Regulation of MAPK-activated Protein Kinase 5 Activity and Subcellular Localization by the Atypical MAPK ERK4/MAPK4*

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MAPK-activated protein kinase 5 (MK5) was recently identified as a physiological substrate of the atypical MAPK ERK3. Complex formation between ERK3 and MK5 results in phosphorylation and activation of MK5, concomitant stabilization of ERK3, and the nuclear exclusion of both proteins. However, ablation of ERK3 in HeLa cells using small interfering RNA or in fibroblasts derived from ERK3 null mice reduces the activity of endogenous MK5 by only 50%, suggesting additional mechanisms of MK5 regulation. Here we identify the ERK3-related kinase ERK4 as a bona fide interaction partner of MK5. Binding of ERK4 to MK5 is accompanied by phosphorylation and activation of MK5. Furthermore, complex formation also results in the relocation of MK5 from nucleus to cytoplasm. However, unlike ERK3, ERK4 is a stable protein, and its half-life is not modified by the presence or absence of MK5. Finally, although knock-down of ERK4 protein in HeLa cells reduces endogenous MK5 activity by ~50%, a combination of small interfering RNAs targeting both ERK4 and ERK3 causes a further reduction in the MK5 activity by more than 80%. We conclude that MK5 activation is dependent on both ERK3 and ERK4 in these cells and that these atypical MAPKs are both physiological regulators of MK5 activity.

The subfamily of mitogen-activated protein kinase-activated protein kinases (MKS)3 comprises three serine/threonine kinases: MK2, MK3, and MK5. These enzymes lie downstream of the MAPK signaling cascades and couple these pathways to key effectors, which mediate cell-type-specific responses. These include cytokine biosynthesis, reorganization of the cytoskeleton, cell motility, cell cycle control, and chromatin remodeling (1–5). Biochemical and genetic studies have revealed that MK2 is the major target of the stress activated MAPks p38α and p38β. MK2-deficient mice have an impaired inflammatory response, which manifests as an increased resistance to endotoxic shock (6). Underlying this resistance is a failure to regulate the stability and translation of a variety of cytokine mRNAs including those encoding tumor necrosis factor and interleukins 6 and 8. Another major target of MK2 is the small heat shock protein Hsp27 (7). Phosphorylation of Hsp27 by MK2 modifies its ability to act as a chaperone, and this latter activity may be involved in stabilizing actin filaments and the regulation of cytoskeletal architecture (2).

Although MK3 is most closely related to MK2 and also lies downstream of the p38 MAPK pathway, its biological functions are still unclear. MK3 may mediate the residual p38-dependent cytokine synthesis and Hsp27 phosphorylation observed in MK2-deficient animals (8). MK3 has also been reported to interact with human polyhomeotic protein 2 and can phosphorylate this protein in vitro (5). Human polyhomeotic protein 2 is a component of polycomb repressive complex 1 and is involved in the maintenance of chromatin structure associated with the stable silencing of certain genes (9). MK5, the final member of this family, was originally identified as a p38-regulated and activated protein kinase (PRAK) (10, 11). Furthermore, like MK2, both the activity and subcellular distribution of MK5 could be regulated by ectopic expression of p38 MAPK (12, 13). However, endogenous MK5 activity is not regulated by stimuli that activate the p38 MAPK pathway, nor can any interaction between endogenous MK5 and p38 be detected (14). Furthermore, p38 protein levels are unchanged in mice deficient in MK5, indicating that unlike MK2, which acts to stabilize endogenous p38 protein, MK5 does not perform a role as a p38 chaperone in vivo (8, 14).

A major breakthrough in understanding how MK5 activity might be regulated came with the finding that the atypical MAPK ERK3 is a physiological binding partner for MK5 (15, 16). Complex formation between ERK3 and MK5 is accompanied by both phosphorylation and activation of MK5 and experiments using siRNA to deplete endogenous ERK3 and in mouse embryo fibroblasts derived from ERK3 null mice demonstrated that MK5 activity is regulated by ERK3 in vivo (16). Finally, experiments performed in mouse embryo fibroblasts derived from MK5-deficient mice and in cells following siRNA-medi-
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ated knock-down of MK5 clearly demonstrated that this protein acts as a chaperone to stabilize the endogenous ERK3 kinase (15, 16).

Despite the proven role for ERK3 in regulating MK5, the loss of ERK3 does not completely ablate MK5 activity, reducing it only by ~50% (16). This suggests the existence of additional regulators of MK5. Possible candidates include p38 MAPK and also the ERK3-related kinase ERK4/p63 MAPK, which is encoded by the gene designated MAPK4 (17). However, preliminary experiments indicated that although a truncated form of ERK4 might act as a binding partner for MK5, unlike ERK3 it was unable to mediate MK5 activation (15). Here we have studied ERK4 in greater detail and report that full-length ERK4 is indeed a physiological binding partner for MK5 and that binding results in the relocalization of MK5 from the nucleus to the cytoplasm. Furthermore, complex formation also results in both the phosphorylation and activation of MK5. Finally, we demonstrate that although siRNA-mediated knock-down of ERK4 protein in HeLa cells reduces endogenous MK5 activity by ~50%, a combination of siRNAs targeting both ERK4 and ERK3 causes a further loss of MK5 activity by more than 80%. We conclude that MK5 activation is dependent on both ERK3 and ERK4 in these cells and that these atypical MAPKs are both physiological regulators of MK5 activity.

EXPERIMENTAL PROCEDURES

Reagents—SB203580 was purchased from Upstate Group, LLC (Lake Placid, NY). Sodium arsenite, sorbitol, bovine serum albumin, and cycloheximide were purchased from Sigma-Aldrich. Redivue IgG, horseradish peroxidase conjugate (12–342) was acquired from Rockland Inc. (Gilbertsville, PA).

DNA Constructs—Construction of the following plasmids has been described previously: pGADT7–ERK3 (18), pEGFP-MK5, pEGFP-MK5K51E, pEGFP-MK5T182A and pGEX4-T3 MK5 (13), and pGEGFP-MK5 1–423 and pGEX5-T3 MK5 1–423 (16). The constructs containing constitutively active MKK6 (MKKK6/E) and FLAG-tagged p38β2 (19, 20) were kindly provided by Dr. J. Han (Scripps Institute). To facilitate the transfer of the human full-length ERK4, ERK4 D168A, and the C-terminal deletion mutants of ERK4 1–330 and ERK4 1–340 into a variety of expression vectors, cDNA constructs were first subcloned into Gateway entry vectors and subsequently transferred into appropriate expression vectors according to the manufacturer's instructions (Invitrogen). Human full-length ERK4 was amplified from an IMAGE clone (IMAGE number 1870004) using primers 5′-caccatgtgctgagaagggtgactgcatcgcc-3′ and 5′-tccaccccttctggagagg-3′ and cloned into pENTR-D-TOPO (Invitrogen). C-terminal deletion mutants of ERK4 1–330 and 1–340 were generated by PCR using the forward primer 5′-gggaccaatgtagttacaaaaagagctgtataaggtgaggtgctcgcag-3′ and the reverse primers 5′-gaggctccgttggagaggagcatcctcgcag-3′ and the reverse primers 5′-ggggcacacttctgaagacagatgggtggtttt-3′ and 5′-ggggacactttttgcaagatgacgatgtggtttg-3′, respectively, and cloned into the pENTR207. A kinase-dead mutant of ERK4 was produced by changing Asp168 to Ala by site-directed mutagenesis on pENTR-D-ERK4 using the QuikChange method (Stratagene) and primers 5′-ggtcctgaattggtgctttcgggttggcaagg-3′ and 5′-tccggcaccagccagaccccaactttggcag-3′. The resulting Gateway entry vectors pENTR-D-ERK4, pENTR207-ERK4 1–330, pENTR207-ERK4 1–340, and pENTR-D-ERK4 D168A were used in recombination reactions with Gateway destination vectors pDESTGAD, pDESTmyc, and pDESTEGFP (21) to construct the expression vectors pEXPADG–ERK4, pEXPMyC–ERK4, and pEXP-EGFP–ERK4, respectively.

The pLP–GBK7 vector was generated by inserting a HindIII (filled in)/BamHI fragment from pLP–EYFP–C1 into the BamHI/SmaI fragment of pGBK7 (Clontech). Creator pDR dual donor vectors containing MK2 and MK3 (accession numbers Y335730 and Y335561, respectively) were purchased from the FLEXGene consortium (Harvard Institute of Proteomics) and cloned into the creator acceptor vector pLP–GBK7 by recombination using the BD Creator pDRN dual cloning kit (Clontech). All of the plasmid constructs described were verified by DNA sequencing using the BigDye Terminator v3.1 cycle sequencing kit (Applied Biosystems). All PCR was performed using Pfx platinum polymerase (Invitrogen) according to the manufacturer’s instructions.

Yeast Two-hybrid Assay—Expression plasmids with GAL4 DNA-binding domain and activation domain fusion were transformed into the Saccharomyces cerevisiae strains P69-2A(MATa) and Y187(MATa), respectively, using the Frozen-EZ yeast transformation II (Zymo Research Corp.) and plated out on suitable drop-out medium, according to the manufacturer’s instructions. The yeast mating procedure was performed as described (16).

Cell Culture and Transfection—HeLa cells were maintained in Eagle’s minimum essential medium supplemented with 1× mouse IgG (610-131-121) and IRDye 800CW conjugated affinity purified anti-rabbit IgG (611-131-122) were acquired from Rockland Inc. (Gilbertsville, PA).
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nonessential amino acids (Invitrogen), 10% fetal bovine serum (Invitrogen), 2 mM L-glutamine, penicillin (100 units/ml), and streptomycin (100 μg/ml). Lipofectamine Plus (Invitrogen) reagent was used to transfect the HeLa cells according to the manufacturer's instructions.

siRNA—All siRNAs were purchased from Ambion. The sequences of the siRNAs used were as follows (sense and antisense): ERK3 siRNA 5′-ggcuuuuacguauacgctt-3′ and 5′-agcguuuaugaaagccctg-3′, MK5 siRNA 5′-ggauagggcagaaag-aucct-3′ and 5′-gauuucuucgcuaauccct-3′, and ERK4 siRNA 5′- ggguagcguuacgctt-3′ and 5′-gacuugacuagcuacccctg-3′. As a control siRNA the Silencer negative control siRNA (Ambion catalog number 4611) was used. Lipofectamine 2000 (Invitrogen) was used to transfect the siRNA into HeLa cells according to the manufacturer's instructions.

Immunoblotting—For detection of epitope-tagged ERK4, ERK3, MK5, and p38β2 in transfected cells and in GST pull-down experiments, the samples were analyzed by SDS-PAGE (4–12% NUPAGE; Invitrogen), transferred to a nitrocellulose membrane (Amersham Biosciences), and probed with either anti-Myc (1:200; 9E10; Cancer Research UK), anti-FLAG (1:1000; M2; Stratagene), anti-GST (1:500; Santa-Cruz Biotechnology), anti-ERK3 (1:1000; Zymed Laboratories Inc.), anti-ERK4 (1:1000), or anti-GFP (1:200; Santa Cruz). Detection and quantification were performed using IRDye 800CW conjugated goat anti-mouse IgG (H&L) or IRDye 800CW conjugated goat anti-rabbit IgG (H&L) (1:5000; Rockland Inc.), Alexa Fluor 680 conjugated goat anti-rabbit IgG (H+L), Alexa Fluor 680 conjugated donkey anti-sheep IgG (H+L) (1:5000; Molecular Probes, Inc., Eugene, OR) and the Odyssey Infrared Imaging System (Li-Cor Biosciences) or horseradish peroxidase-conjugated anti-sheep antibody (1:2000) (Upstate) and the chemiluminiscent substrate Luminol (Santa Cruz) by exposure to Kodak film or X-ray film. Quantification were performed using IRDye 800CW conjugated goat anti-sheep antibody (1:200; 9E10; Cancer Research UK).

Coimmunoprecipitation of Endogenous ERK3 and ERK4 with Endogenous MK5—HeLa cells were transfected with 20 nm of siRNA for either ERK3, ERK4, or MK5 and a scrambled siRNA (Ambion catalog number 4611) was used as a negative control. After 48 h the cells were lysed in a buffer containing 50 mM Tris/HCl, pH 7.5, 1 mM EGTA, 1 mM EDTA, 1% (w/v) Triton X-100, 10 mM β-glycerophosphate, 1 mM sodium orthovanadate, 50 mM sodium fluoride, 5 mM sodium pyrophosphate, and 0.27 mM sucrose) for 1 h at 4 °C. Direct interaction between MK5 and ERK4 was detected by mixing the above HeLa cell lysates or 1 μg of recombinant ERK4 with 3 μg of purified GST-MK5, GST-MK5 1–423, or GST in 300 μl of the above buffer for 2 h at 4 °C. After the addition of 30 μl of glutathione-Sepharose (50% slurry equilibrated in the above buffer), the samples were incubated for an additional hour. The beads were washed five times with the above buffer and 50 μl Tris, pH 7.5, and then resuspended in 40 μl of 2× SDS sample buffer. GST-MK5 and co-precipitated ERK4 was detected by SDS-PAGE and Western blotting using a polyclonal GST antibody (1:500; Z-5; Santa-Cruz Biotechnology) and a polyclonal sheep anti-ERK4 antibody (1:1000) or a monoclonal mouse anti-Myc antibody (1:200; 9E10; Cancer Research UK).

Expression of GST Fusion Proteins in Escherichia coli—GST fusion proteins were expressed in E. coli (BL21) and purified as previously described (16). SDS-PAGE and Coomassie Blue staining were used to analyze both the expression and yield of the fusion proteins.

GST Pull-down Assays—Lysates were made from HeLa cells transfected with Myc-tagged ERK4 wt, ERK4 1–330, or ERK4 1–340. Recombinant ERK4 was preclarified prior to the pull-down using 30 μl of glutathione-Sepharose (Amersham Biosciences AB) (50% slurry equilibrated in a buffer containing 50 mM Tris/HCl, pH 7.5, 1 mM EGTA, 1 mM EDTA, 1% (w/v) Triton X-100, 10 mM β-glycerophosphate, 1 mM sodium orthovanadate, 50 mM sodium fluoride, 5 mM sodium pyrophosphate, and 0.27 mM sucrose) for 1 h at 4 °C. Direct interaction between MK5 and ERK4 was detected by mixing the above HeLa cell lysates or 1 μg of recombinant ERK4 with 3 μg of purified GST-MK5, GST-MK5 1–423, or GST in 300 μl of the above buffer for 2 h at 4 °C. After the addition of 30 μl of glutathione-Sepharose (50% slurry equilibrated in the above buffer), the samples were incubated for an additional hour. The beads were washed five times with the above buffer and 50 μl Tris, pH 7.5, and then resuspended in 40 μl of 2× SDS sample buffer. GST-MK5 and co-precipitated ERK4 was detected by SDS-PAGE and Western blotting using a polyclonal GST antibody (1:500; Z-5; Santa-Cruz Biotechnology) and a polyclonal sheep anti-ERK4 antibody (1:1000) or a monoclonal mouse anti-Myc antibody (1:200; 9E10; Cancer Research UK).

Kinase Assays—GFP-tagged MK5 was immunoprecipitated from HeLa cells using 1 μl of the polyclonal anti-GFP antibody ab290 (Abcam, Cambridge, UK), and the kinase activity of MK5

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Detection of Endogenous ERK4—A polyclonal sheep anti-ERK4 antibody was immobilized by cross-linking to aldehyde-activated gel beads (AminoLink Plus Coupling Gel) using the Seize primary immunoprecipitation kit (45335; Pierce) according to the manufacturer’s instructions. 3.5 mg of clarified lysate were incubated with 20 μl of either cross-coupled anti-MK5 antibody or sheep IgG for 3 h at 4 °C. The beads were washed extensively using CytoSignal spin filters (CytoSignal, Irvine, CA) and a buffer containing 25 mM Tris and 0.15 mM NaCl, pH 7.2. The proteins were eluted from the beads by the addition of 45 μl of 1× SDS sample buffer. The samples were subjected to electrophoresis and then immunoblotted for ERK3, ERK4, and MK5 using a monoclonal anti-ERK3 antibody (1:1000; Zymed Laboratories Inc.), a sheep polyclonal anti-ERK4 antibody (1:1000), and a polyclonal sheep anti-PRAK antibody (1:1000), respectively.
was measured as previously described (13). To measure the endogenous MK5 kinase activity, MK5 was immunoprecipitated using a cross-linked polyclonal rabbit PRAK antiserum (H-180) from HeLa cells transfected with 20 nM of either ERK3 siRNA, ERK4 siRNA, or control siRNA as described above. The kinase activity was assayed as described previously (13). Activity is expressed as the number of cpm incorporated. The experiments were performed three times, and the results are presented as the mean values with associated errors. Where appropriate, the statistical significance of differences in activities under particular experimental conditions was evaluated using Student's t test. Endogenous MK5 and ERK3 from whole cell extracts and immunoprecipitated ERK4 were verified by Western blotting using a monoclonal mouse anti-PRAK antibody (1:500; A 7; Santa Cruz Biotechnology), a rabbit anti-ERK3 antibody (1:1000; 4067; Cell Signaling Technology), and a polyclonal sheep anti-ERK4 (1:1000) respectively.

Cell Staining and Microscopy—Determination of the subcellular localization of GFP fusion proteins, Myc-tagged ERK3, and ERK4 and the visualization of cell nuclei was performed as described previously (16). Images were collected using a Zeiss LSM510 confocal laser-scanning microscope and processed using Adobe Photoshop. For cell counting experiments several fields of cells from each transfection or co-transfection were examined using a Leica Leitz DMIRB inverted microscope (Leica Microsystems), and at least 100 cells were scored for the subcellular localization of the protein(s) of interest.

RESULTS

The MAPK4/ERK4 Gene Encodes a Protein of 587 Amino Acids—ERK4 was first isolated as one of four related human sequences with homology to MAPK and designated p63 MAPK (17). The published p63 MAPK cDNA encodes a protein of 557 amino acid residues (GI:4506089; Fig. 1). However, we obtained
an IMAGE clone (accession number AI245528, similar to human p63 MAPK), and DNA sequencing revealed that this cDNA contains five single base insertions when compared with the original p63 MAPK sequence. These changes result in the substitution of four amino acids (residues 384–388; Fig. 1A) and also in a frameshift within the C terminus of the ERK4 protein. This alters the amino acid sequence from residue 502 and extends the open reading frame from 557 to 587 amino acids (Fig. 1A). The latter sequence is in complete agreement with that predicted by the human genome and is also homologous to a full-length murine MAPK4 cDNA (accession number BC062911), indicating that this represents the correct ERK4/MAPK4 protein sequence. ERK4 is most closely related to the atypical MAPK ERK3 with 73% amino acid sequence identity within the kinase domain (17). In addition, both proteins contain a single phosphoacceptor site (SEG) within the activation loop and the amino acid sequence SPR instead of APE within kinase subdomain VIII (Fig. 1B) (23).

**ERK4 Is a Stable Protein That Is Localized Exclusively in the Cytosol**—ERK3 was recently characterized as an unstable protein, which is localized in both the nucleus and cytoplasm when expressed in mammalian cells (24, 25). To study the localization of full-length ERK4, this was expressed as a Myc epitope-tagged protein in HeLa cells, and its localization was compared with that of ERK3 (Fig. 2). In agreement with previous studies, we observe that ERK3 protein is found in both the nuclear and cytoplasmic compartments. In contrast, ERK4 is localized only in the cytoplasm. We next expressed ERK4 and ERK3 in the presence of cycloheximide and followed the rate of degradation of the Myc epitope-tagged proteins by Western blotting (Fig. 2B). In agreement with previous studies (24), ERK3 was a relatively unstable protein showing significant turnover within the first 3 h after cycloheximide addition. In contrast, levels of ERK4 were completely unchanged over the time course of the experiment (Fig. 2B). To ensure that neither overexpression nor presence of the Myc epitope tag influenced ERK3 and ERK4 stability, we also determined the turnover of endogenous proteins. Again ERK3 levels were reduced at times after 1 h, whereas ERK4 levels were unchanged (Fig. 2C). We conclude that ERK4 differs from its close relative ERK3 in terms of both its stability and its localization in mammalian cells.

**Endogenous ERK4 Is Found in the Cytosol**—To show that the subcellular distribution of overexpressed epitope-tagged ERK4 reflects that of the endogenous protein, we have performed cell fractionation studies (Fig. 3A). In agreement with the results obtained by confocal and fluorescence microscopy, immunoprecipitation followed by Western blotting using a polyclonal ERK4 antibody reveals that endogenous ERK4 is found exclusively in the cytoplasmic fraction (Fig. 3A). We conclude that the localization of epitope-tagged ERK4 proteins mirrors that of endogenous ERK4, indicating that it constitutes an appropriate model system with which to study the mechanism determining the subcellular localization of this protein.

**Extracellular Stimuli That Activate MAPKs Have No Effect on ERK4 Localization**—The classical ERK1 and -2 MAPKs are known to translocate to the nucleus following activation. We tested a range of agents known to induce MAPK activation for their ability to alter the subcellular localization of ERK4. We found that the majority of the ERK4 protein was retained in the cytoplasm in cells following treatment with serum, lysophosphatidic acid, epidermal growth factor, and arsenite (Fig. 3B).
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A

| ERK4 | MEK | UBF |
|------|-----|-----|
|      |     |     |
| T    | N   | C   |
| anti-ERK4 | IP-ERK4 | anti-MEK | anti-UBF |

B

quiescent exponential growth serum 3h EGF 1h LPA 1h arsenite 1h

C

Untreated Leptomycin B

anti-HA

D

% transfected cells with ERK4 in nucleus

![Graph showing % transfected cells with ERK4 in nucleus.]

FIGURE 3. Subcellular localization of ERK4. A, HeLa cells were subjected to subcellular fractionation, and fractions were analyzed by immunoprecipitation followed by Western blotting using an ERK4 specific antibody to detect endogenous ERK4 (top panel). Direct Western blotting with antibodies against either MEK (middle panel) or upstream binding factor (UBF, bottom panel) were used to verify efficient fractionation of cytoplasmic and nuclear proteins, respectively. T, total lysate; C, cytoplasmic fraction; N, nuclear fraction. B, NIH 3T3 fibroblasts were transfected with FLAG-tagged ERK4 and left to grow exponentially or serum-starved for 24 h (quiescent). The cells were then stimulated for 3 h with 10% serum or for 1 h with the following agents: 10 nM epidermal growth factor (EGF), 10 μM lysophosphatic acid (LPA), or 0.5 mM sodium arsenite. The localization of ectopically expressed ERK4 was analyzed by fluorescence microscopy after staining with anti-FLAG antibody. C, ERK4 is exported via a CRM1-dependant mechanism. Exponentially growing NIH 3T3 cells were transfected with HA-tagged ERK4 and treated either with vehicle (0.02% ethanol) or 2 ng/ml LMB for 12 h and fixed for immunofluorescence analysis. D, nuclear import of ERK4 is temperature-sensitive. Exponentially growing cells expressing ERK4 were treated either with vehicle (0.02% ethanol) or vehicle (2 ng/ml) and incubated at 4°C or 37°C for 2 h. The medium was supplemented with 15 mM HEPES, pH 7.4, before incubation. The cellular localization of ERK4 was scored as either mostly cytoplasmic or both nuclear and cytoplasmic. At least 150 cells were counted for each coverslip. The bar graph represents the means ± S.E. of three independent experiments.

of ERK4 in the nucleus (Fig. 3C). After 3 h of treatment, ERK4 was detected in the nucleus of more than 88% of the cells (data not shown).

ERK4 is Imported into the Nucleus by a Temperature-dependent Mechanism—The molecular mass of ERK4 (∼70 kDa) suggests that the protein enters the nucleus by an active transport mechanism. To address this point further, we tested the temperature dependence of ERK4 nuclear import. Exponentially proliferating NIH 3T3 cells transfected with HA-ERK4 were treated with LMB at either 37 or 4°C, and the localization of the protein was monitored by immunofluorescence. As shown in Fig. 3D, ERK4 accumulated in the nucleus of 89% of cells after 1 h of exposure to LMB at 37°C. However, the nuclear accumulation of ERK4 was completely abrogated at low temperature, implying that ERK4 is imported by an active mechanism.

ERK4 Interacts Specifically with MK5 Both in Vitro and in Vivo—We and others recently reported that the ERK3 MAPK is a physiological binding partner of the serine threonine kinase MK5 and that complex formation results in both the phospho-

ylation and activation of MK5 and also the nuclear to cytoplasmic relocalization of both proteins (15, 16). In preliminary experiments, Schumacher et al. (15) found that ERK4 could interact with and relocalize MK5 but found no evidence that ERK4 could activate MK5. However, these studies utilized a truncated form of the mouse ERK4 cDNA, which encodes only the first 471 residues of the protein. To study the ability of full-length ERK4 to interact with MK5 in vitro, we used recombinant ERK4 purified from baculovirus infected SF9 cells and performed GST pull-down assays using purified recombinant GST-MK5 (Fig. 4A). This experiment shows that GST-MK5 but not GST alone is able to interact directly with ERK4. We next examined the ability of endogenous MK5 to interact with both endogenous ERK4 and ERK3 in HeLa cells (Fig. 4B). Both ERK4 and ERK3 are detected in MK5 immunoprecipitates by Western blotting using either a sheep polyclonal antibody against recombinant ERK4 or a polyclonal rabbit antiseraum raised against an ERK3 peptide. Furthermore, pretreatment of HeLa cells with a specific siRNA directed against ERK4 greatly reduces the amount of this protein in complex with MK5, confirming the specificity of our anti-ERK4 antiseraum (Fig. 4B).

Specific binding between ERK3 and MK5 results in the relocalization of MK5 from the nucleus to the cytoplasm (15, 16). To examine the effect of ERK4 expression on MK5 localization, we performed co-transfection experiments in which GFP-MK5 was expressed either alone or with Myc epitope-tagged ERK4. As observed before, GFP-MK5 is localized exclusively in the cell nucleus (Fig. 5A). However, co-expression of ERK4 causes a dramatic relocalization of MK5 into the cytoplasm (Fig. 5B). This is not dependent on the kinase activity of either protein because translocation of MK5 into the cytoplasm was seen irrespective of mutations that abrogate protein kinase activity (Fig. 5C). We next examined the requirement for specific protein-protein interactions in mediating the ERK4-induced translocation of MK5. We previously demonstrated that a region...
FIGURE 4. MK5 interacts specifically with ERK4. A, direct interaction between ERK4 and MK5 in vitro assayed by GST pull-down. Recombinant ERK4 (1 μg) was mixed with 2 μg of either GST-MK5 or GST alone and glutathione-agarose. Bound ERK4 was detected by Western blotting using a polyclonal anti-ERK4 antibody. *, band detected by GST-antibody in the GST-MK5 pull-down because of degradation of GST-MK5. B, HeLa cells were transfected with 20 nM of the indicated siRNAs. The cells were harvested 48 h post-transfection, and MK5 was immunoprecipitated (IP) from the cell lysates using a polyclonal anti-MK5 antibody cross-linked to agarose beads. The immunoprecipitates were analyzed by Western blotting (WB) using a polyclonal ERK4 antibody (top panel), monoclonal ERK3 antibody (middle panel), or a polyclonal PRAK (MK5) antibody (bottom panel). Control immunoprecipitations were performed using preimmune IgG. C, an expression vector encoding full-length, wild type human ERK4 fused to the GAL4 activation domain (GAD) was transformed into the yeast strain Y187. Selected transformants were mated with transformants of yeast strain PJ69–2A expressing the indicated GAL4 DNA-binding domain fusions. Yeast diploids expressing both DNA-binding domain and activation domain fusions were selected on synthetic dropout medium deficient for leucine and tryptophan (DDO, lower panel). Leu/Trp positives were dropped onto SD minus leucine, tryptophan, histidine, and adenine (QDO, upper panel), and protein-protein interactions were assessed by growth on this medium.

FIGURE 5. Expression of ERK4 causes the nuclear export of MK5. A, HeLa cells were transfected with expression vector encoding EGFP-MK5. After 24 h, the cells were fixed, and EGFP fluorescence was visualized directly (green channel on left). The cell nuclei were visualized by DRAQ5 staining (blue channel on right). B, HeLa cells were co-transfected with expression vectors encoding EGFP-MK5 and Myc-ERK4 and fixed 24 h post transfection. EGFP-MK5 was visualized directly (green channel on left), and ERK4 was visualized by staining with an anti-Myc antibody and Alexa 594 anti-mouse antibody (red channel on right). A merged image of the green and red channels is shown (lower left), and the nuclei were visualized by DRAQ5 staining (blue channel on lower right). C, HeLa cells were co-transfected with the indicated expression vectors, and EGFP-MK5 and Myc-ERK4 were visualized as above. In all, 100 cells co-expressing both EGFP-MK5 and ERK4 from three independent transfections were counted, and the distribution of both proteins was scored. The results are presented as the percentages of cells in which EGFP-MK5 was predominantly cytosolic (C>N), and the mean values with the associated errors are shown. In all experiments, several fields of cells were examined, and the representative images are shown.
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A. HeLa cells were transfected with expression vectors encoding Myc-tagged full-length or two C-terminal truncation mutants of ERK4 encoding either amino acids 1–330 or 1–340 of the protein (ERK4 wt, ERK4 1–330, and ERK4 1–340, respectively). The cell lysates were then mixed with either recombinant GST-MK5 or GST alone. Binding of ERK4 was detected by Western blotting using an anti-Myc monoclonal antibody.

B. EGFP-MK5, anti-myc, and DRAQ5.

FIGURE 6. Amino acids 330-340 of ERK4 are required for interaction with and relocalization of MK5. A. HeLa cells were transfected with expression vectors encoding Myc-tagged full-length or two C-terminal truncation mutants of ERK4 encoding either amino acids 1–330 or 1–340 of the protein (ERK4 wt, ERK4 1–330, and ERK4 1–340, respectively). The cell lysates were then mixed with either recombinant GST-MK5 or GST alone. Binding of ERK4 was detected by Western blotting using an anti-Myc monoclonal antibody. B. HeLa cells were co-transfected with vectors encoding EGFP-MK5 and either ERK4 1–330 or 1–340. EGFP-MK5 was visualized directly (green channel in top panels), ERK4 was visualized by staining with an anti-Myc antibody and an Alexa 594-coupled secondary (anti-mouse) antibody (red channel in middle panels) and cell nuclei were visualized by DRAQ5 staining (blue channel in bottom panels). A total of 100 cells expressing either EGFP-MK5 alone or co-expressing both EGFP-MK5 and the indicated mutant of ERK4 from three independent transfections were counted, and the distribution of EGFP-MK5 was scored. The results are presented as the percentages of cells in which: EGFP-MK5 was either predominantly nuclear (N>C) or predominantly cytoplasmic (C>N), and the mean values with the associated errors are shown. In all experiments, several fields of cells were examined, and the representative images are shown.

between residues 330 and 340 in ERK3 is necessary for binding to MK5. Deletion of the corresponding region in ERK4 also prevents interaction between ERK4 and MK5 in vitro (Fig. 6A). Furthermore, ERK4 1–330 can no longer cause relocalization of GFP-MK5, whereas ERK4 1–340 is able to translocate MK5 into the cytoplasm as efficiently as the full-length protein (Fig. 6B). Finally, we also employed a truncated form of MK5 (residues 1–423), which can no longer interact with ERK4 in vitro (Fig. 7A). This protein is still nuclear when expressed as a GFP fusion protein, but unlike wild type MK5-GFP it is not translocated to the cytoplasm in the presence of wild type ERK4 (Fig. 7B). We conclude that, like ERK3, ERK4 interacts specifically and directly with MK5 and that it is this interaction rather than the kinase activity of either protein that is responsible for the ability of ERK4 to cause nuclear export of the MK5 protein.

Co-expression of ERK4 Leads to Phosphorylation and Activation of MK5—Given the ability of ERK3 to activate MK5 and the striking similarities in the abilities of both ERK3 and ERK4 to bind and relocalize MK5, we wanted to determine whether ERK4 was also able to mediate the phosphorylation and activation of MK5.

To examine the ability of ERK4 to activate MK5 in vivo, a series of co-transfection experiments were performed. Firstly wild type ERK4 was co-expressed with wild type MK5, and this led to a 6–7-fold increase in the activity of GFP-MK5 (Fig. 6), failed to activate MK5, whereas ERK4 1–340 efficiently activated MK5 (Fig. 8C). By the same token, the truncated form of MK5 1–423, which was unable to bind to or be relocalized by ERK4 (Fig. 7), was not activated by wild type ERK4 (Fig. 8D). Because the activation of MK5 by ERK4 clearly requires Thr182 within the activation loop of the kinase, we wanted to investigate whether co-expression of ERK4 could also induce the phosphorylation of Thr182 in MK5. To this end, we co-transfected either wild type or kinase-dead ERK4 (D168A) together with either wild type, kinase-dead (K51E) or Thr182 to Ala mutants of MK5. MK5 was then immunoprecipitated from the extracts of transfected cells and immunoblotted with a Thr(P)182-specific antibody. As shown in Fig. 9A, only wt ERK4 is able to induce an increase in Thr182 phosphorylation in both wt and kinase-dead MK5. In the latter case it is important to note the complete lack of Thr(P)182 in this protein in the absence of ERK4. Immunoblot analysis of the same immunoprecipitates for total MK5 level and co-immunoprecipitated ERK4 show that equal amounts of ERK4 and MK5 were present in all the experiments (Fig. 9A, lower and middle panels, respectively).

Similar experiments were also performed with wild type and kinase-dead ERK3. Here we observe that wild type ERK3 induced Thr182 phosphorylation on both wild type and kinase-dead MK5. However, in contrast to kinase-dead MK5 toward the model MK5 peptide substrate PRAKtide (KKLR-RTLSVA, derived from glycogen synthase). In contrast, a kinase-dead mutant of ERK4 (D168A) did not increase the activity of MK5 (Fig. 8A). Activation of MK5 was completely dependent on an intact phosphoacceptor site within the activation loop of MK5 because the T182A mutant was not activated by wild type ERK4. The activity toward PRAKtide was entirely dependent on MK5 activity because a kinase-dead form of MK5 (K51E) showed no activity when co-expressed with wild type ERK4 (Fig. 8A). The activity of MK5 in the presence of wild type ERK4 was not sensitive to the specific p38 inhibitor SB203580, whereas MK5 activity obtained by co-expression of p38β2, and constitutively active MKK6 was inhibited by this drug (Fig. 8B).

To determine whether the specific protein-protein interactions required for ERK4 to relocalize MK5 are also important for activation of MK5 by ERK4, we employed truncated forms of both proteins. ERK4 1–330, which was unable to bind to and relocalize
ERK4, co-expression of kinase-dead ERK3 did lead to a significant increase in Thr\(^{182}\) phosphorylation in wild type MK5 (Fig. 9B). Thus, whereas both phosphorylation and activation of MK5 are strictly dependent on ERK4 kinase activity, in the case of ERK3 MK5 activation may depend on the kinase activities of both proteins.

**MK5 Activity Is Dependent on Both ERK3 and ERK4 in Vivo**

Our results thus far strongly suggest that ERK4 is a physiological activator of MK5 and that in cells that express both ERK4 and ERK3, both kinases should contribute to MK5 activation. We previously demonstrated that either ablation of murine ERK3 by homologous recombination or siRNA-mediated knock-down reduced the activity of endogenous MK5 by \(~40\)–\(~60\)% (16). To examine the respective roles of ERK3 and ERK4 in activating MK5, we have used siRNA mediated knock-down to reduce the levels of each kinase singly and in combination and determined the effect on endogenous MK5 activities in HeLa cells (Fig. 10). siRNA-mediated knock-down of ERK3 significantly reduces the levels of ERK3 without affecting the levels of MK5, and MK5 activity is reduced by \(~70\)%.

**DISCUSSION**

ERK4 was first described as a protein of 45 kDa found in both human brain and rat PC12 cells that cross-reacted with an ERK1-specific antibody (28). To date no genes encoding a 45-kDa ERK homolog have been identified, but a 46-kDa splice variant of ERK1 (designated ERK1b) has been described, and this may account for the previously reported 45-kDa ERK4 (29). Here we have characterized the kinase encoded by the MAPK4 gene as ERK4. The cDNA encoding human ERK4 was originally identified as p63 MAPK in a screen for ERK2 homologs and found to specify a protein of 557 amino acids (17). During our re-cloning of ERK4 based on human expressed sequence tag data bases, we discovered that the original sequence published by Gonzalez et al. (17) contained several errors. Correction of these errors gives rise to several changes in the protein. The most notable of these changes is in the C terminus of ERK4 where the open reading frame is profoundly altered from residue 502 onwards and also extended by a further 30 residues to a total of 587 amino acids. This correction also results in a protein that is homologous to the predicted mouse ERK4 protein. In agreement with this corrected open reading frame, Western blot analysis of both overexpressed and endogenous ERK4 detects a protein with an apparent molecular mass of \(~65\) kDa. ERK4 is more than 70% homologous to the atypical MAPK ERK3 within the kinase domain, and both proteins share distinct features such as the unique SEG motif in the activation loop and the SPR instead of the highly conserved APE sequence in subdomain VIII. This means that ERK4 together with ERK3 comprise a subfamily of atypical MAPKs. Interestingly this is the only subfamily of MAPKs that are found exclusively in vertebrates (23, 30, 31).

Although ERK3 has been shown to be a highly unstable protein, when expressed in exponentially growing cells, here we demonstrate that ERK4 is a stable protein under the same conditions. Sequences within the N-terminal lobe of ERK3 have been identified as responsible for the degradation of ERK3 through the ubiquitin-proteasome pathway (24). Interestingly, the first 83 residues of ERK3 that are responsible for its degradation are less conserved in ERK4 (32).

In contrast to ERK3, which is found in both the nucleus and the cytoplasm, we find that ERK4 is mainly localized in the cytoplasm. No significant change in the subcellular distribution of ERK4 was observed in response to common mitogenic stimuli or chemical stresses. Our data using leptomycin B clearly indicate that the cytoplasmic distribution of ERK4 is dependent on active nuclear export. These differences in the subcellular distribution and half-life of ERK4 when compared with ERK3 may indicate that there are significant differences in the regulatory mechanisms governing the activity of these kinases.
Regulation of MK5 by ERK4/MAPK4

Here we report physiological interaction between the atypical MAPK ERK4 (MAPK4) and the MAPKAP kinase MK5. We find that this interaction leads to both subcellular redistribution and activation of MK5. This is in contrast to a previous report by Schumacher et al. (15), who failed to detect activation of MK5 by ERK4. The reason for this discrepancy may be because Schumacher et al. expressed a truncated form of ERK4. This is supported by recent data obtained by the Matthias Gaestel group in which they have repeated their previous experiments using full-length murine ERK4 and find that this protein is capable of activating MK5.4 Our results together with previous studies of ERK3 clearly demonstrate that the atypical MAPKs are important regulators of both the subcellular distribution and the activity of MK5 in mammalian cells.

The exact mechanism by which ERK3 activates MK5 is unclear. Schumacher et al. (15) concluded that ERK3 kinase activity was not necessary for activation of MK5 and proposed that scaffolding of MK5 by ERK3 is sufficient to stimulate autophosphorylation and activation of MK5. In contrast, we have previously shown in co-transfection experiments that although kinase-dead ERK3 is able to induce a weak activation of MK5, full activation of this kinase also requires ERK3 kinase activity (16). In the present study we have found that the requirement for the kinase activity of ERK4 to activate MK5 is absolute, with little or no activation of MK5 by a kinase-dead mutant. (Fig. 8A).

Just as for activation of MK5 by ERK3, MK5 activation mediated by ERK4 is absolutely dependent on phosphorylation of Thr182 within the activation loop of MK5 and requires specific protein-protein interactions between ERK4 and MK5. Further investigation into the mechanism of both ERK4- and ERK3-mediated activation of MK5 using a phospho-specific antibody against Thr182 clearly shows that wt ERK4 but not a kinase-dead version of ERK4 is capable of inducing Thr182 phosphorylation in MK5 (Fig. 9A). In the case of ERK3 it is true that significant incorporation of phosphate into Thr182 of MK5 occurs in the presence of a kinase-dead mutant of ERK3, but this is greatly enhanced when both kinases are wild type.

4 M. Gaestel, personal communication.
Thus, it is possible that a component of the activation of MK5 by ERK3 is mediated by autophosphorylation of MK5 but that this process is either greatly stimulated or primed by a kinase-dependent function of ERK3.

In conclusion, we have characterized ERK4 as a physiological partner for MK5 and demonstrated that both ERK3 and ERK4 are important for the regulation of MK5 activity in mammalian cells. We have also shown that although both ERK3 and ERK4 are atypical MAPKs sharing considerable sequence homology, they display clear differences both in subcellular distribution and stability. These may indicate differences in the way that these proteins are regulated. The physiological roles of ERK3 and ERK4 are still enigmatic. The generation of knockout models lacking these kinases will be important in this regard. With respect to MK5, Schumacher et al. (15) reported an embryonic lethal phenotype with incomplete penetrance in homozygous MK5 mutant mice. Whether this phenotype is due in any way to the abnormal regulation of the ERK3/MK5 or ERK4/MK5 signaling modules is a critical question. In addition, the downstream effectors and upstream activators of these signaling modules remain to be identified. Our study provides additional insights and tools for further investigation into the function of the signaling pathways defined by the atypical MAPKs ERK3 and ERK4.

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REFERENCES

1. Winzen, R., Kracht, M., Ritter, B., Wilhelm, A., Chen, C. Y., Shyu, A. B., Muller, M., Gaestel, M., Resch, K., and Holtmann, H. (1999) EMBO J. 18, 4969–4980
2. Ehrnsperger, M., Graber, S., Gaestel, M., and Buchner, J. (1997) EMBO J. 16, 221–229
3. Hannigan, M. O., Zhan, L., Ai, Y., Kotlyarov, A., Gaestel, M., and Huang, C. K. (2001) J. Immunol. 167, 3953–3961
4. Manke, I. A., Nguyen, A., Lim, D., Stewart, M. Q., Elia, A. E., and Yaffe, M. B. (2005) Mol. Cell 17, 37–48
5. Voncken, J. W., Niessen, H., Neufeld, B., Rennefahrt, U., Dahlmans, V., Kubben, N., Holzer, B., Ludwig, S., and Rapp, U. R. (2005) J. Biol. Chem. 280, 5178–5187
6. Kotlyarov, A., Neininger, A., Schubert, C., Eckert, R., Birchmeier, C., Volk, H. D., and Gaestel, M. (1999) Nat. Cell Biol. 1, 94–97
7. Stokoe, D., Engel, K., Campbell, D. G., Cohen, P., and Gaestel, M. (1992) FEBS Lett. 313, 307–313
8. Kotlyarov, A., Yannoni, Y., Fritz, S., Laass, K., Telliez, J., Pitman, D., Lin, L. L., and Gaestel, M. (2002) Mol. Cell. Biol. 22, 4827–4835
9. Levine, S. S., King, I. F., and Kingston, R. E. (2004) Trends Biochem. Sci. 29, 478–485
10. New, L., Jiang, Y., Zhao, M., Liu, K., Zhu, W., Flood, L. J., Kato, Y., Parry, G. C., and Han, J. (1998) EMBO J. 17, 3372–3384
11. Ni, H., Wang, X. S., Diener, K., and Yao, Z. (1998) Biochem. Biophys. Res. Commun. 243, 492–496
12. New, L., Jiang, Y., and Han, J. (2003) Mol. Biol. Cell 14, 2603–2616
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13. Seternes, O. M., Johansen, B., Hegge, B., Johannessen, M., Keyse, S. M., and Moens, U. (2002) *Mol. Cell. Biol.* **22**, 6931–6945
14. Shi, Y., Kotlyarov, A., Laabeta, K., Gruber, A. D., Butt, E., Marcus, K., Meyer, H. E., Friedrich, A., Volk, H. D., and Gaestel, M. (2003) *Mol. Cell. Biol.* **23**, 7732–7741
15. Schumacher, S., Laass, K., Kant, S., Shi, Y., Visel, A., Gruber, A. D., Kotlyarov, A., and Gaestel, M. (2004) *EMBO J.* **23**, 4770–4779
16. Seternes, O. M., Mikalsen, T., Johansen, B., Michaelsen, E., Armstrong, C. G., Morrice, N. A., Turgeon, B., Meloche, S., Moens, U., and Keyse, S. M. (2004) *EMBO J.* **23**, 4780–4791
17. Gonzalez, F. A., Raden, D. L., Rigby, M. R., and Davis, R. J. (1992) *FEBS Lett.* **304**, 170–178
18. Slack, D. N., Seternes, O. M., Gabrielsen, M., and Keyse, S. M. (2001) *J. Biol. Chem.* **276**, 16491–16500
19. Jiang, Y., Chen, C., Li, Z., Guo, W., Gegner, J. A., Lin, S., and Han, J. (1996) *J. Biol. Chem.* **271**, 17920–17926
20. Li, Z., Jiang, Y., Ulevitch, R. J., and Han, J. (1996) *Biochem. Biophys. Res. Commun.* **228**, 334–340
21. Lamark, T., Perander, M., Outzen, H., Kristiansen, K., Overvatn, A., Michaelsen, E., Bjorkoy, G., and Johansen, T. (2003) *J. Biol. Chem.* **278**, 34568–34581
22. McMahon, M., Itoh, K., Yamamoto, M., and Hayes, J. D. (2003) *J. Biol. Chem.* **278**, 21592–21600
23. Turgeon, B., Lang, B. F., and Meloche, S. (2002) *Genomics* **80**, 673–680
24. Coulombe, P., Rodier, G., Pelletier, S., Pellerin, J., and Meloche, S. (2003) *Mol. Cell. Biol.* **23**, 4542–4558
25. Julien, C., Coulombe, P., and Meloche, S. (2003) *J. Biol. Chem.* **278**, 42615–42624
26. Kudo, N., Wolff, B., Sekimoto, T., Schreiner, E. P., Yoneda, Y., Yanagida, M., Horinouchi, S., and Yoshida, M. (1998) *Exp. Cell Res.* **242**, 540–547
27. Wolff, B., Sanglier, I., and Wang, Y. (1997) *Chem. Biol.* **4**, 139–147
28. Boulton, T. G., Nye, S. H., Robbins, D. J., Ip, N. Y., Radziejewska, E., Morgenbesser, S. D., DePinho, R. A., Panayotatos, N., Cobb, M. H., and Yancopoulos, G. D. (1991) *Cell* **65**, 663–675
29. Yung, Y., Yao, Z., Hanoch, T., and Seger, R. (2000) *J. Biol. Chem.* **275**, 15799–15808
30. Watanabe, N., Nagamatsu, Y., Gengyo-Ando, K., Mitani, S., and Ohshima, Y. (2005) *Development* **132**, 3175–3184
31. Goldberg, J. M., Manning, G., Liu, A., Fey, P., Pilcher, K. E., Xu, Y., and Smith, J. L. (2006) *PLoS Genet.* **2**, e38
32. Turgeon, B., Saba-El-Leil, M. K., and Meloche, S. (2000) *Biochem. J.* **346**, 169–175