotal role in signal transfer from the cytoplasm into the nucleus.

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