Supplementary Section

Activation of MK5/PRAK by the atypical MAP kinase ERK3 defines a novel signal transduction pathway

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Materials and methods

Reagents

Leptomycin B (LMB) was generously provided by Dr Minoru Yoshida, (University of Tokyo, Japan). SB203580 was purchased from Alexis Corp. Sodium arsenite, Sorbitol and Bovine Serum Albumin were purchased from Sigma Aldrich. [^{32}P] Orthophosphate and [γ-^{32}P] ATP were from Amersham Pharmacia. Recombinant active and inactive MK5(PRAK), recombinant active MK2 and PRAKtide were provided by Prof P. Cohen (MRC Protein Phosphorylation Unit, University of Dundee, Scotland, UK).

Antibodies

Antibodies were raised at the Scottish Antibody Production Unit (SAPU, Lanarkshire, UK). The ERK3 antibody was raised in sheep against the ERK3 protein purified from Sf9 cells and was affinity purified on CH-Sepharose covalently coupled to the protein antigen. The phospho-specific antibody recognizing ERK3 phosphorylated at Ser189

(S189-P) was raised in sheep against peptide HYSHKGHLSEGLVTK corresponding to residues 181-195 of human ERK3 in which the underlined residue is phosphoserine. The antibody was affinity-purified on CH-Sepharose covalently coupled to the phosphorylated peptide and then passed through a column of CH-Sepharose coupled to the non-phosphorylated peptide. Antibody that did not bind to the latter column was selected. The Rabbit polyclonal ERK3 antiserum (E3-CTE4) has been described previously (Coulombe et al., 2003). Polyclonal rabbit MK5 antiserum (H-180) and polyclonal ERK2 antibody (C-14) were purchased from Santa-Cruz, monoclonal p38 antibody (5F11) and polyclonal MK2 antibody (cat# 3042) were purchased from Cell Signaling Technology. A sheep polyclonal anti-MK5 antibody and a phospho-specific antibody, which recognises phosphorylated threonine 182 within the activation loop of MK5, were kindly provided by Prof P. Cohen (MRC Protein Phosphorylation Unit, University of Dundee).

DNA constructs

Construction of the yeast expression plasmids pGADT7-ERK1, pGADT7-ERK2, pGADT7-ERK3, pGBKT7-ERK3, pGADT7-ERK5, pGADT7-ERK7, pGADT7-JNK1, pGADT7-p38α, pGADT7-p38γ, pGADT7-p38δ and the mammalian expression vectors pSG5.ERK2-myc, pSG5.p38α-HA and pSG5.JNK1-HA have been described previously (Slack et al., 2001). The construction of pEGFP-MK5, pEGFP-MK5K51E, pEGFP-MK5T182A, pGEX4-T3 MK5 and pGEX4-T3 MK5mutNLS are described in (Seternes et al., 2002). The pGEXMnk1 construct (Waskiewicz et al., 1997) was a gift from Dr Chris Proud (University of Dundee). The Msk1 cDNA was a gift from Dr Dario Alessi

(University of Dundee). The constructs containing constitutively active MKK6 (MKK6E/E) and FLAG-tagged p38β2 (Jiang et al., 1996; Li et al., 1996) were kindly provided by Dr. J. Han (Scripps Institute, CA, USA).

All plasmid constructs described were verified by DNA sequencing. All PCR was performed using Pfx Platinium polymerase (Invitrogen Corp.) according to the manufacturers instructions. Human ERK3 was amplified from pSK.ERK3 using primers 5'-cggaattcgcaatagtaagggtttcaaaatggc-3' and 5'-gctagcctcgaggttcagatgtttcagaatgc-3' and subcloned as an *EcoRI-XhoI* fragment into a modified pSG5 vector encoding a single myc epitope tag at the C-terminus of the expressed protein (Groom et al., 1996). The vector pGBKT7-ERK3 was digested with *Nde*I/*SalI,* and the fragment ligated into the corresponding sites of a modified pFastBAC1 vector encoding a hexahistidine tag 5´ to the *Nde*I site. The resulting pFastBAC-ERK3 construct was then used to generate recombinant baculovirus using the Bac-to-Bac methodology (Invitrogen, Inc.). MK5 was amplified from pEGFP-MK5 using primers 5'-gaagtcgcatatgtcggaggacagcgacatgg-3' and 5'- ccgctcgagctactggggctcgtggggaag –3' and cloned as a *NdeI*/*XhoI* fragment into the *NdeI*/*SalI* sites of pGBKT7 and into the *NdeI*/*XhoI* sites of pGADT7. MK3 was amplified from an IMAGE clone (2988489, HGMP Hinxton Cambridge) using primers 5'-cggaattcatggatggtgaaacagcagagg-3' and 5'-cgggacctctactggttgttgcagccc-3' and cloned as an *EcoRI*/*BamHI* fragment into pGBKT7. Mnk1 was excised from pGEX-Mnk1 as a *BamHI* fragment and ligated into the *BamHI* site of pGBKT7. Msk1 was excised as a *NdeI*/*SalI* fragment from the vector and ligated into the *NdeI*/*SalI* sites in pGBKT7. The C-terminal deletion mutant 1-423 of MK5 was generated by PCR using the forward primer 5´-gggaattcgtcggaggacagcgacatgg-3´ and the reverse primer 5´-ccgctcgagttaggcctc

ctgcattacctcattcagc-3´ and cloned as an *EcoRI*/*XhoI* fragment into the *EcoRI*/*XhoI* site of pGEX5-T3 (Pharmacia-Amersham) or the *EcoRI*/*SalI* site of pEGFP-C1 (Clontech). The C-terminal deletion mutants of ERK3 1-330 and 1-340 were generated by PCR using the forward primer 5´-cggaattcgcaatagtaagggtttcaaaatggc-3´ and the reverse primers 5´-ccact cgagatcttcaatatgaaaaggatgg-3´ and 5´-ccgctcgaagagtttcatccataagcaaaatatc-3´ respectively and cloned into the modified pSG5 vector as an *EcoRI*/*XhoI* fragment. A kinase dead ERK3 was produced by changing Asp 171 to a Ala by site directed mutagenesis using the Quickchange method (Stratagene) and primers 5'-agggaaatccttgcaaggagaccatccac-3' and 5'-gtaggatggtctccttgcaaggatttccct-3'. Mutation of Ser189 in ERK3 to Glu was made using the primers 5'-ccataagggtcatcttgaagaaggattggttactaaatgg-3' and 5'-ccatttagtaaccaatc cttcttcaagatgacccttat gg-3'

Yeast two-hybrid assay

GAL4 DNA binding domain and activation domain fusion plasmids were transformed into the yeast strains PJ69-2A and Y187, respectively, according to the manufacturer's instructions (Clontech). Semiquantitative analysis of two-hybrid interactions was performed using a β−galactosidase assay according to the manufacturer's instructions (Clontech). The PJ69-2A clone expressing SV40- Large T antigen and the Y187 clone expressing p53 were provided with the Matchmaker II kit (Clontech).

Cell culture and transfection

COS-1 cells were maintained in Dulbecco's modification of Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (Invitrogen Corp.), 2 mM L-glutamine,

penicillin (100 units/ml), streptomycin (100 μg/ml) and transfected by the DEAE-dextran method (McCutchan and Pagano, 1968). HeLa cells were maintained in Eagle's Minimum Essential Medium (MEM) supplemented with 1x non-essential amino acids (Invitrogen Corp.) 10% fetal bovine serum (Invitrogen Corp.), 2 mM L-glutamine, penicillin (100 U/ml), streptomycin (100 μg/ml) and transfected using Lipofectamine plus reagent (Invitrogen Corp.) according to the manufacturer´s instructions. PC12 cells were maintained in RPMI supplemented with 10% heat inactivated horse serum (Invitrogen Corp.), 5% fetal bovine serum (Invitrogen Corp), 2 mM L-glutamine, penicillin (100 U/ml) and streptomycin (100 mg/ml). To induce neuronal differentiation, PC12 cells were plated on collagen type VII (Sigma) and stimulated with NGF (100ng/ml, Invitrogen Corp.) for the times indicated. RAW 264.7 cells were maintained in DMEM supplemented with glucose (4.5 g/l), 10% heat inactivated fetal calf serum (Invitrogen Corp), 2 mM L-glutamine, penicillin (100 U/ml) and streptomycin (100 μg/ml). Primary embryonic fibroblasts cells were maintained in DMEM supplemented with glucose (4.5 g/l), 10% fetal bovine serum (Invitrogen Corp), 2 mM L-glutamine, penicillin (100 U/ml) and streptomycin (100 μg/ml).

SiRNA

All siRNAs were purchased as validated or pre-designed from Ambion. The sequence of the siRNAs used were as follows (sense and antisense): ERK3 siRNAa 5´-ggcuuuucauguaucagcutt –3´ and 5´-agcugauacaugaaaagcctg-3´ (ORF 389-408) , ERK3 siRNAb 5´-ggcaauggcuugguuuuuutt-3´ and 5´-aaaaaaccaagccaagccauugcctc-3´ (ORF 88-108), ERK3 siRNAc 5´-ggaguacauggagacagactt-3´ and 5´-gucugugcuccauguacucctg-3´

(ORF 324-345) and MK5 siRNA 5´-ggauaugcgaagaaagauctt-3 5´-gaucuuucuucgcauauc ctt-3 (ORF 755-774). The underlined nucleotides in the siMK5 ORF represent the differences between the human and mouse sequences. Silencer negative control siRNA (Ambion cat# 1661) was used as the control siRNA. The siRNAs were transfected into HeLa cells using Lipofectamine 2000 according to the manufacturer´s instructions.

Western-blotting

For detection of epitope tagged ERK3, EGFP-MK5, p38α and p38β2 in transfected cells and in GST pulldown experiments, samples were analysed by SDS-PAGE (4-12 % NU-PAGE, Invitrogen corp.) transferred to 0.45mm PVDF membrane (Millipore) and probed with either anti-myc 9E10 (Cancer Research UK), anti-FLAG (M2 Stratagene), anti-HA 12CA5 (Cancer Research UK) or anti-GFP (Santa-Cruz) as described previously (Seternes et al., 1999)

Expression of GST fusion proteins in *E.coli*

GST fusion proteins were expressed in *E.coli* (BL21) by induction with 0.5 μM isopropyl-1-thio-β-D-galactopyranoside, at 23^0 C for 3 h. Proteins were then purified using glutathione-sepharose (Amersham Pharmacia) using standard techniques. Both the expression and yield of these fusion proteins were analysed by SDS PAGE and Coomassie Blue staining.

GST pull down assays

Lysates from COS-1 cells transfected with either HA-tagged p38α, FLAG-tagged p38β2, HA-tagged JNK1, Myc-tagged ERK2 or Myc-tagged ERK3. ERK3 1-330 and ERK3 1-340 were used in GST-pulldown assays as described previously (Seternes et al., 2002). Direct interaction between MK5 and ERK3 was detected by mixing 1 µg of recombinant ERK3 with 2 µg purified GST MK5 or GST in 200 µl of a buffer containing 50 mM Tris/HCl pH 7.5, 1 mM EGTA, 1 mM EDTA, 1% (w/v) Triton-X 100, 1 mM sodium orthovanadate, 50 mM sodium fluoride, 5 mM sodium pyrophosphate and 0.27 M sucrose for 1 h at 4ºC. After addition of 30µl Glutathione-Sepharose (50% slurry equilibrated in the above buffer, Amersham-Pharmacia) the samples were incubated for an additional 30 min. The beads were extensively washed with the above buffer and 50 mM Tris PH 7.5 and then resuspended in 40 µl 2 x SDS-sample buffer. Co-precipitated ERK3 was detected by SDS PAGE and western-blotting using a polyclonal sheep anti-ERK3 antibody

Immunoprecipitation of endogeneous ERK3 and MK5

RAW 264.7 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, 1 mM sodium orthovanadate, 50 mM sodium fluoride, 5 mM sodium pyrophosphate, 0.27 M sucrose, and 'Complete' protease inhibitor cocktail (one tablet/50 ml, Roche). Lysates were clarified by centrifugation at 4ºC for 10 min at 15 000 g. Two milligrams of clarified lysate were incubated with 5µg of mouse monoclonal anti-ERK3 antibody (Zymed) or 2 µg sheep polyclonal MK5

antibody for 1 h at 4ºC, before addition of 30 µl protein G-agarose (50% slurry equilibrated in lysis buffer) and incubated for a further 30 min. The immunoprecipitates were then washed three times in lysis buffer and the beads were resuspended in a volume of 30µl 2xSDS-sample buffer. The samples were subjected to electrophoresis and then immunoblotted for either ERK3, MK5 and p38 using a sheep polyclonal anti-ERK3 antibody, a sheep polyclonal anti-MK5 antibody (gift of Prof P. Cohen) and a monoclonal anti p38 antibody (Cell Signaling Technology) respectively.

Kinase Assays

GFP-tagged MK5 was immunoprecipitated from HeLa cells using 1 µl of the polyclonal anti-GFP antibody ab290 (Abcam, Cambridge, UK). Endogenous MK5 was immunoprecipitated from 500 µg cell lysate using 3 µg anti-PRAK Ab (H-180, Santa-Cruz) and kinase activity was assayed as described previously (Seternes et al., 2002). *In vitro* activation of MK5 by ERK3 was monitored by incubation of inactive MK5 (2 µg) with either 6 µg wild-type ERK3, ERK3 D171A (kinase dead) or ERK3 S189E at 30ºC for 30 min in a total volume of 20 µl containing 50 mM Tris pH 7.5, 10 mM $MgCl_2$, 0.1 mM ATP and 1 µg/µl BSA. Following incubation, 10 µl of this mixture was transferred to a clean eppendorf tube and 40 µl of a buffer containing 50 mM Tris pH 7.5 , 1 µg/µl BSA, 0.1 mM ATP, [γ-^{32}P] ATP(1 µCi, 3,000 Ci/mmol; Amersham Pharmacia), 10 mM $MgCl_2$ and Praktide (30 µM) was added. After 20 min incubation at 30ºC, 20 µl aliquots were spotted onto Whatman p81 paper and washed extensively with 1% phosphoric acid before measurement of radioactive incorporation by scintillation counting. *In vitro* phosphorylation of either ERK3 or Hsp27 by MK2 or MK5 was monitored by incubation

of 5 μg of either ERK3 or HSP27 with 1U/ml of activated MK2 or MK5 or 1μg of inactive MK5 in 40 μl of a buffer containing 50 mM Tris pH 7.5 , 1 μg/μl l BSA, 0.1 mM ATP, [γ-^{32}P] ATP(1 μCi, 3,000 Ci/mmol; Amersham Pharmacia), 10 mM $MgCl_2$ for 20 min at 30ºC. Following incubation, 10 μl 5xSDS-sample buffer was added and the samples were heated to 95ºC for 5 min. Following analysis by SDS-PAGE, phosphorylated proteins were detected by audioradiography. To determine the time course and stoichiometry of ERK3 phosphorylation by activated MK5 *in vitro*, ERK3 (2 μM) was incubated at 30ºC with 3 U/ml of MK5 and 0.1 mM [γ-^{32}P] ATP in a 50 μl reaction volume containing 50 mM Tris/HCL (pH 7.5), 0.1 mM EGTA, 10 mM magnesium acetate and 0.1% 9(v/v) 2-mercaptoethanol. At 3 min, 15 min and 60 min duplicate aliquots were removed and analysed by SDS-PAGE, stained with Coomassie Blue and subjected to autoradiography. The bands were then excised and [^{32}P]-incorporation into ERK3 was assessed by Cerenkov counting. The stoichiometry of phosphorylation at each time point was determined using the molecular mass of ERK3 and the specific activity of the [γ-^{32}P] ATP.

In vivo [^{32}P]- labeling.

Twenty-four hours after transfection of HeLa cells the growth medium was changed to phosphate-free Dulbecco's minimal Eagle's medium supplemented with 10% dialysed fetal bovine serum (Invitrogen Corp.). After 1h incubation, 500 μCi of HCl-free [^{32}P]orthophosphate (Amersham-Pharmacia) was added and cells were incubated for an additional 4 h at 37ºC before lysis.

Cell staining and microscopy

To determine the subcellular localization of GFP fusion proteins and ERK3, cells were seeded onto a chambered coverglass (Nalgene Nunc International) at a density of $3x10^4$ cells per well and incubated at 37ºC in 5% CO_2. After 24h cells were transfected with expression vectors encoding the appropriate MK5 EGFP fusion proteins (50 ng per well) with or without 450 ng of the indicated pSG5-ERK3 expression constructs. After 24h the cells were simultaneously fixed and permeabilized using 4% paraformaldehyde (PFA) and 0.3% Triton X-100 for 5 min and 4% PFA for 20 min. Fixed cells were then incubated for 2 h with 3% Bovine Serum Albumin in phosphate buffered saline at room temperature before incubation with anti-ERK3 antibody at a final concentration of 2 μg/ml for 1 h at room temperature. Immunostaining was developed using an Alexa594 conjugated anti-sheep IgG (Molecular probes) at a dilution of 1:400. The nuclei of cells were visualized by staining with DRAQ5 (Biostatus Ltd, Leicestershire, UK). Images were collected using a Zeiss LSM510 confocal laser-scanning microscope and processed using Adobe Photoshop 7.0. For cell counting experiments several fields of cells from each transfection or co-transfection were examined using a Leitz DMIRB inverted microscope and at least one hundred cells were scored for the subcellular localization of the protein(s) of interest.

Generation of *Erk3* deficient MEFs

The mouse *Erk3* gene (Turgeon et al., 2002) was disrupted by replacing the coding region of exon 2 with the β-galactosidase gene by homologous recombination in embryonic stem cells. Correct targeting of the *Erk3* locus was confirmed by Southern analysis of

genomic DNA. Details of the procedure and analysis of the resulting phenotype will be described elsewhere (Turgeon B and Meloche S, manuscript in preparation). Primary embryonic fibroblasts were prepared from E14.5 embryos obtained from intercrosses of *Erk3* heterozygous mice. Each embryo was dissected separately, and the head, limbs and internal organs were removed. The remaining carcasses were rinsed in phosphate-buffered saline and minced. After serial digestions with a solution of trypsin-EDTA, the cell suspension was filtered using a cell strainer. The cells were cultured in DMEM supplemented with 10% fetal bovine serum, glutamine, and penicillin/streptomycin. The experiments described here were performed on MEFs before passage 6.

Supplementary References

Coulombe, P., Rodier, G., Pelletier, S., Pellerin, J. and Meloche, S. (2003) Rapid turnover of extracellular signal-regulated kinase 3 by the ubiquitin-proteasome pathway defines a novel paradigm of mitogen-activated protein kinase regulation during cellular differentiation. *Mol Cell Biol*, **23**, 4542-4558.

Groom, L.A., Sneddon, A.A., Alessi, D.R., Dowd, S. and Keyse, S.M. (1996) Differential regulation of the MAP, SAP and RK/p38 kinases by Pyst1, a novel cytosolic dual-specificity phosphatase. *Embo J*, **15**, 3621-3632.

Jiang, Y., Chen, C., Li, Z., Guo, W., Gegner, J.A., Lin, S. and Han, J. (1996) Characterization of the structure and function of a new mitogen-activated protein kinase (p38beta). *J Biol Chem*, **271**, 17920-17926.

Li, Z., Jiang, Y., Ulevitch, R.J. and Han, J. (1996) The primary structure of p38 gamma: a new member of p38 group of MAP kinases. *Biochem Biophys Res Commun*, **228**, 334-340.

McCutchan, J.H. and Pagano, J.S. (1968) Enchancement of the infectivity of simian virus 40 deoxyribonucleic acid with diethylaminoethyl-dextran. *J Natl Cancer Inst*, **41**, 351-357.

Seternes, O.M., Johansen, B., Hegge, B., Johannessen, M., Keyse, S.M. and Moens, U. (2002) Both binding and activation of p38 mitogen-activated protein kinase (MAPK) play essential roles in regulation of the nucleocytoplasmic distribution of MAPK-activated protein kinase 5 by cellular stress. *Mol Cell Biol*, **22**, 6931-6945.

Seternes, O.M., Johansen, B. and Moens, U. (1999) A dominant role for the Raf-MEK pathway in forskolin, 12-O-tetradecanoyl-phorbol acetate, and platelet-derived growth factor-induced CREB (cAMP-responsive element-binding protein) activation, uncoupled from serine 133 phosphorylation in NIH 3T3 cells. *Mol Endocrinol*, **13**, 1071-1083.

Slack, D.N., Seternes, O.M., Gabrielsen, M. and Keyse, S.M. (2001) Distinct binding determinants for ERK2/p38alpha and JNK map kinases mediate catalytic activation and substrate selectivity of map kinase phosphatase-1. *J Biol Chem*, **276**, 16491-16500.

Turgeon, B., Lang, B.F. and Meloche, S. (2002) The protein kinase ERK3 is encoded by a single functional gene: genomic analysis of the ERK3 gene family. *Genomics*, **80**, 673-680.

Waskiewicz, A.J., Flynn, A., Proud, C.G. and Cooper, J.A. (1997) Mitogen-activated protein kinases activate the serine/threonine kinases Mnk1 and Mnk2. *Embo J*, **16**, 1909-1920.