Activation of the Protein Kinase ERK5/BMK1 by Receptor Tyrosine Kinases

IDENTIFICATION AND CHARACTERIZATION OF A SIGNALING PATHWAY TO THE NUCLEUS*

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ERK5 (also known as BMK1), a member of the mitogen-activated protein kinase (MAPK) superfamily, was known to be activated strongly by oxidant and osmotic stresses. Here we have found that ERK5 is strongly activated by epidermal growth factor and nerve growth factor, whose receptors are tyrosine kinases. The activation of ERK5 was inhibited by expression of dominant-negative Ras and induced by expression of active Ras in PC12 cells, indicating a requirement for Ras in ERK5 activation. The epidermal growth factor-induced activation of ERK5 was found to be inhibited by PD98059 and U0126 inhibitors, which were previously thought to act specifically on classical MAPK kinase (also known as MEK1) and readily reversed by CLI005 and MKP-3 dual-specificity phosphatases for which classical MAPKs were previously shown to serve as preferred substrates. The reporter assays demonstrated that the serum-induced enhancement of transcription from serum response element was significantly inhibited by expression of a dominant-negative form of MEK5, which was a direct and specific activator for ERK5 and that transcription from serum response element mediated by the Ets-domain transcription factor Sap1a, but not by Elk1, was stimulated by coexpression of ERK5 and active MEK5. In addition, Sap1a was shown to be phosphorylated by ERK5 in vitro and by the activation of the ERK5 pathway in cells. Moreover, the serum-induced c-Fos expression was markedly inhibited by expression of dominant-negative MEK5. These results reveal a novel signaling pathway to the nucleus mediated by ERK5 that functions downstream of receptor tyrosine kinases to induce immediate early genes, in parallel with the classical MAPK cascade.

The classical mitogen-activated protein kinase (MAPK,† also known as ERK) cascade is an evolutionarily conserved module that mediates the signaling from various extracellular stimuli to the nucleus (1–4). Several other members of the MAPK family including c-Jun N-terminal kinase/stress-activated protein kinase (JNK/SAPK) and p38/MAPK2 have been identified. Each member of MAPKs is activated by both tyrosine and threonine phosphorylation catalyzed by a distinct upstream kinase, a member of the MAPK kinase family (1, 3, 5–7). Classical MAPK has a Thr-Glu-Tyr (TEY) sequence in the activation phosphorylation motif and is activated by mitogenic stimuli such as growth factors mainly through the receptor tyrosine kinase-Ras pathway. JNK and p38, which have Thr-Pro-Tyr (TPY) and Thr-Gly-Tyr (TGY) sequences, respectively, respond to a variety of cellular stresses and pro-inflammatory cytokines and may function in apoptosis, immune response, and differentiation. Most recently, ERK5/BMK1 was identified as a novel member of the MAPK family (9, 8). Although ERK5 has a TEY sequence in the activation phosphorylation site like classical MAPK, it is strongly activated by stresses such as oxidant and hyperosmolality, like JNK and p38 (10).

Classical MAPK, JNK, and p38 are known to phosphorylate many of transcription factors to activate their transcriptional activity, which is required for the immediate early gene expression and various cellular responses (11, 12). ERK5 has also been shown to phosphorylate transcription factors such as c-Myc and MEF2C, a member of the MADS box transcription factors (13, 14). The best characterized signaling target of MAPK, JNK, and p38 involves the serum response element (SRE) on the c-fos promoter. At the SRE, the dimer of serum response factor (SRF) forms a ternary complex with a ternary complex factor (TCF) to bring full SRE function. Two TCF proteins, Elk1 and Sap1a, which belong to the Ets transcription factor family, are phosphorylated and activated by classical MAPKs, JNK, and p38 (11, 15–18). It has remained unclear, however, whether ERK5 functions at the element, although serum stimulation was shown to induce ERK5 activation (14).

MEK5, a member of the MAPKK family, is a direct and specific activator of ERK5 (8, 14, 19). It has been reported that ERK5 somehow senses the signals from Src and Ras (13, 20). However, activation of ERK5 by Ras or Raf-1, a downstream target of Ras, has not been demonstrated (13). Thus, the signaling pathways for the MEK5-ERK5 cascade are largely undefined.

Here we have shown that ERK5 is strongly activated by epidermal growth factor (EGF) and nerve growth factor (NGF) through Ras and that the ERK5 pathway is blocked by several inhibitors known as the classical MAPK pathway inhibitors. The reporter gene assays have demonstrated that c-fos SRE is regulated by the MEK5-ERK5 cascade via Sap1a activation. In addition, Sap1a is shown to be phosphorylated by ERK5 in vitro and by the activation of the ERK5 pathway in cells. The serum-induced c-Fos expression is inhibited by expression of

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The abbreviations used are: MAPK, mitogen-activated protein kinase; ERK, extracellular signal-regulated kinase; MAPKK, MAPK kinase; MEK, MAPK/ERK kinase; EGF, epidermal growth factor; NGF, nerve growth factor; SRE, serum response element; SRF, serum response factor; TCF, ternary complex factor; HA, hemagglutinin; MBP, myelin basic protein; TPA, 12-O-tetradecanoylphorbol-13-acetate; PCR, polymerase chain reaction; DMEM, Dulbecco’s modified Eagle’s medium; dnRas, dominant-negative Ras; JNK, c-Jun N-terminal kinase; SAPK, stress-activated protein kinase.
dominant-negative MEK5. These findings reveal a novel signaling pathway from the plasma membrane to the nucleus that is mediated by the MEK5-ERK5 pathway.

**EXPERIMENTAL PROCEDURES**

Screening of cDNA Library and Expression Plasmids—A mouse brain cDNA library (Stratagene) was screened with a 1-kilobase pair-cDNA fragment encoding the kinase domain of the human ERK5-BMK1. Under high stringency washing conditions, a positive clone, mouse ERK5 cDNA, was isolated. The mouse MEK5 cDNA was obtained in a process of the MKK7 cDNA screening (21). Each of the coding regions was subcloned into pcDL-Sre456 and pSRo-HAI (22). The mutagenic primers (5'-CAGCTGTGGATCTGATGACAGGCTTATATTACAAAAAC-3' and 5'-GTACTCAACATACACTTGTAGTATACAGCA-3') were used to obtain MEK5(D), in which Ser-311 and Thr-315 were replaced by Ala and Val, respectively. The primers (5'-CAGCTGTGGATCTGATGACAGGCTTATATTACAAAAAC-3' and 5'-GTACCTCAACATACACTTGTAGTATACAGCA-3') were used to obtain MEK5(D), in which Ser-311 and Thr-315 were replaced by Asp and Glu, respectively. The primers (5'-CAGCTGTGGATCTGATGACAGGCTTATATTACAAAAAC-3' and 5'-GTACCTCAACATACACTTGTAGTATACAGCA-3') were used to obtain MEK5(D), in which Ser-311 and Thr-315 were replaced by Cys and His, respectively. The primers (5'-CAGCTGTGGATCTGATGACAGGCTTATATTACAAAAAC-3' and 5'-GTACCTCAACATACACTTGTAGTATACAGCA-3') were used to obtain MEK5(D), in which Ser-311 and Thr-315 were replaced by Val and Leu, respectively. The primers (5'-CAGCTGTGGATCTGATGACAGGCTTATATTACAAAAAC-3' and 5'-GTACCTCAACATACACTTGTAGTATACAGCA-3') were used to obtain MEK5(D), in which Ser-311 and Thr-315 were replaced by Ala and Val, respectively. The primers (5'-CAGCTGTGGATCTGATGACAGGCTTATATTACAAAAAC-3' and 5'-GTACCTCAACATACACTTGTAGTATACAGCA-3') were used to obtain MEK5(D), in which Ser-311 and Thr-315 were replaced by Asp and Glu, respectively. The primers (5'-CAGCTGTGGATCTGATGACAGGCTTATATTACAAAAAC-3' and 5'-GTACCTCAACATACACTTGTAGTATACAGCA-3') were used to obtain MEK5(D), in which Ser-311 and Thr-315 were replaced by Cys and His, respectively. The primers (5'-CAGCTGTGGATCTGATGACAGGCTTATATTACAAAAAC-3' and 5'-GTACCTCAACATACACTTGTAGTATACAGCA-3') were used to obtain MEK5(D), in which Ser-311 and Thr-315 were replaced by Val and Leu, respectively.

Cell Culturing—COS7 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum. Rat PC12 cells were cultured in DMEM supplemented with 0.3% glucose, 5% heat-inactivated horse serum, and 10% fetal calf serum. HeLa and NIH3T3 cells were maintained in DMEM with 10% calf serum. Transient Transfection—All plasmids used in transfection were prepared with Qiagen-tip 500. 24 h before transfection, 1.5 × 10^6 COS7 and PC12 cells were plated on a 60-mm dish, and 0.5 × 10^6 HeLa and NIH3T3 cells were plated on a 35-mm dish. For cell staining, cells were plated onto coverslips. Transfection for COS7, PC12, and NIH3T3 cells was carried out using a LipofectAMINE (Life Technologies, Inc.) with 4.5-μg total amounts of plasmids on 60-mm dish or 1.5 μg on a 35-mm dish. After 24 h, the complete medium was replaced by a low serum medium containing 10% calf serum for COS7 and NIH3T3 cells or DMEM with 0.5% calf serum for COS7 and NIH3T3 cells. The transfection efficiency was 8–12% in COS7 cells, 5–10% in PC12 and NIH3T3 cells, and 4–5% in HeLa cells.

Kinase Assays—48 h after transfection, COS7 or PC12 cells in the low serum medium were treated with or without each stimuli: 30 nM EGF, 10% fetal calf serum, and 100 ng/ml NGF for 5 min and 10 μM lysophosphatidic acid, 1 mM H_2O_2, 100 ng/ml TPA, 100 J/m^2 UV, incubation at 45 °C (heat shock), 100 nM A23187, and 2 μg/ml anisomycin for 30 min. For kinase assays of endogenous ERK5, subconfluent cells on a 100-mm dish in the low serum medium were treated with or without the growth factors as described above. Then the cells were washed once in ice-cold Heps-buffered saline, scraped in a lysis buffer consisting of 20 mM Tris-Cl (pH 7.5), 5 mM EGTA, 25 mM β-glycerophosphate, 1% Triton X-100, 2 mM dithiothreitol, 1 mM vanadate, 1 mM phenylmethylsulfonyl fluoride, and 1% aprotinin (200 μl of buffer/10^6 cells) and disrupted by vortexing 20 times, followed by centrifugation at 12,000 rpm for 20 min. The supernatant was incubated with 45 μl of a 1:1 slurry of protein A-Sepharose beads (Amersham Pharmacia Biotech) and antibody (10 μl (200 μg/ml) of rabbit anti-HA antibody Y-11 (Santa Cruz), 20 μl (100 μg/ml) of goat anti-ERK5 antibody C-20 (Santa Cruz), or 10 μl (200 μg/ml) of mouse anti-Myc antibody 9E10 (Santa Cruz) for 1 h at 4 °C. The immune complex on beads was washed twice with a solution containing 0.5 M NaCl, 2 mM dithiothreitol, and 0.05% Tween 20. Then, the immunoprecipitate was divided into two parts, and one part was used for the kinase assay, and the other was used for immunoblotting. To detect the kinase activity, the immune complex was washed once with a reaction buffer containing 20 mM Tris-Cl (pH 7.5), 2 mM EGTA, 2 mM dithiothreitol, and 1 mM phenylmethylsulfonyl fluoride and incubated for 30 min at 30 °C with the substrate (final volume, 15 μl) containing 20 mM Tris-Cl (pH 7.5), 10 mM MgCl_2, and 100 μM ATP (2 μCi of γ-^32P]ATP). 10 μg of myelin basic protein (MBP) and 10 μg of GST-ERK5(KN) were used as substrate for ERK5 and MEK5, respectively. The immune complex kinase assays for classical MAPK, JNK, and p38 were carried out using 3 μg of MBP, GST-c-Jun (1–79), or GST-ATF-2 as a substrate (21). After SDS-polyacrylamide gel electrophoresis, the radioactivity was analyzed with an image analyzer (Bio-Rad).

Cell Staining—48 h after transfection, NIH3T3 cells in DMEM with 0.5% calf serum were treated with or without 15% fetal calf serum for 2 h. Then the cells on the coverslip were fixed with 3.7% paraformaldehyde in phosphate-buffered saline for 10 min at 37 °C, permeabilized in 0.5% Triton X-100 in phosphate-buffered saline for 10 min, rinsed with phosphate-buffered saline containing 3% bovine serum albumin (Sigma) for 30 min, and then stained with rabbit anti-human c-Fos antibody (Upstate Biotechnology Inc.) for 16 h at 4 °C. After washing three times with phosphate-buffered saline, the cells were stained with 4,6-diamidino-2-phenylindole and tetramethyl rhodamine isothiocyanate-labeled anti-rabbit IgG antibody (Cappel) for 1 h at room temperature.

Luciferase Assays—40 h after transfection, HeLa cells in DMEM with 0.5% calf serum were treated with or without 15% fetal calf serum and incubated for 8 h. Then the cells were washed once in ice-cold Heps-buffered saline, scraped in 150 μl of reporter lysis buffer (Promega), and centrifuged at 12,000 rpm for 20 min after vortexing for 15 s. The supernatant was used as a cell extract to detect the luciferase activity. The luciferase assay was carried out using a luciferase assay system (Promega). In brief, 20 μl of the room-temperature cell extract was mixed with 100 μl of room temperature luciferase assay reagent containing the substrate. The reaction was performed and measured in a luminometer (LB9507, Berthold). The protein concentration was determined with a protein assay kit (Bio-Rad) and used for normalization of the luciferase assays. All the experiments were repeated at least three times.

Phosphorylation of Sap1a and Elk1—For in vitro analysis, GST-Sap1a and GST-Elk1 were prepared by using the expression vector pGEX-6P (Amersham Pharmacia Biotech), expressed in Escherichia coli, and purified by affinity chromatography on glutathione-Sepharose 4B (Amersham Pharmacia Biotech). For in vivo phosphorylation, 6 h after transfection of Myc-tagged Sap1a or Elk1, COS7 cells were labeled for 24 h with 0.5 μCi/ml [^32P]orthophosphate in phosphate-free DMEM with 10% fetal calf serum. Then, cell lysates were prepared and subjected to immunoprecipitation with anti-myc antibody (Santa Cruz).

RESULTS

Isolation of Mouse ERK5 and MEK5 cDNA Clones—We isolated the ERK5 and MEK5 cDNA clones from mouse brain cDNA library and determined the nucleotide sequences containing their open reading frames (Fig. 1). The mouse ERK5 is deduced to contain 506 amino acids and shows 82% identity to human ERK5 (8, 9). The kinase domain, which is located in the N-terminal half is highly conserved, including the dual phosphorylation site (Thr-219 and Tyr-221) in the TYK sequence (Fig. 1A). The mouse MEK5 is predicted to have 448 amino acids and displays 99 and 93% identity with the rat and human sequences, respectively (8, 19). The activating phosphorylation sites Ser-311 and Thr-315 are conserved.
ERK5 Activation Induced by Growth Factors—To determine the characteristics of ERK5 activation, we compared the kinase activity in response to several extracellular stimuli. The epithelial-like COS7 cells transfected with HA-tagged mouse ERK5 were exposed to various stimuli; EGF, lysophosphatidic acid, serum, phorbol ester TPA, UV, heat shock, A23187 Ca\textsuperscript{2+} ionophore, and anisomycin. As a control, the cells were stimulated by H\textsubscript{2}O\textsubscript{2}, which was previously reported to induce ERK5 activation (10). The immune complex kinase assays revealed that ERK5 was strongly activated by EGF, TPA, or lysophosphatidic acid as well as by H\textsubscript{2}O\textsubscript{2} (Fig. 2A). Although fetal calf serum (10%) and heat shock at 45 °C activated ERK5 moderately, UV, A23187, or anisomycin did not. In the phaeochromocytoma PC12 cells, ERK5 activation by serum was very weak, whereas the activation by EGF or by NGF was strong (Fig. 2B). The EGF-induced activation of HA-tagged ERK5 was seen in other cell lines, including C2C12 and NIH3T3 cells (data not shown). The EGF-induced activation of ERK5 in PC12 cells peaked at 5 min and then declined rather rapidly (Fig. 2B). The activation by NGF also peaked at about 5 min and persisted longer than that by EGF (Fig. 2B). The activation of ERK5 by H\textsubscript{2}O\textsubscript{2}, TPA, or heat shock was slow and peaked at about 30 min or later (data not shown). We then measured the activity of endogenous ERK5 by an immune complex kinase assay with anti-ERK5 antibody C-20 (Santa Cruz) and found that endogenous ERK5 was activated by EGF in PC12 cells and COS7 cells and by NGF in PC12 cells (Fig. 2C). The EGF-induced activation of endogenous ERK5 was also observed in NIH3T3 cells and HeLa cells (data not shown).

Ras-dependent Activation of ERK5—Low molecular weight GTPases such as the Ras family and the Rho family proteins have been shown to function in the signaling pathways of growth factors or stress stimuli to activate the downstream kinase cascades (7, 12, 26, 27). To test the effect of activation of these GTPases on the activation of ERK5, cells were cotransfected with plasmids expressing HA-ERK5 and each of dominant-active forms of GTPases: RhoV14, RacV12, Cdc42V12, or RasV12. The immune complex kinase assay revealed that ERK5 as well as classical MAPK (also known as ERK1 and ERK2, simply called MAPK hereafter) was strongly activated by RasV12 in PC12 cells (Fig. 3A) and in C2C12 cells (data not shown). In agreement with a recent report (13), however, ERK5 was not markedy activated by RasV12 in COS7 cells, whereas
MAPK was activated (Fig. 3B). RacV12 and Cdc42V12, which were able to activate JNK in COS7 cells and PC12 cells (data not shown), were not potent activators of ERK5 (Fig. 3, A and B). RhoV14 also failed to activate ERK5. Because activation of Ras was found to be able to induce ERK5 activation, at least in several types of cells including...
PC12 cells, we next tested the requirement of Ras for ERK5 activation. PC12 cells were cotransfected with HA-ERK5 and dominant-negative Ras (dnRas). After NGF stimulation, the kinase activity of ERK5 was measured by immune complex kinase assay. The NGF-induced activation of ERK5 was inhibited by expression of dnRas (Fig. 3C, left). The EGF-induced activation of ERK5 in PC12 cells (data not shown) and in COS7 cells (Fig. 3C, right) was also inhibited by dnRas. Furthermore, to assess the requirement of Ras for endogenous ERK5 activation, we used a PC12 cell line in which dominant negative Ras (N17) was stably expressed (28). In the N17Ras-expressing PC12 cell line, the EGF- or NGF-induced activation of endogenous ERK5 was markedly reduced, although the amount of endogenous ERK5 was almost equivalent to that in wild-type PC12 cells (Fig. 3D). These results, therefore, indicate that Ras is important for the activation of ERK5 induced by NGF and EGF.

**Fig. 3. Ras-dependent activation of ERK5.** PC12 cells (A) or COS7 cells (B) were transfected with HA-ERK5 or HA-MAPK together with an empty vector or expression vector encoding RhoV14, RacV12, RasV12, or Cdc42V12. 48 h after transfection, the cells were harvested, and the immune complex kinase assay was performed. C, HA-ERK5 was transfected into PC12 cells with an empty vector or a plasmid expressing dnRas. Then, 48 h after transfection, the cells were treated by 100 ng/ml NGF for 0, 5, or 30 min, and ERK5 activity was measured by the immune complex kinase assay. In A, B, and C, the kinase activity toward MBP is shown (MBP), and it was confirmed by immunoblotting with anti-HA antibody (aHA) that nearly equal amounts of ERK5 were precipitated in each lane. D, PC12 cells (left) and the stable transfectant with a dominant-negative (N17) Ras (dnRas, right) were stimulated with 30 nM EGF or 100 ng/ml NGF for 5 min, and the endogenous ERK5 activity was measured by immune complex kinase assay (MBP). The amount of endogenous ERK5 in each immune complex was determined by immunoblotting with anti-ERK5 antibody (aERK5).

**PD98059, U0126, and MAPK Phosphatases Inhibit ERK5 Activation.** The growth factor-induced Ras-dependent activation of ERK5 and its having the TEY sequence are the same as the characteristics of MAPK. We tested the effect of some well known inhibitors of the classical MAPK cascade on ERK5 activation. COS7 cells were pretreated with increasing concentrations of PD98059, a MAPKK/MEK1 inhibitor that does not inhibit the JNK or p38 pathway (29, 30), and stimulated by EGF. Another MAPKK inhibitor, U0126 (31), was also tested. The kinase assay showed that PD98059 and U0126 inhibited the EGF-induced ERK5 activation in a concentration-dependent manner (Fig. 4A). The inhibitory effect of these drugs on ERK5 activation was similar to, or slightly more potent than, that on MAPK activation (29–31) (Fig. 4A). It is assumed that the target of PD98059 and U0126 may be MEK5, analogous to the action of the drugs on the MAPK cascade, because the MEK5-ERK5 cascade and the classical MAPKK-MAPK cascade are analogous but independent. In fact, expression of dominant-negative MAPKK, MKK(A), which inhibited MAPK activity, did not inhibit ERK5 activation. Similarly, the ERK5 activation, but not the activation of MAPK, JNK, or p38, was inhibited by expression of dominant-negative MEK5, MEK5(A) (Fig. 4E). We then tested the effect of these drugs on MEK5 in vitro. We utilized MEK5(D), a dominant-active form of a direct activator for ERK5. Myc-tagged MEK5(D) expressed in COS7 cells was immunoprecipitated, and the in vitro kinase assay
was performed with PD98059 or U0126 added directly in the reaction mixture. As shown in Fig. 4B, U0126 inhibited the activity of MEK5(D) in a concentration-dependent manner, whereas PD98059 did not inhibit completely the activity of MEK5(D), although some variable inhibition was observed. It is possible that PD98059 may inhibit phosphorylation and activation of MEK5 (see “Discussion”).

We then examined the action on ERK5 of two dual-specificity phosphatases, CL100 (MKP-1) and MKP-3 (Pyst-1). It has been reported that CL100 acts on classical MAPKs, JNK/SAPK, and p38 (23, 32, 33), whereas MKP-3 acts on classical MAPKs more potently than on JNK/SAPK or p38 (24, 34, 35). The obtained result showed that expression of either of the two phosphatases potently inhibited the EGF-induced activation of ERK5 (Fig. 4, C and D).

The MEK5-ERK5 Pathway Is Involved in Transcriptional Activation of the SRE—One of the best characterized downstream targets of MAPKs is the SRE on the c-fos promoter. To address the possible role of the MEK5-ERK5 pathway in this transcriptional activation, we used the SRE-dependent reporter gene for luciferase assay, which contains the binding elements for both TCF and SRF. We examined the effect of MEK5(A), a dominant-negative construct, on the serum-induced transcription via SRE. In HeLa cells, ERK5 was activated by serum (Fig. 5A), and the expression of MEK5(A) specifically inhibited the ERK5 activation (Fig. 5A). The transcriptional activation from the SRE was inhibited significantly but not completely by expression of MEK5(A) (Fig. 5B, left, closed bars). Similarly, the transcriptional activation was inhibited markedly but partially by expression of MKK(A) (Fig. 5B, left, closed bars), a dominant-negative construct of MAPKK that specifically inhibits the MAPK activation (see Fig. 4E). Expression of CL100, which inhibits the activation of both ERK5 and MAPK (see Fig. 4C), suppressed the transcriptional activation almost completely (Fig. 5B, left, closed bars). We next tested whether the inhibitory effect of MEK5(A) on the SRE was via TCF or via SRF. The SRE activation by expression of dominant-active Rho (RhoV14), which is an activator for SRF but not for TCF (36), was not inhibited by MEK5(A) (Fig. 5B, right, closed bars). We then utilized a reporter construct having the binding element for SRF alone whose transcriptional activation was induced by serum treatment or by expression of RhoV14 (Fig. 5B, left and right, open bars). MEK5(A) did not inhibit either of the SRF-dependent transcriptional activations (Fig. 5B, open bars). These results suggest that the MEK5-ERK5 pathway is involved in the signaling to TCF but not to SRF.

To clarify the role of the MEK5-ERK5 pathway in the signaling to TCF, we tested the effect of activation of ERK5 on the transcriptional activation from the SRE in the presence of the TCF transcription factor, Sap1a or Elk1. Coexpression of ERK5 and MEK5(D) enhanced the Sap1a-dependent transcriptional activation to nearly the same extent as did coexpression of JNK and MKK7(DE), a constitutively active form of MKK7 (Fig. 5C, Sap1a). In contrast, activation of the ERK5 pathway did not enhance the Elk1-dependent activation, but activation of the JNK pathway did (Fig. 5C, Elk1). Thus, the MEK5-ERK5 pathway may be targeted to Sap1a but not to Elk1. That the MEK5-ERK5 pathway is not involved in the activation of Elk1 activity was reported previously (14).

Phosphorylation of Sap1a In Vitro and in Vivo—Phosphorylation of the Ets transcription factors has been shown to enhance their transactivation activity (11, 15–18). The above results suggested the possibility that ERK5 activation may lead to phosphorylation of Sap1a. To address this, we tested whether or not active ERK5 is able to phosphorylate Sap1a or Elk1.

Fig. 5. The MEK5-ERK5 pathway is involved in transcriptional activation of the SRE. A, HeLa cells were cotransfected with HA-ERK5, HA-MAPK, HA-JNK, or HA-p38 together with an empty vector or a plasmid encoding MEK5(A). 48 h after transfection, the cells were stimulated with or without 15% fetal calf serum for 30 min or 2 μg/ml anisomycin for 60 min. These cells were harvested, and the immune complex kinase assay was performed. The amount of HA-MAPKs in each immune complex was determined by immunoblotting. B, in HeLa cells, the reporter plasmid, which has the binding elements for both TCF and SRF (pSRE-Luc, closed bars) or the element for SRF alone (pSRF-Luc, open bars), was transfected with an empty vector, MEK5(A), MKK(A), CL100, or RhoN19 as indicated. 40 h after transfection, the cells were treated with or without 15% fetal calf serum for 8 h (indicated by Serum) (left panel). HeLa cells were transfected with a plasmid encoding an active form of Rho (indicated by RhoV14) and either pSRE-Luc or pSRF-Luc together with an empty vector or a plasmid encoding MEK5(A) (right panel). C, HeLa cells were transfected with pSRE-Luc and either the plasmid encoding Sap1a (left panel) or Elk1 (right panel) together with an empty vector or expression vectors encoding ERK5, ERK5 and MEK5(D), JNK, or JNK and MKK7(DE) as indicated. In B and C, 48 h after transfection, the cells were harvested, and the luciferase activity was measured as described under “Experimental Procedures.” The average relative luciferase activities were calculated relative to the activity of the control vector, which was set at 1.
The amount of HA-ERK5 and HA-JNK in each immune complex was determined by immunoblotting (Fig. 7A), and the expression of MEK5(A) and MKK(A) specifically inhibited the ERK5 activation and MAPK activation, respectively (Fig. 7A). NIH3T3 cells expressing each of the constructs, control vector, MEK5(A), MKK(A), or RhoN19, were stimulated by serum, and 2 h later the c-Fos induction was assayed by anti-Fos immunostaining. The strong c-Fos staining in the nucleus was detected in about 75% of nontransfected or control vector-transfected cells after serum stimulation (Fig. 7, B and C). This c-Fos induction was partially but significantly inhibited in MEK5(A)-expressing cells; the percentage of the cells showing the nuclear c-Fos staining was decreased to about 44% of the cells (Fig. 7, B and C). The extent of the inhibition of the c-Fos induction by MEK5(A) was similar to that caused by expression of MKK(A) or RhoN19 (Fig. 7, B and C). In addition, the c-Fos induction was not inhibited by expression of wild-type MEK5 (data not shown). Therefore, the MEK5-ERK5 pathway as well as the classical MAPK pathway is involved in the c-Fos induction.

**DISCUSSION**

Activation of ERK5 by EGF and NGF—Here we have shown that ERK5 is strongly activated by EGF and NGF whose receptors are tyrosine kinases. This is the starting point of this study. After the completion of this study, a paper of Kato et al. (38) appeared, which showed the activation of ERK5 by EGF in HeLa cells. Previously it was thought that ERK5 was not activated by growth factors such as platelet-derived growth factor (10). This apparent discrepancy might arise from the difference in the cells and growth factors used and/or the subtle difference in the kinase assay. In fact, we were able to see EGF-induced activation of ERK5 in many types of cells including PC12, COS7, C2C12, and NIH3T3 cells. In PC12 cells, ERK5 activation by EGF was more transient than that by NGF. This observation, together with the activation profile of ERK5 by various stimuli such as the activation by TPA, is reminiscent of the mode of classical MAPK activation that is known to often be mediated by Ras. Our experiments with dominant-negative Ras indicated that EGF- or NGF-induced activation of ERK5 requires Ras. Moreover, the activation of Ras was capable of activating ERK5 in PC12 cells and C2C12 cells. Thus, ERK5 activation by receptor tyrosine kinase may be largely mediated by Ras. It has been reported, however, that in 293 cells and HeLa cells activation of Ras did not induce ERK5 activation (13, 38) and that expression of dominant-negative Ras did not significantly interfere with the EGF-induced activation of ERK5 in HeLa cells (38). In our experiments, the extent of the inhibition of the EGF-induced ERK5 activation by dominant-negative Ras varied, depending on the cell type; in PC12 cells, the inhibition was almost complete, whereas in COS7 cells it was about 50%. Therefore, it may be suggested that there are Ras-dependent and -independent pathways for the activation of ERK5 and that the extent of contribution of each pathway to the growth factor-induced ac-
activation of ERK5 depends on the cell type. In different cell types, the amounts and kinds of the signaling molecules lying upstream and downstream from Ras may differ significantly, and basal activities of other signaling pathways may also differ markedly. These differences may give rise to different results in different cell types.

Inhibitors of the Classical MAPK Pathway Suppress the MEK5-ERK5 Pathway—Our results reveal that the MEK5-ERK5 pathway is activated potently by the dual-specificity phosphatases CL100 (MKP-1) and MKP-3 and the drugs PD98059 and U0126. MKP-3 has previously been shown to act preferentially on classical MAPK (ERK1 and ERK2) (34), and PD98059 and U0126 inhibit the classical MAPK pathway but not the JNK or p38 pathway by blocking the activation of classical MAPKK, MEK1, and by directly inhibiting the activated activity of MEK1 and MEK2, respectively (30, 31). The present finding, however, is not unpredictable, because ERK5 has a TEY sequence and is most similar to classical MAPK (ERK1 and ERK2) (8, 9) and because MEK5 is most related to MAPKK MEK1 with 48% identity (8, 19). MKP-3 and PD98059 were previously used as tools to show the requirement of the classical MAPK pathway for biological processes studied. However, the present finding together with a recent report that cyclooxygenase 1 and 2 may be targets of PD98059 (37) suggests that we should use these tools with caution. To show the requirement of ERK5 or MAPK unambiguously, dominant-negative MEK5 or dominant-negative MAPKK may be useful as they inhibit their own pathway alone (see Figs. 4E, 5A, and 7A).

PD98059 is known to block MEK1 activation by c-Raf and other upstream activators and does not strongly inhibit the activity of the activated, phosphorylated form of MEK1 (29). In contrast, U0126 strongly inhibits the activity of the activated MEK1 and MEK2 (31). On the other hand, these two drugs were reported to inhibit dominant-active mutants of MEK1 (29, 31). In this study, it was not possible to test the effect on MEK5 activation for lack of information about the upstream MAPKKs, which activate MEK5. Therefore, we utilized a dominant-active mutant of MEK5, MEK5(D), to detect the possible inhibitory effect of the drugs. U0126 inhibited the MEK5(D) activity in a dose-dependent manner, whereas PD98059 did not strongly inhibit the activity. It should be noted that activation of ERK5 in vivo was about 100-fold more sensitive to U0126 than the activity of MEK5(D) (Fig. 4, A and B). It is possible that these two drugs may be much more effective on wild-type MEK5 than on the mutant form of MEK5. Moreover, PD98059 may preferentially act on the inactive, dephosphorylated form of MEK5.

The Downstream Target of the MEK5-ERK5 Pathway—It is known that the SRE is responsive to several extracellular stimuli: serum, growth factors, phorbol esters, and oxidants. We have here demonstrated that ERK5 is activated by growth factors and phorbol esters as well as by serum and stress (10, 14) and that the MEK5-ERK5 pathway is involved in the SRE transcriptional activation. On the c-fos promoter, three major elements are known; sis-inducible element (SIE), cAMP response element, and SRE. The SRE receives the major input of signals and is essential for the c-fos gene expression (15, 36). We have found that the endogenous c-fos induction is prevented significantly by inhibition of ERK5 activation. This and the results of the SRE reporter gene assay may suggest that the MEK5-ERK5 pathway acts on the endogenous SRE to express the c-fos gene. Furthermore, our results have shown that the MEK5-ERK5 pathway is able to activate SRE via Sap1a, the TCF/Ets transcription factor. We have also shown that the activated ERK5 is able to phosphorylate Sap1a, but not Elk1, in vitro and that activation of the ERK5 pathway results in the enhanced phosphorylation of Sap1a in cells. In contrast, classical MAPK is known to phosphorylate and activate both Sap1a and Elk1 (17). It has been reported that ERK5,
but not MAPK, is targeted to the MADS-box transcription factor MEF2C (14). The activated MEF2C acts on c-jun promoter. Thus, we can suggest that the MEK5-ERK5 pathway and the classical MAPK pathway have overlapping but distinct functions to regulate the expression of the immediate early genes.

**Signaling Pathways from Receptor Tyrosine Kinases to the c-fos SRE Are Mediated by the MEK5-ERK5 Pathway—Activation of ERK5 by MEK5(D) has been shown to result in transcription of ERK5 from the cytoplasm to the nucleus (14). We confirmed this observation in several cell lines (data not shown). The pathway that is mediated by ERK5 is analogous to the receptor tyrosine kinase/Ras/MAPK pathway, the major signal transduction pathway suggested to function in control of cell proliferation, differentiation, and development. Kato et al. (38) reported that ERK5 was required for the EGF-induced S phase entry of cells. The present finding that the ERK5 pathway is involved in the activation of Sap1a activity and the c-Fos expression may underlie the mechanisms by which EGF and other growth factors stimulate cell proliferation. Future studies should clarify not only regulatory mechanisms of this new pathway but also its functions in detail.

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