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Prolonged Nuclear Retention of Activated Extracellular Signal-regulated Kinase 1/2 Is Required for Hepatocyte Growth Factor-induced Cell Motility*

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We examined the signaling pathway by which hepatocyte growth factor (HGF) induces cell motility, with special focus on the role of extracellular signal-regulated kinase (ERK) in the nucleus. We used Madin-Darby canine kidney cells overexpressing ERK2 because of their prominent motility response to HGF. HGF stimulation of the cells induces not only a rapid, marked, and sustained activation and rapid nuclear accumulation of ERK1/2, but also a prolonged nuclear retention of the activated ERK1/2. Interruption of the ERK1/2 activation by PD98059 treatment of the cells 30 min after HGF stimulation abolishes the HGF-induced cell motility. Enforced cytoplasmic retention of the activated ERK1/2 by the expression of an inactive form of MKP-3 cytoplasmic phosphatase inhibits the cell motility response. Although epidermal growth factor stimulation of the cells induces the activation and nuclear accumulation of ERK1/2, it does not induce the prolonged nuclear retention of the activated ERK1/2, and fails to induce cell motility. In the nucleus, activated ERK1/2 continuously phosphorylate Elk-1, leading to the prolonged expression of c-*fos*, which results in the expression of several genes such as matrix metalloproteinase *(mmp)-9*; MMP-9 activity is required for the induction of the cell motility response. Our results indicate that the sustained activity of ERK1/2 in the nucleus is required for the induction of HGF-induced cell motility.

Hepatocyte growth factor (HGF),[1] also known as scatter factor, is a multifunctional cytokine capable of inducing a wide variety of biological effects in normal and neoplastic cells. These include proliferation of hepatocytes as well as many types of epithelial and endothelial cells, dissociation of epithelial cell colonies into individual cells and stimulation of their motility, induction of epithelial morphogenesis, induction of angiogenesis, and promotion of the invasion of extracellular matrices. *In vivo*, HGF has been shown to be involved in embryological development, tissue regeneration, tumorigenesis, and metastasis (reviewed in Refs. 1 and 2).

HGF exerts its diverse effects through a high-affinity HGF receptor, which has been identified as the c-*met* proto-oncogene product (reviewed in Ref. 3). The mature form of the c-Met receptor is a heterodimeric protein consisting of a 50-kDa extracellular α-subunit and a transmembrane 145-kDa β-subunit containing a catalytic tyrosine kinase domain. Binding of HGF triggers phosphorylation of the receptor on Tyr^{1349} and Tyr^{1356} of the β-subunit; these phosphorylated tyrosine residues function as docking sites for Src homology 2 domain-containing signal transducers such as PI 3-kinase, phospholipase-Cγ, Grb2, Shc, $pp60^{c\text{-}src}$, and Gab1. As a result of these interactions, HGF induces the activation of various signaling molecules, including Ras, PI 3-kinase, phospholipase-Cγ, and 41-/43-kDa mitogen-activated protein kinases (ERK2 and ERK1, respectively) (3, 4). However, the precise signaling pathways downstream of c-Met that mediate each of the diverse biological effects of HGF remain largely obscure.

Cell motility is a fundamental process required during normal embryonic development, wound repair, inflammatory response, and tumor metastasis (5). Recently, the molecular mechanisms involved in the regulation of cell motility have been examined using several lines of normal and neoplastic cells in culture. Among them is the Madin-Darby canine kidney (MDCK) epithelial cell line. HGF initially induces centrifugal spreading of MDCK cells in colonies and subsequently stimulates cell-cell dissociation, allowing each cell to detach from colonies and to migrate independently of other cells (cell scattering). Using the HGF/MDCK cell system, Ras function has been shown to be necessary for the induction of the cell motility response (6, 7). Ras functions as a molecular switch controlled by GDP/GTP cycling. Once in the active GTP-binding state, Ras activates multiple signal transduction pathways such as the ERK and PI 3-kinase pathways (8). We have recently shown that the ERK pathway plays an essential role in inducing the motility response of MDCK cells to HGF (9). Similar results suggesting a crucial role for the ERKs in the regulation of cell motility have been reported in a variety of cell systems (10–14).

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[1] The abbreviations used are: HGF, hepatocyte growth factor; MDCK, Madin-Darby canine kidney; ERK, extracellular signal-regulated kinase; MAP kinase, mitogen-activated protein kinase; PI 3-kinase, phosphatidylinositol 3-kinase; MKP, MKP kinase phosphatase; EGF, epidermal growth factor; MEK, MAP kinase/ERK kinase; MMP, matrix metalloproteinase, GFP, green fluorescent protein; DMEM, Dulbecco's modified Eagle's medium; EGCG, epigallocatechin-3-galate; AP-1, activator protein-1.

The activation of the ERK pathway is thus required downstream of Ras for the induction of the cell motility response.

Activation of the ERK pathway is essential for cell proliferation (15, 16), and rapid translocation of the activated ERKs from the cytoplasm to the nucleus is observed in mitogen-stimulated cells (17). Furthermore, it has recently been shown that the nuclear accumulation of the activated ERKs and their subsequent phosphorylation of nuclear targets is required for mitogen-induced gene expression and cell cycle re-entry (18). On the contrary, ERK promotion of cell motility in a transcription-independent manner has been reported. For example, phosphorylation of myosin light chain kinase by activated ERKs has been shown to increase its ability to phosphorylate the myosin light chain, which promotes the cytoskeletal contraction necessary for cell movement (10, 12). Activated ERKs phosphorylate and suppress the function of specific integrins (19) and may promote rapid disassembly of focal contacts (20), allowing more effective cytoskeletal contraction at the rear of migrating cells (5).

In this study, we have examined whether or not ERK activity in the nucleus is required for the induction of the cell motility response. We used MDCK cell clones overexpressing ERK2 because of their prominent motility response to HGF. Our results demonstrate that in addition to a rapid, marked, and sustained activation of ERK1/2 and its rapid accumulation in the nucleus, a prolonged nuclear retention of the activated ERK1/2 is required for the induction of the cell motility response.

EXPERIMENTAL PROCEDURES

Materials—Human recombinant HGF (21) was kindly provided by Dr. Toshikazu Nakamura (Osaka University). EGF purified from mouse submaxillary glands was purchased from Toyobo Co. (Osaka, Japan). The polyclonal anti-ERK1/2 antibody has been described previously (22, 23). The polyclonal anti-GFP antibody was obtained from CLONTECH. The monoclonal anti-c-Myc antibody (9E10) and the polyclonal anti-c-Myc antibody (A-14) were from Santa Cruz Biotechnology. The monoclonal anti-phospho-ERK1/2 antibody and the monoclonal anti-FLAG antibody (M2) were from Sigma. The monoclonal anti-β-catenin antibody was from Transduction Laboratories. 2-(2-Amino-3-methoxyphenyl)chromone, which is identical to the published compound PD98059 (24), was synthesized as described (9). GM6001 was purchased from Calbiochem, whereas epigallocatechin-3-galate (EGCG) was kindly provided by Dr. Isao Kouno (Nagasaki University). Other chemicals and reagents were of the purest grade available.

Cell Culture—MDCK cells obtained through the Japanese Cancer Resources Bank were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum. MDCK cell clones stably overexpressing ERK2 were cultured in DMEM supplemented with 10% fetal calf serum and 200 μg/ml Geneticin (Invitrogen).

Plasmids and Transfection—The expression vectors for MKP-3 (pMT-SM-myc-MKP-3) (25) and an inactive form of MKP-3 (pMT-SM-myc-MKP-3(CS), the catalytic site Cys^{293} was replaced with serine) (26) were kindly provided by Dr. Steve Arkinstall (Serono Pharmaceutical Research Institute), and the vectors for a kinase-negative form of MEK1 (pcDNAIneo-myc-MEK1(AA), the phosphorylation sites Ser^{218} and Ser^{222} were replaced with alanine) and a constitutively active form of MEK1 (pcDNAIneo-myc-MEK1 (EE), the phosphorylation sites Ser^{218} and Ser^{222} were replaced with glutamic acid) were kindly provided by Dr. Emmanuel Van Obberghen (INSERM Unit 145, France). Transfection of these plasmids into MDCK cells was carried out using LipofectAMINE 2000 reagent (Invitrogen) according to the manufacturer's protocol.

For the establishment of MDCK cell clones stably overexpressing ERK2, ERK2 cDNA (a kind gift from Dr. Michael J. Weber, University of Virginia Health Science Center) was subcloned into the *Eco*RI site of the pEGFP-C1 expression vector (CLONTECH) and transfected into the cells using LipofectAMINE 2000 reagent. After selection in growth medium supplemented with 400 μg/ml Geneticin, individual resistant colonies were isolated to obtain ERK2-MDCK cell clones.

Cell Lysis and Immunoblotting—MDCK or ERK2-MDCK cells, mock-treated or treated with 4 ng/ml HGF, were washed twice with ice-cold phosphate-buffered saline, scraped off the plates into a hypotonic cell lysis buffer (25 mM Tris-HCl, pH 7.4, 25 mM NaCl, 1 mM sodium orthovanadate, 10 mM NaF, 10 mM sodium pyrophosphate, 25 mM β-glycerophosphate, 25 mM *p*-nitrophenyl phosphate, 20 nM okadaic acid, 0.2 mM sodium molybdate, 0.5 mM EGTA, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, and 1% aprotinin), and then flash-frozen in liquid nitrogen (27, 28). After three freeze-thaw cycles, the cells were lysed by sonication for 30 s. Lysates were cleared by centrifugation at 12,000 × *g* for 30 min, and protein concentrations were determined using the BCA protein assay reagent (Pierce). Cell lysates (10 μg of protein) were then separated by SDS-PAGE, electrophoretically transferred to an Immobilon-P membrane (Millipore Corp.), and subjected to immunodetection using the appropriate primary antibody and horseradish peroxidase-conjugated secondary antibody (Promega). Proteins were visualized with the enhanced chemiluminescence system (Amersham Biosciences) (9, 22).

Protein Kinase Assay—ERK activity was measured in an immune complex kinase assay as described previously (9, 27, 28). Briefly, cell lysates prepared as described above (10 μg of protein) were immunoprecipitated by incubation for 3 h at 4 °C with the anti-ERK1/2 antibody or the anti-GFP antibody preabsorbed to Protein A-Sepharose (Amersham Biosciences). After washing twice with kinase buffer (50 mM Tris-HCl, pH 8.0, 25 mM $MgCl_2$, 0.5 mM EGTA, 1 mM dithiothreitol, and 0.1 mM sodium orthovanadate), each immunoprecipitate was incubated for 30 min at 30 °C with 20 μM ATP, 1 μCi of [γ-^{32}P]ATP (Amersham Biosciences), and 15 μg of myelin basic protein (Sigma) in 30 μl of the kinase buffer. Radioactivity incorporated into myelin basic protein was determined by liquid scintillation spectrometry.

Cell Staining—Cells grown on glass coverslips were fixed with 3.7% paraformaldehyde and then permeabilized with 0.25% Triton X-100. After blocking the nonspecific sites with 2.5% bovine serum albumin in phosphate-buffered saline, cells were immunostained with the monoclonal anti-c-Myc antibody (dilution: 1/250), the polyclonal anti-c-Myc antibody (1/250), the monoclonal anti-phospho-ERK1/2 antibody (1/250), the polyclonal anti-ERK1/2 antibody (1/250), or the monoclonal anti-β-catenin antibody (1/250) as the primary antibody, and the Alexa™ 488 goat anti-rabbit IgG conjugate (A-11034, Molecular Probes) or the Alexa™ 546 goat anti-mouse IgG conjugate (A-11030, Molecular Probes) as the secondary antibody (28).

Northern Blot Analysis—Poly(A)$^+$ RNA was isolated from HGF-stimulated ERK2-MDCK cells by the use of Quickprep mRNA Purification Kits (Amersham Biosciences). One microgram of poly(A)$^+$ RNA was electrophoresed on a 1.2% agarose gel containing 2.2 M formaldehyde, and then transferred to a positively charged nylon membrane (Roche Molecular Biochemicals). The filter-bound poly(A)$^+$ RNA was hybridized for 16 h at 50 °C with cDNA probes that were labeled with digoxigenin-11–2′-deoxyuridine-5′-triphosphate by PCR. The filter was then washed under stringent conditions, and chemiluminescent detection with a DIG Luminescent Detection Kit was performed according to the DIG System user's guide (Roche Molecular Diagnostics). Rehybridization was effected on membranes that had been stripped by boiling in 0.1% SDS. cDNA probes were generated by PCR from human TIG3 fibroblast cDNA using specific primers against the published sequence of human *mmp-9* from nucleotide 118 to 640 (EMBL accession number J05070), human *rhoE* from nucleotide 257 to 627 (GenBank™ accession number S82240), human *ezrin* from nucleotide 146 to 470 (GenBank™ accession number X51521), and human *β-actin* from nucleotide 216 to 625 (GenBank™ accession number X00351). The amplified products were verified by DNA sequencing. The c-*fos* cDNA probe was prepared as described previously (27). Northern blot analysis of ERK2-MDCK cell poly(A)$^+$ RNA, by hybridization with each of the specific cDNA probes, showed one prominent transcript of 2.8, 3.5, 3.2, 1.8, or 2.2 kb that corresponded in size to mRNA encoding human *mmp-9*, *rhoE*, *ezrin*, *β-actin*, or c-*fos*, respectively.

Construction of the FLAG-tagged Elk-1 Expression Vector—To generate an expression plasmid encoding FLAG-tagged Elk-1, a cDNA fragment was amplified from human TIG3 fibroblast cDNA by PCR using the following primers: forward, 5′-CCAGCGATGGACCCATCTGTG-3′, and reverse, 5′-TCACTTGTCGTCGTCGTCCTTGTAGTCTGGCTTCTGGGGCCCTGG-3′ (the underlined segment corresponds to the antisense sequence of the COOH-terminal FLAG epitope). The PCR product was cloned into pGEM-TEasy vector (Promega), verified by sequencing, digested with *Not*I, and ligated to *Not*I-digested pcDNA3 vector (Invitrogen).

Gelatin Zymography—HGF-induced production of MMP-9 in ERK2-MDCK cells was analyzed by gelatin zymography. This technique resolves gelatinases by their molecular mass and is appropriate for detection of both the latent (precursor) and active (processed) forms of the enzymes; in the presence of SDS, the enzymes are denatured to expose

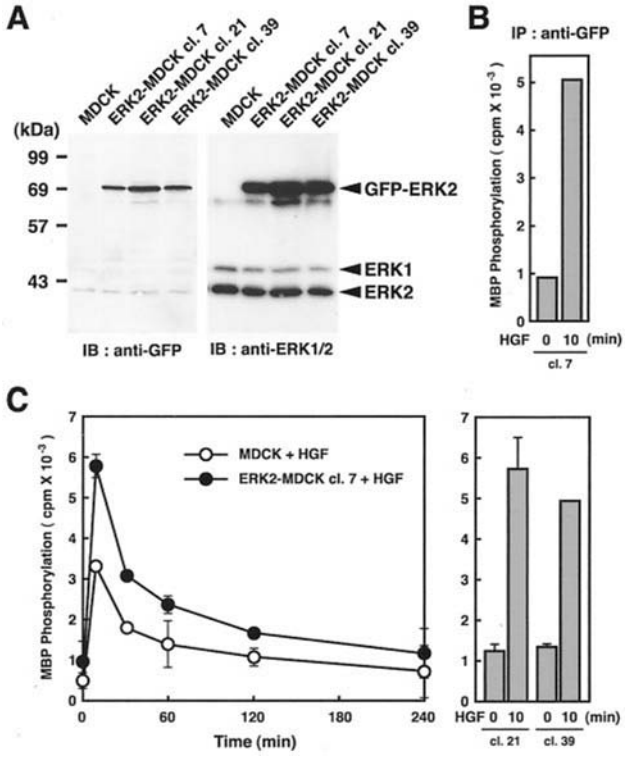

FIG. 1. **HGF-induced activation of ERK1/2 in MDCK and ERK2-MDCK cells.** *A*, expression of exogenously introduced GFP-ERK2 was examined by immunoblot analysis. Cell lysates (10 μg of protein) of MDCK cells and ERK2-MDCK cells (*clones 7*, *21*, or *39*) were resolved by SDS-PAGE, blotted, and probed with the anti-GFP or anti-ERK1/2 antibody, followed by enhanced chemiluminescence detection. *B*, ERK2-MDCK cells (*clone 7*) were treated with 4 ng/ml HGF for 10 min, or were left untreated. ERK assay was performed by incubating cell lysates (30 μg of protein) with the anti-GFP antibody followed by the kinase reaction. Radioactivity incorporated into myelin basic protein (*MBP*) was determined. *C*, MDCK and ERK2-MDCK cells (*clones 7*, *21*, or *39*) were treated with 4 ng/ml HGF for the indicated periods of time. ERK assay was performed by incubating cell lysates (10 μg of protein) with the anti-ERK1/2 antibody followed by the kinase reaction. Radioactivity incorporated into myelin basic protein was determined. Each value represents the mean ± S.D. of triplicate determinations of a representative experiment. Data shown are representative of three to four separate experiments that gave essentially the same results.

their active site, which permits both the latent and active forms of the gelatinases to exhibit gelatinolytic activity after partial renaturation (29).

ERK2-MDCK cells were incubated in serum-free medium (DMEM containing 2 mg/ml bovine serum albumin, 1 μg/ml insulin, 2 μg/ml transferrin, 30 nM Na_2SeO_3, and 10 mM Hepes, pH 7.4) for 6 h, and then stimulated with 4 ng/ml HGF for the indicated periods of time. In some experiments, the cells were treated with 50 μM PD89059 30 min before or 30 min after HGF stimulation. Conditioned media were mixed with 1/5 volume of 312 mM Tris-HCl, pH 6.8, containing 10% SDS and 0.1% bromphenol blue, and loaded on 7.5% SDS-polyacrylamide gels that had been co-polymerized with 0.1% gelatin (Sigma). Electrophoresis was performed under nonreducing conditions. Gels were washed twice for 60 min in wash buffer (50 mM Tris-HCl, pH 7.5, containing 1% Triton X-100) to remove SDS, and were incubated in reaction buffer (50 mM Tris-HCl, pH 7.5, containing 1% Triton X-100, 10 mM $CaCl_2$, 150 mM NaCl, and 0.01% NaN_3) for 24 h at 37 °C. Gels were stained with 0.2% Coomassie Blue in 30% methanol, 10% acetic acid for 60 min at room temperature and destained in 30% methanol, 10% acetic acid. The presence of MMPs was indicated by an unstained proteolytic zone in the substrate.

Matrigel Invasion Assay—The ability of cells to move through Matrigel-coated filters was measured in a Boyden chamber. Eight-micrometer Transwell filters coated with 50 μg/cm^2 Matrigel (BD PharMingen) were used as described (30). DMEM supplemented with 10% fetal calf serum was placed in the lower chamber. ERK2-MDCK cells (3.5×10^4 per well) were placed into the upper chamber in 0.5 ml of DMEM supplemented with 10% fetal calf serum. After 24 h, the cells were treated with HGF for 24 h. Cells that had not penetrated the filter were wiped away with cotton swabs, and cells that had migrated to the lower surface of the filter were fixed with 70% ethanol, stained by the Giemsa method, and examined by bright field microscopy. Values for invasion were determined by calculating the average number of migrated cells per mm^2 over three fields per assay and were expressed as an average of triplicate determinations.

RESULTS

Overexpression of ERK2 Enhances the Motility Response of MDCK Cells to HGF—We and others have recently shown that activation of the ERK pathway is required for the induction of the cell motility response (9–14). This conclusion has been drawn from experiments that used a specific inhibitor of the ERK pathway, PD98059, which inhibits MEK activity (24). To elucidate the direct causal link between the ERK pathway and cell motility, we established MDCK cell clones stably overexpressing GFP-ERK2. Immunoblot analysis of cell lysates using the anti-GFP antibody and the anti-ERK1/2 antibody revealed that the expression levels of GFP-ERK2 were severalfold greater than the endogenous ERK2 level in many of the ERK2-MDCK cell clones isolated (Fig. 1*A*). HGF treatment of these ERK2-MDCK cells stimulated the ability of anti-GFP immunoprecipitates to phosphorylate myelin basic protein, indicating that the expressed GFP-ERK2 was functionally active (Fig. 1*B*). Accordingly, total ERK activity in HGF-stimulated ERK2-MDCK cells was ~2 times higher than that in the parental MDCK cells (Fig. 1*C*). Although overexpression of GFP-ERK2 induced a slight increase in the basal ERK activity, the overexpression did not affect the morphology or growth of the cells, *i.e.* ERK2-MDCK cells proliferated as discrete colonies when seeded sparsely as did the parental MDCK cells (Fig. 2). Importantly, however, the motility of ERK2-MDCK cells was markedly enhanced in response to HGF stimulation as compared with that observed in MDCK cells. Upon treatment with HGF, the parental MDCK cell colonies began to spread and dissociate by ~6 h, and scattering of the cells was observed only after ~12 h. On the other hand, the ERK2-MDCK cell colonies spread and dissociated within the first 3 h, and marked scattering of these cells was observed as early as 6 h after HGF stimulation (Fig. 2). The close correlation between the elevated ERK activity and enhanced scattering response observed in HGF-stimulated ERK2-MDCK cells suggests that the ERK pathway plays an essential role in the induction of cell motility. In the following experiments, we used ERK2-MDCK cells (clone 7) because of their prominent motility response to HGF.

Expression of a Kinase-negative Form of MEK1 Inhibits HGF-induced Cell Motility, Whereas Expression of a Constitutively Active Form of MEK1 Induces Cell Motility without HGF stimulation—To further confirm the essential role of the ERK pathway in HGF-induced cell motility, ERK2-MDCK cells were transiently transfected with an expression vector encoding either the kinase-negative form or the constitutively active form of MEK1. The transfection efficiency of these cDNAs into ERK2-MDCK cells was ~25% in repeated experiments. Because the effect of exogenous expression of the mutant forms of MEK1 on ERK activity could not be determined precisely in such a mixed cell population, we only analyzed the morphological changes of cells in which expression of the cDNA was confirmed by immunofluorescent microscopy using the anti-c-Myc (epitope) antibody.

ERK2-MDCK cells not expressing the kinase-negative form of MEK1, MEK1(AA), showed ERK activation predominantly in the nuclei 30 min after treatment with HGF, and scattering of the cells was clearly observed after 6 h as revealed by loss of cell-cell contact and spindle-shaped/fibroblastic cell morphology (Fig. 3). In contrast, ERK2-MDCK cells expressing

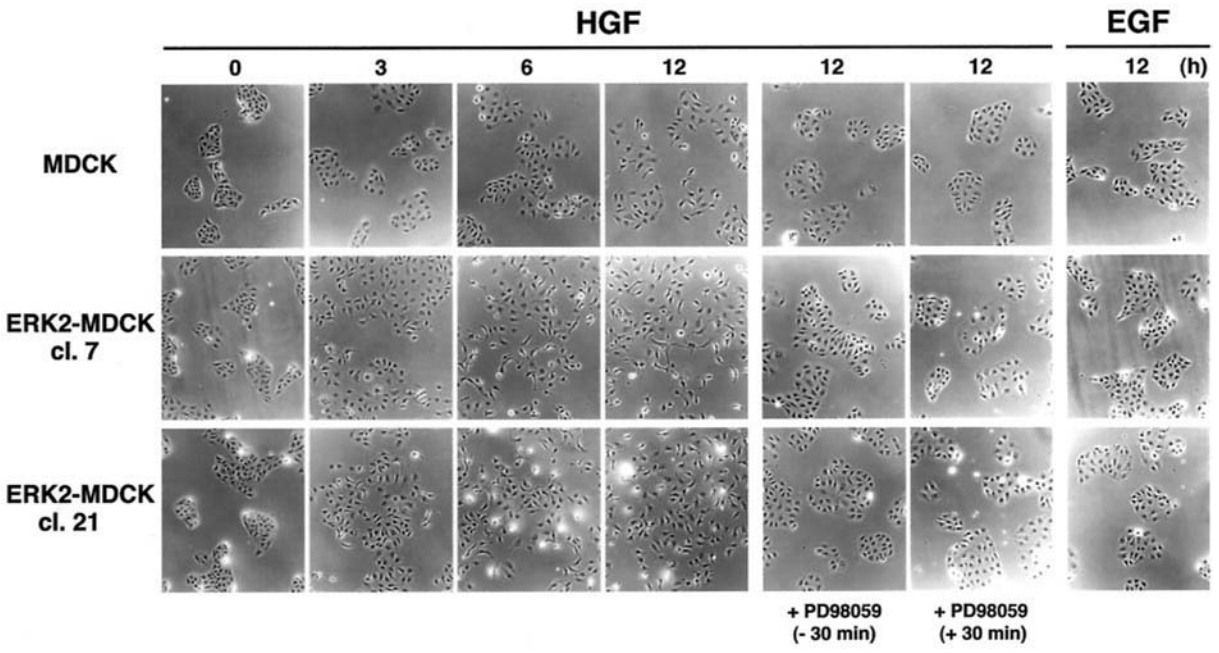

FIG. 2. **Amplification of the ERK pathway by the overexpression of ERK2 enhances the motility response of MDCK cells to HGF.** MDCK and ERK2-MDCK cells (*clones 7* or *21*) were stimulated with 4 ng/ml HGF or 10 ng/ml EGF for the indicated periods of time. In some experiments, the cells were preincubated with 50 μM PD98059 for 30 min followed by HGF stimulation (*−30 min*), or the cells were treated with 50 μM PD98059 30 min after HGF stimulation to interrupt the ERK activation (*+30 min*). Data shown are representative of three separate experiments that gave essentially the same results.

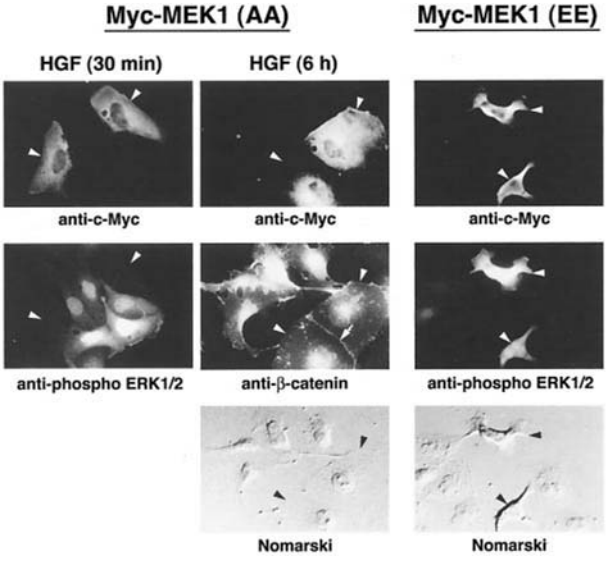

FIG. 3. **Expression of MEK1(AA) in ERK2-MDCK cells inhibits HGF-induced ERK activation and cell motility, whereas expression of MEK1(EE) induces cell motility without HGF-stimulation.** ERK2-MDCK cells (clone 7) were transfected with an expression vector encoding Myc-tagged MEK1(AA) or Myc-tagged MEK1(EE). After incubation in the growth medium for 24 h, the MEK1(AA)-transfected cells were stimulated with 4 ng/ml HGF for the indicated periods of time. Cells expressing either MEK1(AA) or MEK1(EE) were detected by immunofluorescence using the anti-c-Myc antibody. Localization of the activated ERK1/2 was revealed by immunofluorescence using the anti-phospho-ERK1/2 antibody, and localization of β-catenin was determined with the anti-β-catenin antibody. The corresponding interference contrast images are also shown (*Nomarski*). *Arrowheads* indicate cells expressing either Myc-MEK1(AA) or Myc-MEK1(EE), and the *arrow* indicates the localization of β-catenin at an adherens junction in the cells. Data shown are representative of three separate experiments that gave essentially the same results.

MEK1(AA) did not show any ERK activation in response to HGF; these cells retained a flat, polygonal morphology and maintained cell-cell contact even after HGF treatment for 6 h. Immunofluorescence staining showed that the adherens junction components, such as β-catenin and E-cadherin (data not shown), were localized at intercellular junctions in the cells that expressed the kinase-negative form of MEK1.

ERK2-MDCK cells were then transfected with an expression vector encoding the constitutively active form of MEK1, MEK1(EE). As shown in Fig. 3, ERK2-MDCK cells expressing MEK1(EE) showed ERK activation throughout the cells in the absence of HGF stimulation; these cells had disrupted cell-cell contact and exhibited spindle-shaped morphology, characteristics of scattering cells. In contrast, ERK2-MDCK cells not expressing the constitutively active form of MEK1 retained the flat, polygonal morphology. These results clearly support our previous observation that activation of the ERK pathway was necessary for HGF-induced motility of MDCK cells (9).

HGF Induces Prolonged Nuclear Retention of Activated ERK1/2 That Is Required for the Induction of the Cell Motility Response—HGF treatment induced a rapid and sustained activation of ERK1/2 in MDCK and ERK2-MDCK cells (Figs. 1 and 4*A*). To examine the potential significance of such sustained activation of ERKs for the induction of the cell motility response, ERK2-MDCK cells were stimulated with HGF for 30 min to fully activate ERK1/2 and then treated with 50 μM PD98059 to interrupt the ERK activation. This PD98059 treatment rapidly diminished the ERK activity to the basal level (Fig. 4, *A* and *B*), which resulted in the complete abolishment of HGF-induced cell scattering, as had PD98059 treatment prior to HGF stimulation (Fig. 2). In this context, EGF treatment also induced a sustained activation of ERK1/2 in ERK2-MDCK cells; the kinetics and degree of ERK activation were quite similar to those observed in HGF-treated cells (Fig. 4*A*). However, EGF did not induce the motility response (Fig. 2), but did induce a moderate mitogenic response and increased the cell number by 10–20% over that of the untreated cells in 24–48 h (data not shown). These results suggest that sustained activation of ERK1/2 is necessary but not sufficient for the induction of the cell motility response.

It has recently been shown that mitogen-induced activation

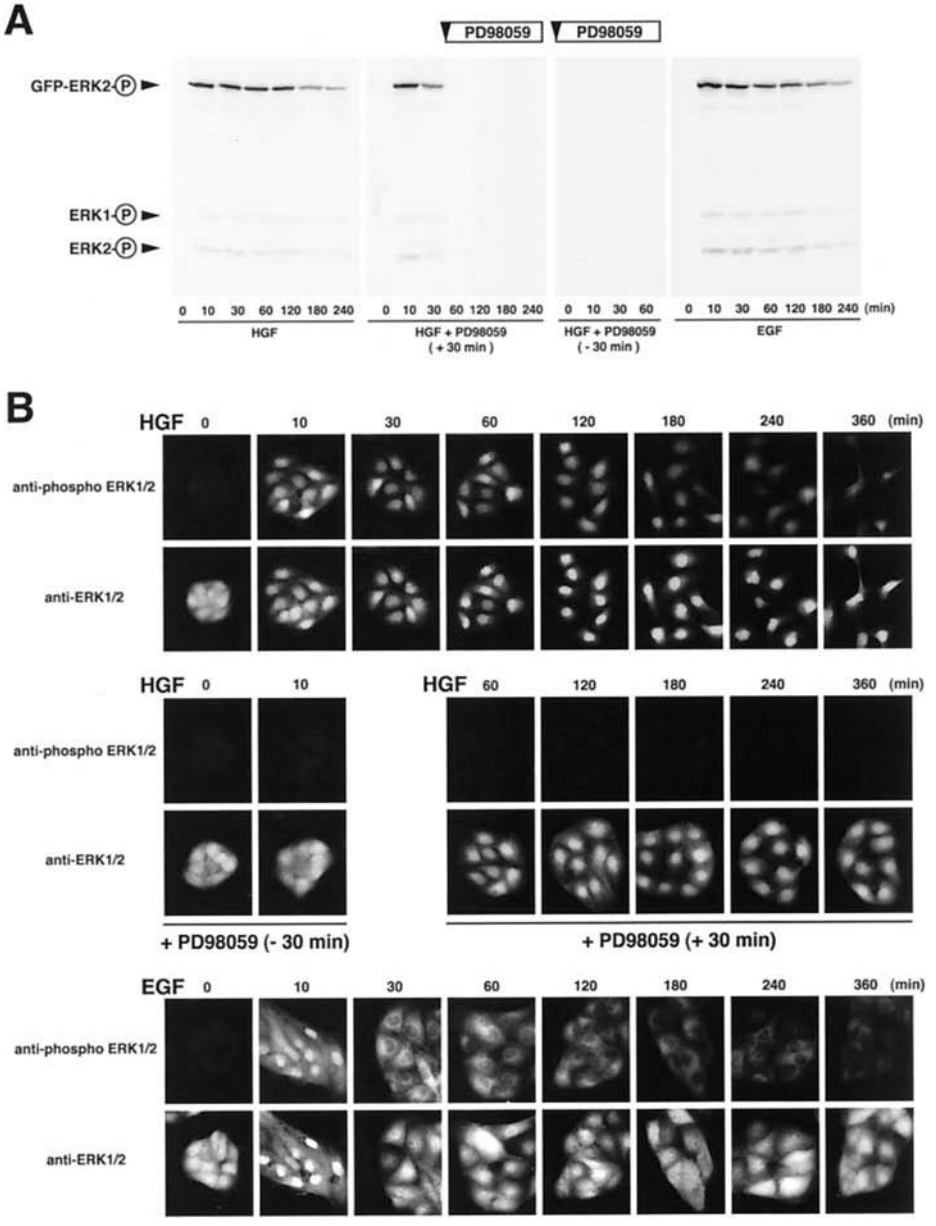

FIG. 4. **HGF-induces a sustained activation of ERK1/2, a nuclear accumulation of ERK1/2, and a prolonged nuclear retention of the activated ERK1/2 in ERK2-MDCK cells.** ERK2-MDCK cells (clone 7) were stimulated with 4 ng/ml HGF or 10 ng/ml EGF for the indicated periods of time. In some experiments, the cells were preincubated with 50 μM PD98059 for 30 min followed by HGF stimulation (*−30 min*), or the cells were treated with 50 μM PD98059 30 min after HGF stimulation to interrupt the ERK activation (*+30 min*). *A*, cell lysates (20 μg of protein) were resolved by SDS-PAGE, followed by immunoblot analysis using the anti-phospho-ERK1/2 antibody. *B*, localization of the activated ERK1/2 was determined by immunofluorescence using the anti-phospho-ERK1/2 antibody, whereas localization of total (activated plus unactivated) ERKs was revealed with the anti-ERK1/2 antibody. Data shown are representative of three to four separate experiments that gave essentially the same results.

of ERKs plays a key role in triggering the nuclear accumulation of ERKs (31). In accordance with this notion, activation of ERK1/2 was accompanied by their rapid nuclear accumulation both in EGF- and HGF-treated ERK2-MDCK cells, which was totally inhibited by pretreatment of the cells with PD98059 (Fig. 4*B*). However, the nuclear retention pattern of total (activated plus unactivated) ERK1/2 in HGF-treated cells was remarkably different from that in EGF-treated cells. In HGF-stimulated cells, ERK immunostaining was observed predominantly in the nucleus even after 6 h and was not affected significantly by PD98059 treatment 30 min after HGF stimulation to interrupt the ERK activation. In contrast, ERK immunostaining was observed throughout the cells as early as 30–60 min after EGF stimulation, a pattern that was very similar to the one observed in unstimulated control cells.

The response to HGF and EGF were more markedly different in the intracellular distribution of activated ERK1/2 in ERK2-MDCK cells. As shown in Fig. 4*B*, stimulation of ERK2-MDCK cells with either HGF or EGF rapidly induced phospho(activated)-ERK1/2 immunostaining in both the cytoplasmic compartment and nucleus. However, the phospho-ERK1/2 immunostaining in HGF-stimulated cells was most prominent in the nucleus; after reaching the maximal level within 10 min, the phospho-ERK1/2 immunostaining in the nucleus declined slowly and could still be detected after 6 h. In contrast, the phospho-ERK1/2 immunostaining in the nucleus of EGF-treated cells declined rapidly and was barely detectable after 1–2 h, whereas that in the cytoplasmic compartment declined slowly and could still be detected after 6 h. Taken together, these results suggest that prolonged nuclear retention of the activated ERK1/2 is required for the induction of the cell motility response.

Enforced Cytoplasmic Retention of the Activated ERK1/2 Abolishes the HGF-induced Cell Motility—To further examine the essential role of nuclear ERK activity in inducing the cell motility response, we attempted to retain ERK1/2 in the cytoplasm without affecting their phosphorylation/activation states. To this end, we used an inactive form of MKP-3 in which the crucial cysteine of the catalytic site had been replaced by serine (MKP-3(CS)) (26). MKP-3 displays a unique characteristic of being localized in the cytoplasm and being able to form a specific complex with ERK1/2, to the exclusion of the other MKP family members (25, 32). Furthermore, MKP-3(CS) has been shown to form specific complexes with ERK1/2 and to

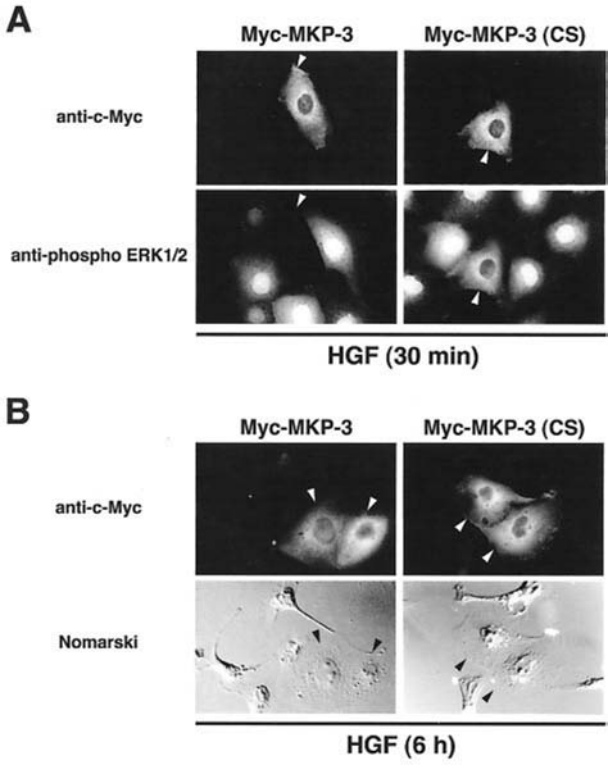

FIG. 5. **Enforced cytoplasmic retention of activated ERK1/2 abolishes HGF-induced cell motility.** ERK2-MDCK cells (clone 7) were transfected with an expression vector encoding Myc-tagged MKP-3 or the Myc-tagged MKP-3(CS), and then incubated in the growth medium for 24 h before HGF stimulation. *A*, the cells were stimulated with 4 ng/ml HGF for 30 min. Cells expressing either MKP-3 or MKP-3(CS) were detected by immunofluorescence using the anti-c-Myc antibody. Localization of activated ERK1/2 was detected by immunofluorescence using the anti-phospho-ERK1/2 antibody. *B*, the cells were stimulated with 4 ng/ml HGF for 6 h. Cells expressing either MKP-3 or MKP-3(CS) were detected by immunofluorescence using the anti-c-Myc antibody. The corresponding interference contrast images are shown (*Nomarski*). *Arrowheads* indicate the cells expressing either Myc-MKP-3 or Myc-MKP-3 (CS). Data shown are representative of three separate experiments that gave essentially the same results.

anchor them in the cytoplasm without affecting their activation state (18).

ERK2-MDCK cells were transiently transfected with an expression vector encoding Myc-tagged MPK-3 or MKP-3(CS), and we determined the effect of these constructs on the activation state and localization of ERKs by double immunofluorescence using an anti-c-Myc antibody and an anti-phospho-ERK1/2 antibody. Both the wild-type and inactive forms of MKP-3 were localized in the cytoplasm (Fig. 5, *A* and *B*). Expression of wild-type MKP-3 completely prevented ERK activation upon HGF stimulation of the cells, which was consistent with its phosphatase activity. In contrast, expression of MKP-3(CS) inhibited the HGF-stimulated activation of ERK1/2 specifically in the nucleus but not in the cytoplasm, presumably by preventing the nuclear accumulation of activated ERK1/2.

Importantly, not only ERK2-MDCK cells expressing wild-type MKP-3 but also those expressing MKP-3(CS) remained in flat and polygonal morphology and maintained cell-cell contact even 6 h after HGF treatment, by which time ERK2-MDCK cells not expressing these constructs lost cell-cell contact and exhibited the spindle-shaped morphology characteristic of scattering cells (Fig. 5*B*). These results strengthen the idea that ERK activity in the nucleus is necessary for eliciting the HGF-induced cell motility response.

Prolonged Nuclear Retention of Activated ERK1/2 Induces the Sustained Phosphorylation of Elk-1 and the Prolonged Expression of c-fos That Leads to the Expression of Matrix Metalloproteinase-9, rhoE, and Ezrin—In the nucleus of mitogen-stimulated cells, activated ERKs phosphorylate ternary complex factors such as Elk-1 (33), which together with serum response factor binds to the cis-acting regulatory element (serum response element) of c-*fos*, resulting in the induction of c-*fos* gene transcription. c-Fos, a transcription factor, then heterodimerizes with the Jun family members of transcription factors to form the activator protein-1 (AP-1), which binds to the 12-*O*-tetradecanoylphorbol-13-acetate response element of certain genes that are crucial for cellular proliferation and thereby activates their transcription (34). Thus, we next examined the phosphorylation of Elk-1, the expression of c-*fos*, and the expression of *mmp-9*, *rhoE*, and *ezrin* in HGF-stimulated ERK2-MDCK cells. *mmp-9* contains an AP-1 binding site in its promoter region (35), whereas expression of the *rhoE* and *ezrin* genes is regulated in a ERK pathway-dependent manner (36, 37). Furthermore, functions of all these gene products have been suggested to be involved in the regulation of cell motility (36, 38–41).

HGF stimulation of FLAG-Elk-1-transfected ERK2-MDCK cells induced a rapid and sustained phosphorylation of FLAG-Elk-1, as revealed by the appearance of a slower migrating band that was sensitive to protein phosphatase-1 treatment and thus represented the phosphorylated form of FLAG-Elk-1 (Fig. 6*A* and data not shown). Treatment of the cells with PD98059 30 min before HGF stimulation to prevent ERK activation abolished the phosphorylation of FLAG-Elk-1, whereas PD98059 treatment 30 min after HGF stimulation to interrupt the ERK activation rapidly decreased the phosphorylation of FLAG-Elk-1, so that within 30 min none of the phosphorylated form was detected. Although EGF stimulation of the cells also induced a rapid phosphorylation of FLAG-Elk-1, the phosphorylation was rather transient: the unphosphorylated form of FLAG-Elk-1 started to reappear as early as 30 min after EGF stimulation. These results suggest that phosphorylation of FLAG-Elk-1 is well correlated with the nuclear retention of activated ERK1/2 but not with their activation state.

HGF stimulation of ERK2-MDCK cells induced a rapid and prolonged expression of c-*fos* (Fig. 6*B*). HGF also induced the expression of the *mmp-9*, *rhoE*, and *ezrin* in ERK-2 MDCK cells but with rather slow kinetics; the expression of these genes was apparent only after 3–6 h (Fig. 6*C*). Pretreatment of the cells with PD98059 to inhibit the activation of ERK1/2 totally abolished the expression of all these genes. Interruption of the ERK activation by PD98059 treatment 30 min after HGF stimulation rapidly diminished the c-*fos* expression, and importantly, completely inhibited the HGF-induced expression of *mmp-9*, *rhoE*, and *ezrin*. Although EGF stimulation of the cells also induced rapid expression of c-*fos*, the expression was rather modest and transient; expression of *mmp-9*, *rhoE*, and *ezrin* was not induced by EGF stimulation. These results suggest that the prolonged expression of c-*fos* is required for the cells to induce the expression of *mmp-9*, *rhoE*, and *ezrin*.

HGF-induced expression of MMP-9 was further analyzed using zymography to determine the gelatinase activity of media conditioned by HGF-stimulated ERK2-MDCK cells. Consistent with the increase in MMP-9 mRNA level (Fig. 6*C*), HGF stimulation but not EGF stimulation of the cells increased the gelatinase activity at both 92 (corresponding to pro-MMP-9) and 83 kDa (the active MMP-9) (Fig. 6*D*). An increase in enzyme activity was clearly observed 6 h after HGF stimulation, and was totally inhibited by treatment of the cells with

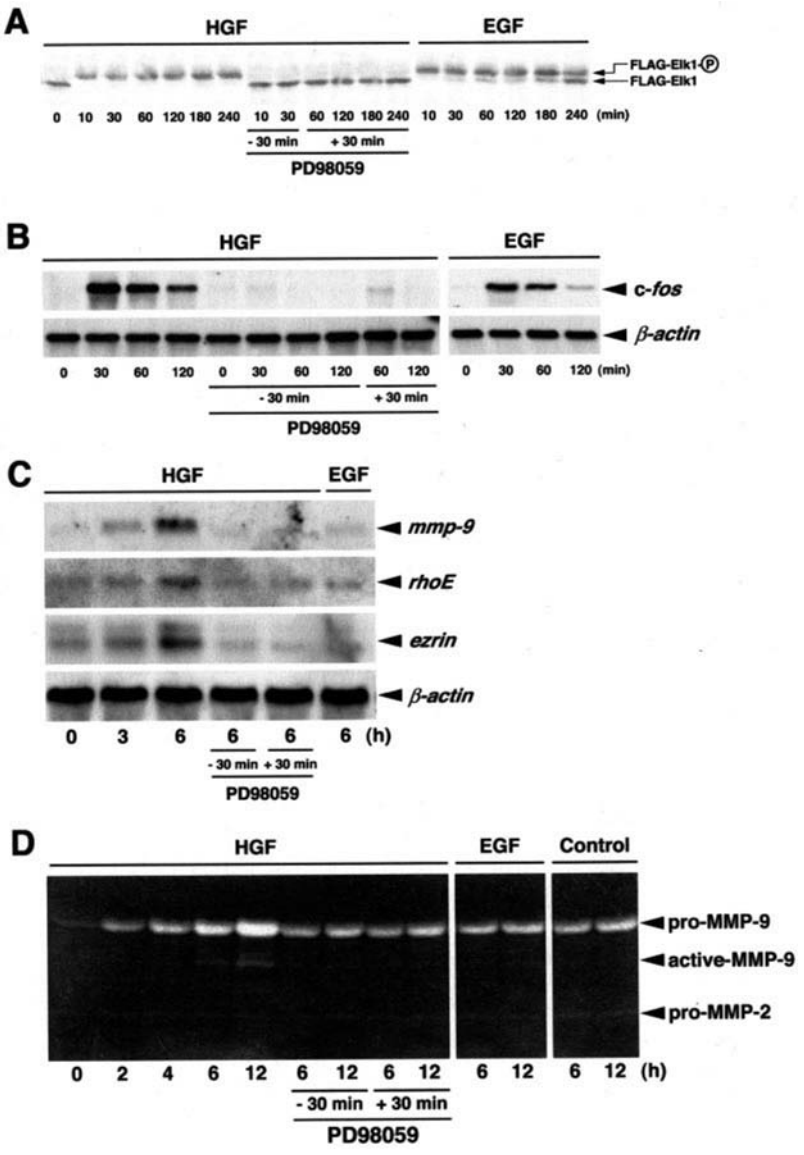

FIG. 6. **HGF induces the sustained phosphorylation of FLAG-Elk-1 and the prolonged expression of c-*fos*, which leads to the expression of *mmp-9*, *rhoE*, and *ezrin* in ERK2-MDCK cells.** *A*, ERK2-MDCK cells (clone 7) were transfected with an expression vector encoding FLAG-tagged Elk-1. After incubation in the growth medium for 24 h, the cells were stimulated with 4 ng/ml HGF or 10 ng/ml EGF for the indicated periods of time. In some experiments, the cells were preincubated with 50 μM PD98059 for 30 min followed by HGF stimulation (*−30 min*), or the cells were treated with 50 μM PD98059 30 min after HGF stimulation to interrupt the ERK activation (*+30 min*). Cell lysates (40 μg of protein) were resolved by SDS-PAGE, followed by immunoblot analysis using the anti-FLAG antibody. *B* and *C*, ERK2-MDCK (clone 7) cells were stimulated with 4 ng/ml HGF or 10 ng/ml EGF for the indicated periods of time. In some experiments, the cells were preincubated with 50 μM PD98059 for 30 min followed by HGF stimulation (*−30 min*), or the cells were treated with 50 μM PD98059 30 min after HGF stimulation to interrupt the ERK activation (*+30 min*). The expression of the c-*fos*, *mmp-9*, *rhoE*, and *ezrin* was analyzed by Northern blot analysis as described under "Experimental Procedures." The expression of the *β-actin* is shown as an internal standard. *D*, ERK2-MDCK cells (clone 7) were incubated in the serum-free medium for 6 h. The cells were then mock-treated (*Control*), or stimulated with 4 ng/ml HGF or 10 ng/ml EGF for the indicated periods of time. In some experiments, the cells were preincubated with 50 μM PD98059 for 30 min followed by HGF stimulation (*−30 min*), or the cells were treated with 50 μM PD98059 30 min after HGF stimulation to interrupt the ERK activation (*+30 min*). Gelatin zymography was performed on media conditioned by the treated cells. Data shown are representative of two to four separate experiments that gave essentially the same results.

PD98059 either 30 min before or 30 min after HGF stimulation. These results suggest that HGF induces not only expression but also activation of MMP-9 in ERK2-MDCK cells. Conversion of latent MMP-9 to active MMP-9 is achieved via proteolytic cleavage mainly by MMP-3 (42). As *mmp-3* contains AP-1 binding sites in its promoter region (43), it seems very likely that HGF stimulation of MDCK cells also induces the expression of *mmp-3*. HGF stimulation of the cells did not increase the gelatinase activity at 72 kDa, which corresponds to pro-MMP-2.

Matrix Metalloproteinase-9 Activity Is Required for the Induction of the Cell Motility Response—Finally, we examined whether or not expression of *mmp-9* is required for HGF-induced cell motility; an important role for MMP-9 in the induction of cell motility has been reported in several other cell systems (38–40). We examined the effect of two MMP inhibitors, GM6001 and EGCG, on the HGF-induced ability of ERK2-MDCK cells to invade through reconstituted extracellular matrix (Matrigel). GM6001, a peptide hydroxamic acid-based compound, is a general inhibitor of MMPs (44), whereas EGCG, the main flavonol of green tea, potently inhibits the activity of MMP-2 and -9 (45). HGF stimulation of ERK2-MDCK cells markedly induced their ability to invade through Matrigel, which was completely inhibited by PD98059 treatment of the cells either 30 min before or after HGF addition (Fig. 7, *A* and *B*). GM6001 and EGCG inhibited the HGF-induced motility of ERK-MDCK cells through Matrigel in a dose-dependent manner; an ~85% inhibition was observed with 100 μM GM6001 and ~80% inhibition was observed with 1 μM EGCG. Possible interference of these inhibitors with enzymes other than MMPs cannot be excluded. However, these inhibitors did not affect the HGF-induced ERK activation nor *mmp-9* expression in the cells under our experimental conditions (Fig. 7*C*). Thus, MMP-9 activity appears to be required for the induction of HGF-stimulated cell motility response.

DISCUSSION

Amplification of the ERK pathway by the overexpression of ERK2 enhanced the motility response of MDCK cells to HGF (Figs. 1 and 2). Expression of a kinase-negative form of MEK1 totally inhibited the HGF-induced ERK activation and cell motility, whereas expression of a constitutively active form of MEK1 induced ERK activation and cell motility in the absence of HGF stimulation (Fig. 3). These results clearly support our previous observation that activation of the ERK pathway is necessary for HGF-induced motility of MDCK cells (9).

HGF stimulation of ERK2-MDCK cells induced a rapid, marked, and sustained activation of ERK1/2 (Fig. 4*A*) and rapid accumulation of ERK1/2 in the nucleus (Fig. 4*B*). En-

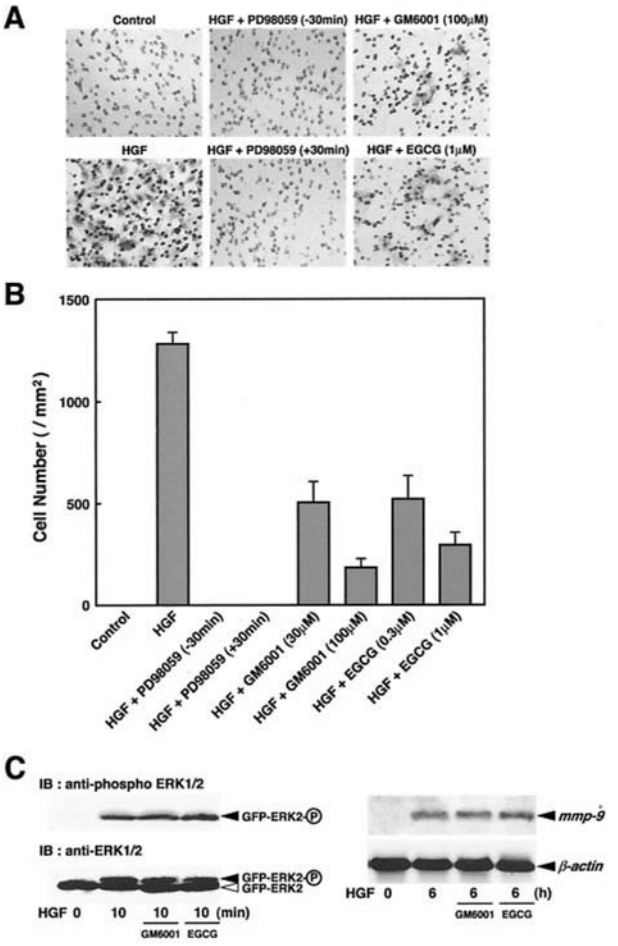

FIG. 7. **MMP-9 activity is required for the induction of the cell motility response.** The effects of several inhibitors on the HGF induced ability of ERK2-MDCK cells (clone 7) to invade through Matrigel-coated filters was examined in a Boyden chamber. The cells placed into the upper chamber were mock-treated (*Control*) or treated with 50 μM PD98059 (*−30 min*), 30 or 100 μM GM6001, or 0.3 or 1 μM EGCG for 30 min, followed by stimulation with 4 ng/ml HGF. In some experiments, cells were treated with PD98059 30 min after HGF stimulation to interrupt the ERK activation (*+30 min*). After 24 h, cells that had migrated to the lower surface of the filter were fixed, stained, and photographed. *A*, representative images of the lower surfaces of the respective filters. *B*, values for invasion were determined by calculating the average number of migrated cells per mm^2 over three fields per assay and were expressed as average ± S.D. for triplicate determinations of a representative experiment. *C*, ERK2-MDCK cells (clone 7) were mock-treated or treated with GM6001 (100 μM) or EGCG (1 μM) for 30 min, followed by stimulation with 4 ng/ml HGF for the indicated periods of time. Cell lysates (10 μg of protein) were resolved by SDS-PAGE followed by immunoblot analysis using the anti-phospho-ERK1/2 antibody or anti-ERK1/2 antibody. The activation profile of the GFP-ERK2 is shown (*left panel*). The expression of *mmp-9* was examined by Northern blot analysis. The expression of *β-actin* is shown as an internal standard (*right panel*). Data shown are representative of two to three separate experiments that gave essentially the same results.

forced cytoplasmic retention of the activated ERK1/2 by the expression of MKP-3(CS), an inactive form of MKP-3, inhibited the HGF-induced cell motility (Fig. 5). Furthermore, interruption of the ERK activation by PD98059 treatment of the cells 30 min after HGF stimulation resulted in the complete abolishment of HGF-induced cell motility (Figs. 2 and 4*A*). The duration of ERK activation is a crucial determinant for the induction of growth factor-mediated cellular responses. For example, the essential role of the sustained activation of ERK1/2 has been demonstrated in inducing nerve growth factor-mediated neuronal differentiation of PC12 cells (46), 12-*O*-tetradecanoylphorbol-13-acetate-mediated megakaryocytic differentiation of K562 cells (47), EGF-mediated migration of SCC-12F (11), HGF-mediated migration of MDCK cells (48), and extracellular calcium-mediated proliferation of osteoblasts (49). Also, nuclear accumulation of ERK1/2 has been shown to be required for the induction of a variety of cellular processes in response to growth factors (18). Our present results, however, clearly show that the sustained activation and nuclear accumulation of ERK1/2 by itself is not sufficient, but prolonged nuclear retention of the activated ERK1/2, and thus sustained activity of ERK1/2 in the nucleus, is another important prerequisite for the induction of the cell motility response. This conclusion can be drawn from the observation that HGF stimulation of ERK2-MDCK cells induces a rapid, marked, and sustained activation of ERK1/2, rapid nuclear accumulation of ERK1/2, and further a prolonged retention of the activated ERK1/2 in the nucleus, which results in the induction of cell motility response. On the contrary, although EGF stimulation of the cells also induces a rapid, marked, and sustained activation of ERK1/2 and rapid nuclear accumulation of ERK1/2, it does not induce the prolonged retention of the activated ERK1/2 in the nucleus, which results in the failure in inducing the cell motility response (Figs. 2 and 4). Why HGF and EGF stimulation of the cells induces such distinct nuclear retention profiles of ERK1/2, irrespective of their activation state, remains unknown. Although nuclear accumulation of ERKs is mediated by their phosphorylation and subsequent dimerization (50), nuclear retention of ERKs occurs independently of ERK-dependent phosphorylation events (31). It may be that HGF activates a putative nuclear retention machinery of ERKs (31) more potently than EGF. HGF- and EGF-stimulated MDCK cells would provide a good experimental system for analyzing the nuclear retention mechanism of ERKs.

In the nucleus of HGF-stimulated cells, activated ERK1/2 continuously phosphorylate ternary complex factors such as Elk-1, which results in the prolonged expression of c-*fos*, an immediate early gene (Fig. 6, A and *B*). The sustained ERK1/2 activity in the nucleus may then contribute to the phosphorylation of c-Fos to enhance its transcription activity. Thus ERK1/2 activity is suggested to be required first for the induction of c-*fos* and second for the phosphorylation of c-Fos to ensure the AP-1-dependent expression of several genes whose products are necessary for the induction of the cell motility response. These sequential roles of ERK activity may explain why prolonged nuclear retention of activated ERK1/2 is required for the induction of HGF-induced cell motility. It remains to be determined whether or not prolonged nuclear retention of the activated ERKs is required for the induction of growth factor-mediated cellular responses other than cell motility.

We show in this report that HGF stimulation of ERK2-MDCK cells induces the expression of *mmp-9*, *rhoE*, and *ezrin* in a manner that is totally dependent on the prolonged nuclear retention of activated ERK1/2 (Fig. 6*C*). Our results also suggest that the activity of MMP-9 is required for the induction of HGF-stimulated cell motility (Fig. 7). Sustained activation of the ERK signaling pathway has been shown to be required for receptor tyrosine kinase-dependent MMP-9 induction and cell migration (11). Very recently, the sustained activation of ERKs was shown to be required for inducing integrin α_2 expression and cell scattering in HGF-stimulated MDCK cells (48). These results indicate that the ERK pathway-dependent expression of several genes, such as *mmp-9* and integrin α_2, is essential for the induction of the cell motility response.

HGF stimulation of ERK2-MDCK cells, however, induces ERK activation not only in the nucleus but also in the cytoplasmic compartment (Fig. 4*B*). In this respect, ERK pathway-

mediated cell motility has been reported to occur in a transcription-independent manner. For example, phosphorylation of myosin light chain kinase by activated ERK1/2 increases the ability of the kinase to phosphorylate myosin light chain, which promotes the cytoskeletal contraction necessary for cell movement (10, 12). Phosphorylation of specific integrins by activated ERK1/2 has been linked with cell migration (5, 19, 20), yet ERK activity in the cytoplasm alone is unable to induce a cell motility response (Fig. 5). Also, the expression of integrin α_2 (48) or MMP-9[2] alone does not induce the motility response in MDCK cells. It thus seems very likely that ERK activity is necessary both in the nucleus (to induce transcriptional events) and in the cytoplasmic compartment (to phosphorylate cytoplasmic, cytoskeletal, and membrane substrates) (51) to induce the cell motility response.

ERK2-MDCK cells expressing the active mutant of MEK1 have disrupted cell-cell contacts and exhibit a spindle-shaped scattering-cell morphology in the absence of HGF stimulation (Fig. 3). Although a requirement for PI 3-kinase activity, but not ERK activity, has been reported for HGF-induced scattering of MDCK cells (52–54), our present results imply that activation of the ERK pathway alone can induce the cell motility response. Furthermore, wortmannin inhibition of PI 3-kinase did not affect the HGF-induced scattering of MDCK cells at all in our repeated experiments (9). It might be possible, however, that activation of both the ERK pathway and the PI 3-kinase pathway is required for the full induction of the cell motility response (55).

In conclusion, we have demonstrated in this report that not only sustained activation and nuclear accumulation of ERK1/2 but also prolonged retention of the activated ERK1/2 in the nucleus are required for the induction of HGF-induced cell motility. In the nucleus, the activated ERK1/2 continuously phosphorylates Elk-1, which leads to the prolonged expression of the c-*fos* gene. This early transcriptional event is then followed by the expression of several genes, such as *mmp-9*, *rhoE*, and *ezrin*; of these, the activity of at least MMP-9 is required for the induction of cell motility response.

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