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Mitogen- and stress-activated protein kinase-1 (MSK1) is directly activated by MAPK and SAPK2/p38, and may mediate activation of CREB

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We have identified a novel mitogen- and stress-activated protein kinase (MSK1) that contains two protein kinase domains in a single polypeptide. MSK1 is activated *in vitro* by MAPK2/ERK2 or SAPK2/p38. Endogenous MSK1 is activated in 293 cells by either growth factor/phorbol ester stimulation, or by exposure to UV radiation, and oxidative and chemical stress. The activation of MSK1 by growth factors/phorbol esters is prevented by PD 98059, which suppresses activation of the MAPK cascade, while the activation of MSK1 by stress stimuli is prevented by SB 203580, a specific inhibitor of SAPK2/p38. In HeLa, PC12 and SK-N-MC cells, PD 98059 and SB 203580 are both required to suppress the activation of MSK1 by TNF, NGF and FGF, respectively, because these agonists activate both the MAPK/ERK and SAPK2/p38 cascades. MSK1 is localized in the nucleus of unstimulated or stimulated cells, and phosphorylates CREB at Ser133 with a K_m value far lower than PKA, MAPKAP-K1(p90Rsk) and MAPKAP-K2. The effects of SB 203580, PD 98059 and Ro 318220 on agonist-induced activation of CREB and ATF1 in four cell-lines mirror the effects of these inhibitors on MSK1 activation, and exclude a role for MAPKAP-K1 and MAPKAP-K2/3 in this process. These findings, together with other observations, suggest that MSK1 may mediate the growth-factor and stress-induced activation of CREB.

Keywords: CREB/MAP kinase/MSK1/p38 MAP kinase/signal transduction

Introduction

Ten mitogen-activated protein kinase (MAPK) family members have been identified in mammalian cells (reviewed in Cohen, 1997). Two of these, MAPK1/ERK1 and MAPK2/ERK2, are strongly activated by polypeptide growth factors whose receptors are protein tyrosine kinases, and by tumour-promoting phorbol esters. They are more weakly activated (in most cell contexts) by stress stimuli and proinflammatory cytokines. In contrast, the other MAPK family members are strongly activated by stress signals and proinflammatory cytokines, but only weakly (in most cell contexts) by poly-peptide growth factors and phorbol esters. For this reason, they are frequently referred to as stress-activated protein kinases (SAPKs).

A major challenge in this field is to identify the physiological substrates and roles of each of the MAPKs and SAPKs, but a problem is presented by the finding that a number of the substrates are themselves protein kinases which are likely to have numerous physiological roles. Thus, MAPK1/ERK1 and MAPK2/ERK2 activate three closely related protein kinases known as MAPK-activated protein kinases-1a, -1b and -1c (MAPKAP-K1a/b/c; also known as RSK1/2/3) (Sturgill *et al*., 1988; Zhao *et al*., 1995), while SAPK2a/p38 and SAPK2b/p38β activate two closely related enzymes termed MAPKAP-K2 (Freshney *et al*., 1994; Rouse *et al*., 1994) and MAPKAP-K3 (Clifton *et al*., 1996; McLaughlin *et al*., 1996). Several lines of evidence indicate that the MAPKAP-K1 isoforms are *in vivo* substrates for the MAPK/ERKs and that MAPKAP-K2/K3 are *in vivo* substrates for the SAPK2/p38 isoforms. For example, the drug PD 98059, which suppresses the activation of MAPK/ERKs by preventing activation of their upstream activator MAPK kinase-1 (MKK1), also inhibits activation of the MAPKAP-K1 isoforms (Alessi *et al*., 1995), but not MAPKAP-K2/K3 (Clifton *et al*., 1996). Conversely, the drug SB 203580, a specific inhibitor of SAPK2a/p38 and SAPK2b/p38β, prevents the activation of MAPKAP-K2/K3 (Cuenda *et al*., 1995; Clifton *et al*., 1996).

The MAPKAP-K1 isoforms are unusual in that they each contain two protein kinase domains within a single polypeptide, and one role of the C-terminal kinase domain is to activate the N-terminal kinase domain, allowing the latter to phosphorylate exogenous substrates (Bjorbaek *et al*., 1995; Vik and Ryder, 1997; Dalby *et al*., 1998). The phorbol-ester-induced activation of MAPKAP-K1a in COS1 cells is accompanied by the phosphorylation of six residues (Ser222, Thr360, Ser364, Ser381, Thr574 and Ser733), four of which (Ser222, Ser364, Ser381 and Thr574) are critical for activation. MAPK/ERKs phosphorylate Thr574 in the C-terminal domain and Thr360 and Ser364, which are located between the two kinase domains. The phosphorylation of Thr574 activates the C-terminal kinase domain, which then phosphorylates Ser381. The combined phosphorylation of Ser364 and Ser381 triggers activation of the N-terminal domain, provided that Ser222 is also phosphorylated (Dalby *et al*., 1998). The identity of the Ser222 kinase is unclear.

The importance of MAPKAP-K1 in cell function is indicated by the finding that inactivating mutations in the MAPKAP-K1b (RSK2) gene are the cause of Coffin-Lowry Syndrome, a disease associated with progressive skeletal abnormalities and severe mental retardation (Trivier *et al*., 1996). However, although the MAPKAP-K1 isoforms phosphorylate many proteins *in vitro*, their physiological role(s) has yet to be defined. MAPKAP-K1

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phosphorylates the transcription factor CREB *in vitro* and at the residue (Ser133) known to trigger activation *in vivo* (Xing *et al*., 1996). MAPKAP-K1b is also reported to be the major kinase acting on CREBtide (a synthetic peptide corresponding to the sequence surrounding Ser133) that can be detected in lysates prepared from NGF-stimulated PC12 cells (Ginty *et al*., 1994; Xing *et al*., 1996). Moreover, the phosphorylation of CREB at Ser133 is induced by signals that activate the MAPK/ERK cascade and prevented by PD 98059 (Pende *et al*., 1997). These findings suggest that CREB may be a physiological substrate for MAPKAP-K1, but the possibility that CREB is phosphorylated by another protein kinase that lies downstream of MAPK/ERKs is not excluded.

MAPKAP-K2/K3 also phosphorylates CREB at Ser133 *in vitro* (Tan *et al*., 1996) and MAPKAP-K2 is the major CREBtide kinase detected in lysates prepared from SK-N-MC cells that have been stimulated with fibroblast growth factor or stressed by incubation with sodium arsenite (Tan *et al*., 1996). Moreover, these stimuli induce the phosphorylation of CREB at Ser133 in SK-N-MC cells and phosphorylation is suppressed by SB 203580 (Tan *et al*., 1996). These findings suggest that CREB may be a physiological substrate for MAPKAP-K2/K3, but the possibility that CREB is phosphorylated by another protein kinase downstream of SAPK2/p38 is not excluded by these data.

MAPK1/ERK1 and MAPK2/ERK2 phosphorylate MAPKAP-K1 (but not MAPKAP-K2/K3) isoforms *in vivo*, and SAPK2a/p38 and SAPK2b/p38β phosphorylate MAPKAP-K2/K3 (but not MAPKAP-K1) *in vivo*. More recently however, two closely related protein kinases have been identified that are activated *in vitro* and *in vivo* by both MAPK/ERKs and SAPK2/p38. For these reasons they have been termed MAPK-integrating kinases-1 and -2 (MNK1 and MNK2, respectively) (Fukunaga and Hunter, 1997; Waskiewicz *et al*., 1997). Like MAPKAP-K2/K3, MNK1 and MNK2 are single kinase domain enzymes. One physiological substrate of MNK1 may be the protein synthesis initiation factor eIF4E. MNK phosphorylates eIF4E at Ser209 *in vitro* (Waskiewicz *et al*., 1997), the residue whose phosphorylation is induced by growth factors or phorbol esters *in vivo*. Growth-factor-induced phosphorylation of Ser209 is prevented by PD 98059, while stress-induced phosphorylation of Ser209 is suppressed by SB 203580 (Wang *et al*., 1998).

In this paper we describe the identification and characterization of two novel protein kinases, that resemble the MAPKAP-K1 isoforms in containing two protein kinase domains within a single polypeptide. However, unlike MAPKAP-K1 (but like MNKs) they are activated *in vitro* and *in vivo* by either MAPK/ERKs or SAPK2/p38, and for this reason they have been termed mitogen- and stress-activated protein kinases-1 and -2 (MSK1 and MSK2, respectively). We also present evidence which suggests that MSK1 (and/or MSK2), rather than MAPKAP-K1 or MAPKAP-K2/K3, mediates activation of the transcription factors CREB and ATF1 by either growth factors or stress signals.

Results

Identification of MSK1 as a novel MAPKAP-K1-related kinase

In order to identify novel members of the protein kinase subfamily that are related to p70 S6 kinase, we used the DNA sequence encoding the N-terminal kinase domain of MAPKAP-K1 to interrogate the NCBI EST database. This search identified one EST (AA15 8571) which upon sequencing was found to encode a full-length cDNA clone of a novel member of this subfamily, hereafter termed MSK1. The open reading frame encoded a protein of 802 amino acids with a molecular mass of 89.9 kDa. There is a stop codon immediately 5′ to the predicted initiating ATG codon. The MSK1 polypeptide possessed two protein kinase domains (Figure 1), both of which contained the 11 subdomains characteristic of all protein kinases (Hanks *et al*., 1988). MSK1 showed greatest similarity to the three isoforms of MAPKAP-K1, which also possess two kinase domains (Figure 2A). The N-terminal and C-terminal kinase domains of MSK1 were 54 and 44% identical to the corresponding kinase domains of MAPKAP-K1. The overall identity between MAPKAP-K1 and MSK1 was 43%. We identified 14 human EST clones encoding fragments of MSK1 that are derived from many tissues (Table I), indicating that MSK1 is a widely expressed enzyme. Northern blot analysis of human tissues revealed that MSK1 was expressed as a 4 kb transcript in all tissues examined (heart, brain, placenta, lung, liver, kidney and pancreas) with the highest levels observed in brain, muscle and placenta (data not shown). An alignment of the N- and C-terminal kinase domains of MSK1 with other protein kinases that are activated by MAPK family members is shown in Figure 2B.

We also found EST cDNA clones (Table I) encoding a further protein kinase whose amino acid sequence was 75% identical to MSK1, but only 40% identical to MAPKAP-K1, and which we have termed MSK2. The near full-length coding sequence of murine MSK2 and the partial sequence of human MSK2 aligned with MSK1 are presented in Figure 2C. The murine and human MSK2 sequences share 90% amino acid sequence identity. We identified eight human EST cDNA clones encoding fragments of MSK2 that were derived from a number of cells and tissues. Six of these were tumour cell lines. Northern blot analysis of human tissues revealed that MSK2 was expressed as a 3 kb transcript with a similar distribution to that of MSK1 (results not shown).

Activation of MSK1 in vitro by MAPK2/ERK2 and SAPK2/p38

The four key activating phosphorylation sites present in MAPKAP-K1a are conserved in MSK1 and MSK2 (Figure 2). Two of the four sites in MSK1 (Ser360 and Thr581) are followed by proline residues suggesting that MSK1 and MSK2 may be activated by one or more MAPK. In order to compare the activation of MSK1 and MAPKAP-K1a by MAPKs, both enzymes were expressed in human embryonic kidney 293 cells as fusion proteins with glutathione *S*-transferase (GST) at the N-terminus (hereafter termed GST–MSK1 and GST–MAPKAP-K1a). Both proteins were purified on glutathione–Sepharose and showed a single major Coomassie Blue-staining band when subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE; Figure 3A). The apparent molecular mass of GST–MSK1 estimated by SDS–PAGE (116 kDa) was slightly larger than GST–MAPKAP-K1a

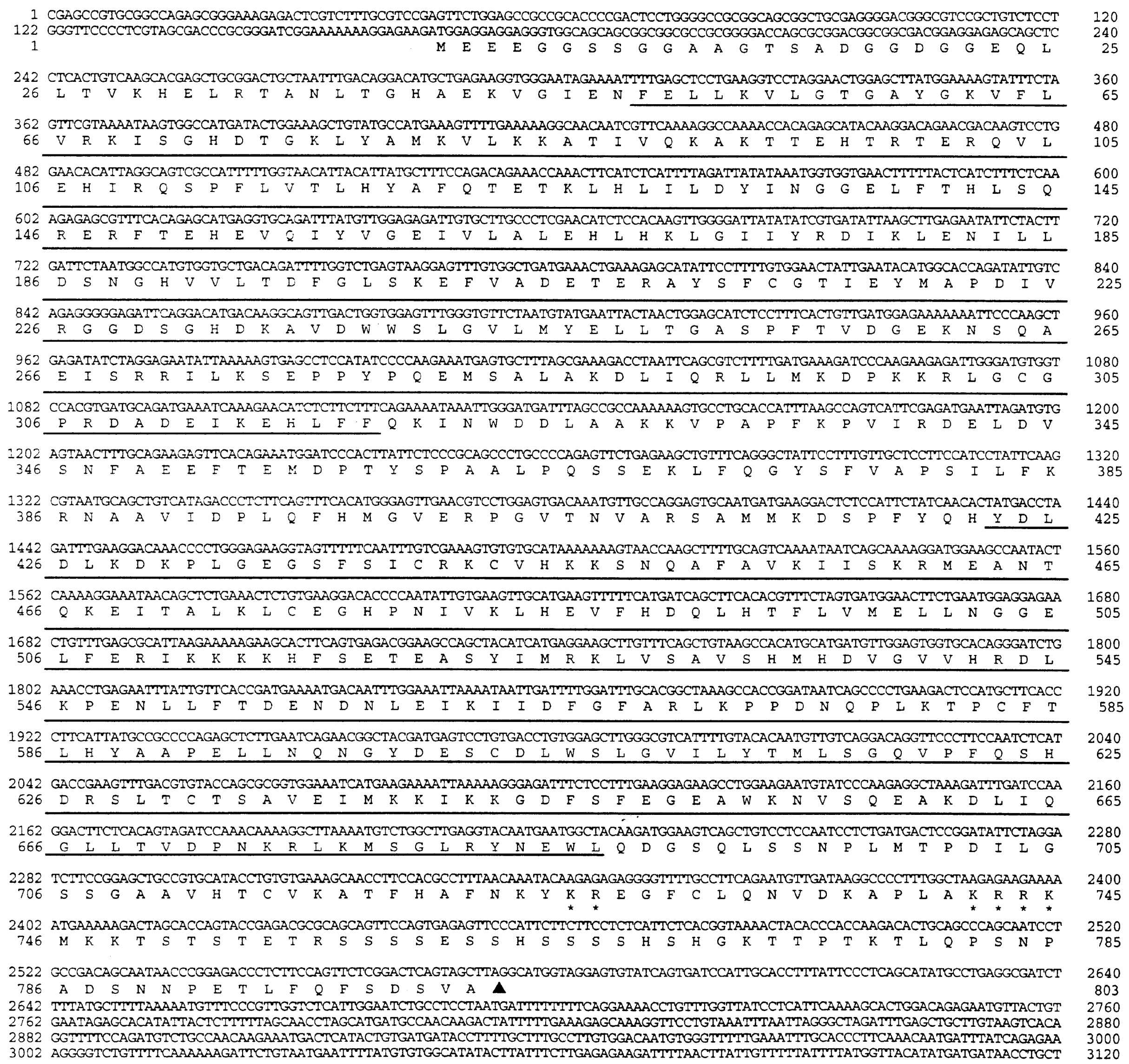

Fig. 1. Nucleotide and deduced amino acid sequence of human MSK1. The underlined residues correspond to the kinase domains. The putative bipartate nuclear localization signal (Robins *et al.*, 1991) between residues 726 and 745 (which is not present in MAPKAP-K1a/b) is denoted with asterisks. The stop codon is denoted by a solid triangle. The DDBJ/EMBL/GenBank accession number for MSK1 is AF074393.

(Figure 3A), which is consistent with the latter enzyme being 67 amino acids shorter (Figure 2A).

MAPKAP-K1 is known to have a high activity towards the peptide named Crosstide (GRPRTSSFAEG) (Alessi *et al.*, 1996a). GST–MSK1 and GST–MAPKAP-K1a purified from 293 cells that had been serum-starved overnight possessed a low activity towards this substrate (2–4 U/mg), which was enhanced >100-fold by incubation with MgATP and activated MAPK2/ERK2 (Figure 3B and C). GST–MSK1 could also be similarly activated by SAPK2a/p38 and SAPK2b/p38β (Figure 3B), whereas MAPKAP-K1a could not (Figure 3C). SAPK1/JNK1γ and SAPK3/p38γ did not activate either GST–MSK1 or GST–MAPKAP-K1a. SAPK4/p38δ was a weak activator of GST–MSK1 and did not activate GST–MAPKAP-K1a. The ability of MAPK2/ERK2 and SAPK2/p38 (Figure 3D) to activate GST–MSK1 correlated with the extent of phosphorylation of this enzyme.

Endogenous MSK1 is activated in vivo by EGF and TPA through the MAPK/ERK pathway and by stressful stimuli through the SAPK2/p38 pathway

The experiments presented in Figure 3 raised the possibility that MSK1 might be activated in response to stimuli that activate the SAPK2/p38 isoforms, as well as by stimuli that activate the classical MAPK/ERK cascade. In order to examine this possibility three MSK1 antibodies were raised, one against residues 26–44 (antibody 'A'), a second against residues 384–402 (antibody 'B') and a third against residues 716–734 (antibody 'C'). All three antibodies immunoprecipitated MSK1 quantitatively (not shown) and immunoprecipitation of expressed MSK1 (Figure 4A) or endogenous MSK1 in cell lysates (not shown) was prevented by incubation with the appropriate peptide immunogen.

The MAPKAP-K1 antibody used in this study immunoprecipitated both MAPKAP-K1a and MAPKAP-K1b

A

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MSK1                           MEEEGGSSGGAAGTSADGGDGGEQLLTVKHELRTANLTGHA----EKVGIENFELLKVLGTGAYGKVFLVRKISGHDTGKLYAMKVLKKATIVQKAKTTE 96
MAPKAP-K1a           MPLAQLKEPWPLMELVPLDPENGQTSGEEAGLQPSKDEGVLKEISITHHVKAGSEKADPSHFELLKVLGQGSFGKVFLVRKVTRPDSGHLYAMKVLKKATL--KVRDRV 107
MAKKAP-K1b    MPLAQLADPWQKMAVESPSDSAENGQQIMDEPMGEEEINPQTEEVSIKEIAITHHVKEGHEKADPSQFELLKVLGQGSFGKVFLVKKISGSDARQLYAMKVLKKATL--KVRDRV 113
MAPKAP-K1c                  MDLSMKKFAVRRFFSVYLRRKSRSKSSSLSRLEEEGVVKEIDISHHVKEGFEKADPSQFELLKVLGQGSYGKVFLVRKVKGSDAGQLYAMKVLKKATL--KVRDRV 104
                                                                                                                                  *
MSK1        HTRTERQVLEHIRQSPFLVTLHYAFQTETKLHLILDYINGGELFTHLSQRERFTEHEVQIYVGEIVLALEHLHKLGIIYRDIKLENILLDSNGHVVLTDFGLSKEFVADETERAYSFCGT 216
MAPKAP-K1a  RTKMERDILADVNH-PFVVKLHYAFQTEGKLYLILDFLRGGDLFTRLSKEVMFTEEDVKFYLAELALGLDHLHSLGIIYRDLKPENILLDEEGHIKLTDFGLSKEAIDHEK-KAYSFCGT 225
MAKKAP-K1b  RTKMERDILVEVNH-PFIVKLHYAFQTEGKLYLILDFLRGGDLFTRLSKEVMFTEEDVKFYLAELALALDHLHSLGIIYRDLKPENILLDEEGHIKLTDFGLSKESIDHEK-KAYSFCGT 231
MAPKAP-K1c  RSKMERDILAEVNH-PFIVKLHYAFQTEGKLYLILDFLRGGDLFTRLSKEVMFTEEDVKFYLAELALALDHLHSLGIIYRDLKPENILLDEEGHIKITDFGLSKEAIDHDK-RAYSFCGT 222

MSK1        IEYMAPDIVRGGDSGHDKAVDWWSLGVLMYELLTGASPFTVDGEKNSQAEISRRILKSEPPYPQEMSALAKDLIQRLLMKDPKKRLGCGPRDADEIKEHLFFQKINWDDLAAKKVPAPFK 336
MAPKAP-K1a  VEYMAPEVVN--RQGHSHSADWWSYGVLMFEMLTGSLPFQGKDRKETMTLI----LKAKLGMPQFLSTEAQSLLRALFKRNPANRLGSGPDGAEEIKRHVFYSTIDWNKLYRREITPPFK 339
MAKKAP-K1b  VEYMAPEVVN--RRGHTQSADWWSFGVLMFEMLTGTLPFQGKDRKETMTMI----LKAKLGMPQFLSPEAQSLLRMLFKRNPANRLGAGPDGVEEIKRHSFFSTIDWNKLYRREIHPPFK 345
MAPKAP-K1c  IEYMAPEVVN--RRGHTQSADWWSFGVLMFEMLTGSLPFQGKDRKETMALI----LKAKLGMPQFLSGEAQSLLRALFKRNPCNRLGAGIDGVEEIKRHPFFVTIDWNTLYRKEIKPPFK 336
                               *                *
MSK1        PVIRDELDVSNFAEEFTEMDPTYSPAALP-QSSEKLFQGYSFVAPSILFKRNAAVIDPLQFHMGVERPGVTNVARSAMMKDSPFYQHYDLDLKDKPLGEGSFSICRKCVHKKSNQAFAVK 455
MAPKAP-K1a  PAVAQPDDTFYFDTEFTSRTPKDSPGIPPSAGAHQLFRGFSFVATGLMEDDGKPRAPQAPLHS---------VVQQLHGKNLVFSDGYVVK---ETIGVGSYSECKRCVHKATNMEYAVK 447
MAKKAP-K1b  PATGRPEDTFYFDPEFTAKTPKDSPGIPPSANAHQLFRGFSFVAITSDDE--SQAMQTVGVHS---------IVQQLHRNSIQFTDGYEVK---EDIGVGSYSVCKRCIHKATNMEFAVK 451
MAPKAP-K1c  PALGRPEDTFHFDPEFTARTPTDSPGVPPSANAHHLFRGFSFVASSLIQEPSQQDLHKVPVHP---------IVQQLHGNNIHFTDGYEIK---EDIGVGSYSVCKRCVHKATDTEYAVK 444

MSK1        IISKRMEANTQKEITALKLCEGHPNIVKLHEVFHDQLHTFLVMELLNGGELFERIKKKKHFSETEASYIMRKLVSAVSHMHDVGVVHRDLKPENLLFTDENDN-LEIKIIDFGFARLKPP 574
MAPKAP-K1a  VIDK-SKRDPSEEIEIEILLRYGQHPNIITLKDVYDDGKHVYLVTELMRGGELLDKILRQKFFSEREASFVLHTIGKTVEYLHSQGVVHRDLKPSNILYVDESGNPECLRICDFGFAKQLRA 566
MAKKAP-K1b  IIDK-SKRDPTEEIEILLRYGQHPNIITLKDVYDDGKYVYVVTELMKGGELLDKILRQKFFSEREASAVLFTITKTVEYLHAQGVVHRDLKPSNILYVDESGNPESIRICDFGFAKQLRA 570
MAPKAP-K1c  IIDK-SKRDPSEEIEILLRYGQHPNIITLKDVYDDGKFVYLVMELMRGGELLDRILRQRYFSEREASDVLCTITKTMDYLHSQGVVHRDLKPSNILYRDESGSPESIRVCDFGFAKQLRA 563
               *
MSK1        DNQPLKTPCFTLHYAAPELLNQNGYDESCDLWSLGVILYTMLSGQVPFQSHDRSLTCTSAVEIMKKIKKGDFSFEGEAWKNVSQEAKDLIQGLLTVDPNKRLKMSGLRYNEWLQDGSQLS 694
MAPKAP-K1a  ENGLLMTPCYTANFVAPEVLKRQGYDEGCDIWSLGILLYTMLAGYTPFANGPSD----TPEEILTRIGSGKFTLSGGNWNTVSETAKDLVSKMLHVDPHQRLTAKQVLQHPWVTQKDKLP 682
MAKKAP-K1b  ENGLLMTPCYTANFVAPEVLKRQGYDAACDIWSLGVLLYTMLTGYTPFANGPDD----TPEEILARIGSGKFSLSGGYWNSVSDTAKDLVSKMLHVDPHQRLTAALVLRHPWIVHWDQLP 686
MAPKAP-K1c  GNGLLMTPCYTANFVAPEVLKRQGYDAACDIWSLGILLYTMLAGFTPFANGPDD----TPEEILARIGSGKYALSGGNWDSISDAAKDVVSKMLHVDPHQRLTAMQVLKHPWVVNREYLS 679

MSK1        SNPLMTP-DILGSSGAAVHTCVKATFHAFNKYKREGFCLQNVDKAPLAKRRKMKKTSTSTETRSSSSESSHSSSSHSHGKTTPTKTLQPSNPADSNNPETLFQFSDSVA 802
MAPKAP-K1a  QSQLSHQ-DLQLVKGAMA-----ATYSALNSS-KPTPQLKPIESSILAQRR-VRKLPSTTL 735
MAKKAP-K1b  QYQLNRQDAPHLVKGAMA-----ATYSALNRN--QSPVLEPVGRSTLAQRRGIKKITSTAL 740
MAPKAP-K1c  PNQLSRQD-VHLVKGAMA-----ATYFALNRTP-QAPRLEPVLSSNLAQRRGMKRLTSTRL 733
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B

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MSK1-N     (46)  IENFELLKVLGTGAYGKVFLVRKISGHDTGKLYAMKVLKKATIVQKAKTTEHTRTERQVLEHIRQSPFLVTLHYAFQTETK----LHLILDYINGGELFTHLSQR--ERFTEHEVQIYVG
MSK1-C    (423)  YDLDLKDKPLGEGSFSICRKCVHKK---SNQAFAVKIISKRM---EANT----QKEITALKLCEGHPNIVKLHEVFHDQLH----TFLVMELLNGGELFERIKKK--KHFSETEASYIMR
MAPKAP-K2 (43)   YKVT--SQVLGLGINGKVLQIFNKR---TQEKFALKMLQDCP---KA------RREVELHWRASQCPHIVRIVDVYENLYAGRKCLLIVMECLDGGELFSRIQDRGDQAFTEREASEIMK
MAPKAP-K3 (63)   YQLS--KQVLGLGVNGKVLECFHRR---TGQKCALKLLYDSP---KA------RQEVDHHWQASGGPHIVCILDVYENMHHGKRCLLIIMECMEGGELFSRIQERGDQAFTEREAAEIMR
MNK1      (48)   YKLT--SELLGEGAYAKVQGAVSLQ---NGKEYAVKIIEKQAGHSRSRV----FREVETLYQCQGNKNILELIEFFEDDTR----FYLVFEKLQGGSILAHIQKQ--KHFNEREASRVVR

MSK1-N           EIVLALEHLHKLGIIYRDIKLENILLDS---NGHVVLTDFGLSKEFVADET------ERAYSFCGTIEYMAPDIVRGGDSG---HDKAVDWWSLGVLMYELLTGASPFT-VDGEKNSQ--
MSK1-C           KLVSAVSHMHDVGVVHRDLKPENLLFTDENDNLEIKIIDFGFARLKPPDNQ-------PLKTPCFTLHYAAPELLN--QNG---YDESCDLWSLGVILYTMLSGQVPFQSHDRSLTCTS-
MAPKAP-K2        SIGEAIQYLHSINIAHRDVKPENLLYTSKRPNAILKLTDFGFAKETTSHN--------SLTTPCYTPYYVAPEVLG--PEK---YDKSCDMWSLGVIMYILLCGYPPFYSNHGLAISP--
MAPKAP-K3        DIGTAIQFLHSHNIAHRDVKPENLLYTSKEKDAVLKLTDFGFAKETT-QN--------ALQTPCYTPYYVAPEVLG--PEK---YDKSCDMWSLGVIMYILLCGFPPFYSNTGQAISP--
MNK1             DVAAALDFLHTKGIAHRDLKPENILCESPEKVSPVKICDFDLGSGMKLNNSCTPITTPELTTPCGSAEYMAPEVVEVFTDQATFYDKRCDLWSLGVVLYIMLSGYPPFVGHCGADCGWDR

MSK1-N           --------AEISRRILKSEPPYP----QEMSALAKDLIQRLLMKDPKKRLGCGPRDADEIKEHLFFQKINWDDLAAKK
MSK1-C           -------AVEIMKKIKKGDFSFEGEAWKNVSQEAKDLIQGLLTVDPNKRLKMSGLRYNEWLQDGSQLSSNPLMTPDIL
MAPKAP-K2        G---------MKTRIRMGQYEFPNPEWSEVSEEVKMLIRNLLKTEPTQRMTITEFMNHPWIMQSTKVPQTPLHTSRVL
MAPKAP-K3        G---------MKRRIRLGQYGFPNPEWSEVSEDAKQLIRLLLKTDPTERLTITQFMNHPWINQSMVVPQTPLHTARVL
MNK1             GEVCRVCQNKLFESIQEGKYEFPDKDWAHISSEAKDLISKLLVRDAKQRLSAAQVLQHPWVQG--QAPEKGLPTPQVL
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C

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MSK1   MEEEGGSSGGAAGTSADGGDGGEQLLTVKHELRTANLTGHAEKVGIENFELLKVLGTGAYGKVFLVRKISGHDTGKLYAMKVLKKATIVQKAKTTEHTRTERQVLEHIRQSPFLVTLHYA 120
mMSK2  -------------HASGDEDEDEGCAVELQITEANLTGHEEKVSVENFALLKVLGTGAYGKVFLVRKTGGHDAGKLYAMKVLRKAALVQRAKTQEHTRTERSVLELVRQAPFLVTLHYA 106
MSK2   ------------------------------------------------------------------------------------------------------------------------
                                                                                                        *
MSK1   FQTETKLHLILDYINGGELFTHLSQRERFTEHEVQIYVGEIVLALEHLHKLGIIYRDIKLENILLDSNGHVVLTDFGLSKEFVADETERAYSFCGTIEYMAPDIVRGGDSGHDKAVDWWS 240
mMSK2  FQTDAKLHLILDYVSGGEMFTHLYQRQYFKEAEVRVYGGEIVLALEHLHKLGIIYRDLKLENVLLDSEGHIVLTDFGLSKEFLTEEKERTFSFCGTIEYMAPEIIRS-KAGHGKAVDWWS 225
MSK2   ------------------------------------------------------------------------------------------------------------------------

MSK1   LGVLMYELLTGASPFTVDGEKNSQAEISRRILKSEPPYPQEMSALAKDLIQRLLMKDPKKRLGCGPRDADEIKEHLFFQK-INWDDLAAKKVPAPFKPVIRDELDVSNFAEEFTEMDPTY 359
mMSK2  LGILLFELLTGASPFTLEGERNTQAEVSRRILKCSPPFPLRIGPVAQDLLQRLLCKDPKKRLGAGPQGAQEVKSHPFFRVWTGW-ALAARKIPAPFRPQIRSELDVGNFAEEFTRLEPVY 344
MSK2   ----------------------QTEVSRRILKCSPPFPPRIGPVAQDLLQRLLCKDPKKRLGAGPQGAQEVRNHPFFQGL-DWVALAARKIPAPFRPQIRSELDVGNFAEEFTRLEPVY 96
       *         *
MSK1   SPAALPQSSE-KLFQGYSFVAPSILFKRNAAVI-DPLQFHMGVERPGVTNVARSAMMKDSPFYQHYDLDLKDKPLGEGSFSICRKCVHKKSNQAFAVKIISKRMEANTQKEITALKLCEG 477
mMSK2  SPAGSPPPGDPRIFQGYSFVAPSILFDHNNAVMADVLQAPGAGYRPGRAAVARSAMMQDSPFFQQYELDLREPALGQGSFSVCRRCRQRQSGQEFAVKILSRRLEENTQREVAALRLCQS 464
MSK2   SPPGSPPPGDPRIFQGYSFVAPSILFDHNNAVMTDGLEAPGAGDRPGRAAVARSAMMQDSPFFQQYELDLREPALGQGSFSVCRRCRQRQSGQEFAVKILSRRLEANTQREVAALRLCQS 216
                                                                                                                  *
MSK1   HPNIVKLHEVFHDQLHTFLVMELLNGGELFERIKKKKHFSETEASYIMRKLVSAVSHMHD-VGVVHRDLKPENLLFTDENDNLEIKIIDFGFARLKPPD-NQPLKTPCFTLHYAAPELLN 595
mMSK2  HPNVVNLHEVLHDQLHTYLVLELLRGGELLEHIRKKRLFSESEASQILRSLVSAVSFMHEEAGVVHRDLKPENILYADDTPGAPVKIIDFGFARLRPQSPAEPMQTPCFTLQYAAPELLA 584
MSK2   HPNVVNLHEVHHDQLHTYLVLELLRGGELLEHIRKKRHFSESEASQILRSLVSAVSFMHEEAGVVHRDLKPENILYADDTPGAPVKIIDFGFARLRPQSPGVPMQTPCFTLQYAAPELLA 336

MSK1   QNGYDESCDLWSLGVILYTMLSGQVPFQSHDRSLTCTSAVEIMKKIKKGDFSFEGEAWKNVSQEAKDLIQGLLTVDPNKRLKMSGLRYNEWLQDGSQLSSNPLMTPDILGSSGAAVHTCV 715
mMSK2  QQGYDESCDLWSLGVILYMMLSGQVPFQGASGQGGQSQAAEIMCKIREGRFSLDGEAWQGVSEEAKELVRGLLTVDPAKRLKLEGLRSSSWLQDGSARSSPPLRTPDVLESSGPAVRSGL 704
MSK2   QQGYDESCDLWSLGVILYMMLSGQVPFQGASGQGGQXQAAEIMCKIREGRFSLDGEAWQGVSEEAKELVRGLLTVDPAKRLKLEGLRGSSWLQDGSARSSPPLRTPDVLESSGPAVRSGL 456

MSK1   KATFHAFNKYKREGFCLQNVDKAPLAKRRKMKKTSTSTETRSSSSESSHSSSSHSHGKTTPTKTLQPSNPADSNNPETLFQFSDSVA 802
mMSK2  NATFMAFNRGKREGFFLKSVENAPLAKRRKQKLRSA 740
MSK2   NATFMAFNRGKREGFFLKSVENAPLNCHFMDCLCNYVHQRPVLGVLKERPWGTL 510
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Fig. 2. Alignment of the amino acid sequences of MSK1 with closely related kinases. The alignment was carried out using the Clustal W program (Thompson *et al*., 1994). Identities are shaded in black. (**A**) Alignment of MSK1 with human MAPKAP-K1 isoforms. The residues corresponding to the key activating phosphorylation sites on MAPKAP-K1a are marked with asterisks. (**B**) Alignment of the N-terminal (MSK1-N) and C-terminal (MSK1-C) kinase domains of MSK1 with the kinase domains of MAPKAP-K2/3 and MNK1. There is no significant homology in the non-catalytic regions of these kinases. (**C**) Alignment of human MSK1 with partial mouse (mMSK2) and human MSK2 sequences. The putative activating phosphorylation sites are indicated with asterisks. The DDBJ/EMBL/GenBank accession numbers for murine and human MSK2 are AF074714 and AF074715, respectively. Revised sequences will be deposited as soon as they become available.

Table I. DDBJ/EMBL/GenBank accession numbers

Accession numbers	Tissue from which EST is derived
Human MSK1 ESTs	
AA314565, AA305163	colon carcinoma
AA134359	colon
AA699729, R11235, T97584, T97538	fetal liver spleen
W04930	fetal lung
AA158571	pancreas
AA322270	cerebellum
H09985, F05701	infant brain
N31641, N57096	placenta
Human MSK2 ESTs	
H41647	adult brain
R17109	fat cell
T19765	cardiovascular system
AA505842	breast tumour
AA443601	ovary tumour
AA678670	Gessler Wilms tumour
AA576979	colon tumour
AA857431	pharynx carcinoma
Mouse MSK2 ESTs	
AA472165	mammary gland
AA389168	embryo
AA06016	fetus
AA657108	myotubes

(Figure 4B and C; Alessi *et al.*, 1995), but did not immunoprecipitate MSK1 (Figure 4A). Furthermore, none of the MSK1 antibodies immunoprecipitated MAPKAP-K1a or MAPKAP-K1b (Figure 4B and C). The peptide sequence used to raise antibody A is not conserved in MSK2, and the 19 peptide residues used to raise antibodies B and C possess only nine and 12 conserved residues with MSK2, respectively. All three MSK1 antibodies immunoprecipitated similar levels of MSK1 activity in both 293 cells exposed to EGF, TPA or stresses, or in HeLa cells stimulated with TNF (data not shown). These results indicate that MSK2 is not immunoprecipitated with MSK1.

Endogenous MSK1 was immunoprecipitated with antibody A from the lysates of 293 cells previously stimulated with EGF or TPA, or exposed to cellular stresses (sodium arsenite, UV radiation or hydrogen peroxide). These experiments demonstrated that MSK1 was potently activated by all of these stimuli (Figure 5A). Interestingly, the activation of MSK1 by EGF and TPA was largely prevented by PD 98059, a specific inhibitor of the activation of MKK1 (Alessi *et al.*, 1995), but not by SB 203580, a specific inhibitor of SAPK2a/p38 and SAPK2b/p38β (Cuenda *et al.*, 1995). In contrast, the activation of MSK1 by stressful stimuli was largely inhibited by SB 203580, but not by PD 98059 (Figure 5A). Identical results were obtained when antibody B or antibody C were used instead of antibody A (data not shown).

EGF- and TPA-stimulation of cells potently activated MAPKAP-K1a/b in 293 cells and, like the activation of MSK1, this was largely suppressed by PD 98059, but not by SB 203580 (Figure 5B). Stressful stimuli did not induce significant activation of MAPKAP-K1a/b (Figure 5B) but induced a large activation of MAPKAP-K2 that was prevented by SB 203580, but not by PD 98059 (Figure 5C). MAPKAP-K2 was not activated significantly by EGF or TPA (Figure 5C).

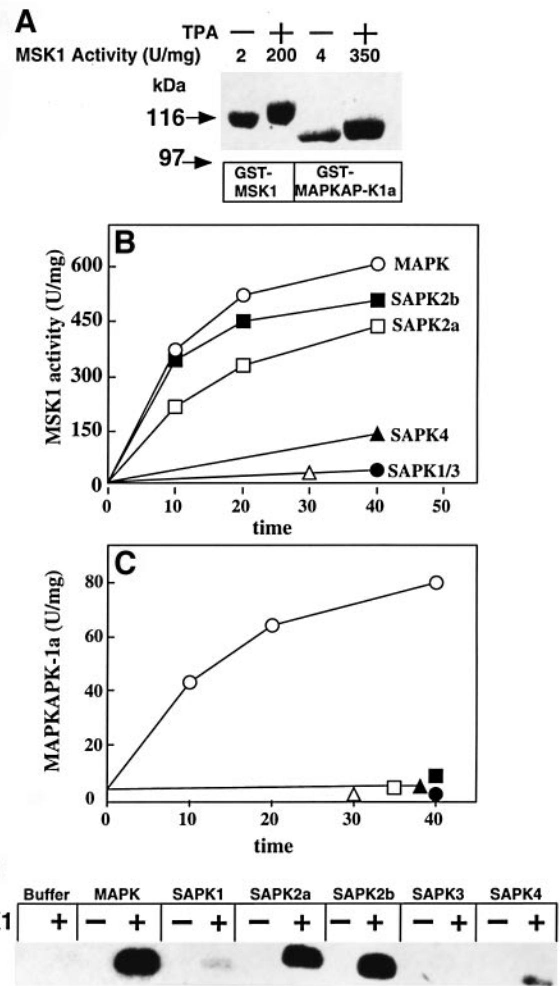


Fig. 3. Purification of GST–MSK1 and activation by MAPK2/ERK2 and SAPK2. (**A**) GST–MSK1 (2 μg protein) and GST–MAPKAP-K1a (2 μg protein) purified from unstimulated and TPA-stimulated cells were electrophoresed on a 7.5% SDS–PAGE and stained with Coomassie Blue. The position of the molecular mass markers, β-galactosidase (116 kDa) and glycogen phosphorylase (97 kDa) are shown. The activity of each protein was assayed using the peptide substrate Crosstide (GRPRTSSFAEG). (**B**) GST–MSK1 or (**C**) GST-MAPKAP-K1a derived from unstimulated cells was incubated with either MAPK2/ERK2 (8 U/ml; open circles), SAPK1/JNK1γ (1 U/ml; closed circles), SAPK2a/p38 (1 U/ml; open squares), SAPK2b/p38β (1 U/ml; closed squares), SAPK3/p38γ (1 U/ml; open traingles) or SAPK4/p38δ (1 U/ml; closed triangles), 10 mM $Mg(Ac)_2$ and 100 μM unlabelled ATP in Buffer B. At the times indicated, aliquots were removed, diluted 10-fold in Buffer B containing 1 mg/ml BSA and assayed for activity towards Crosstide. In parallel experiments the activity of MAPK2/ERK2, and all the SAPK enzymes except SAPK1/JNK1γ were assayed using myelin basic protein. SAPK1/JNK1γ was assayed using ATF2 (data not shown). The results are presented as ± SEM for six determinations (two independent experiments). The error for each point is <15%. (**D**) GST–MSK1 was incubated with MAPK2/ERK2 or SAPK enzymes as above except that [γ-^{32}P]ATP was used. After 20 min, the reactions were terminated by the addition of SDS, the samples electrophoresed on a 7.5% polyacrylamide gel and the Coomassie-Blue-staining bands corresponding to GST–MSK1 autoradiographed. Similar results were obtained in two separate experiments.

The activation of MSK1 by TPA and UV radiation requires both kinase domains

In order to establish which kinase domain of MSK1 was required for its activation *in vivo*, we transfected 293 cells with DNA expression constructs encoding wild-

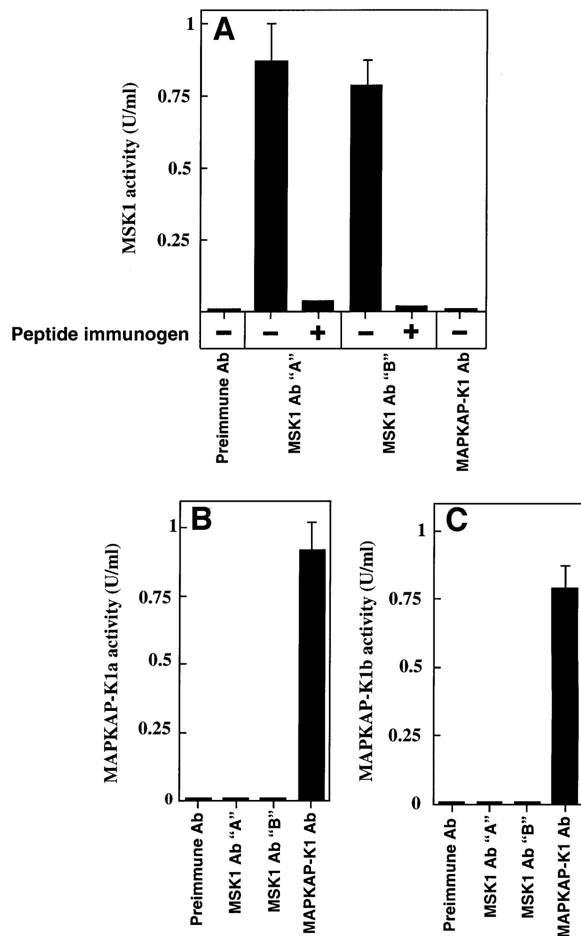


Fig. 4. Generation of antibodies that immunoprecipitate MSK1 specifically. (**A**) Activated GST–MSK1 purified from TPA stimulated 293 cells (50 µl at 1.0 U/ml) was incubated for 30 min at 4°C on a shaking platform with protein G–Sepharose beads (5 µl) coupled to the indicated antibodies (5 µg) in the presence or absence of the indicated peptide immunogens (1 mM), and then centrifuged for 1 min at 13 000 *g*. The beads were washed as described in Materials and methods and assayed for activity towards Crosstide. (B and C) Same as (A), except that activated GST–MAPKAP-K1a derived from TPA-stimulated 293 cells (**B**) or MAPKAP-K1b derived from rabbit muscle (**C**) were immunoprecipitated with the indicated antibodies, and the washed immunoprecipitates assayed for activity towards Crosstide.

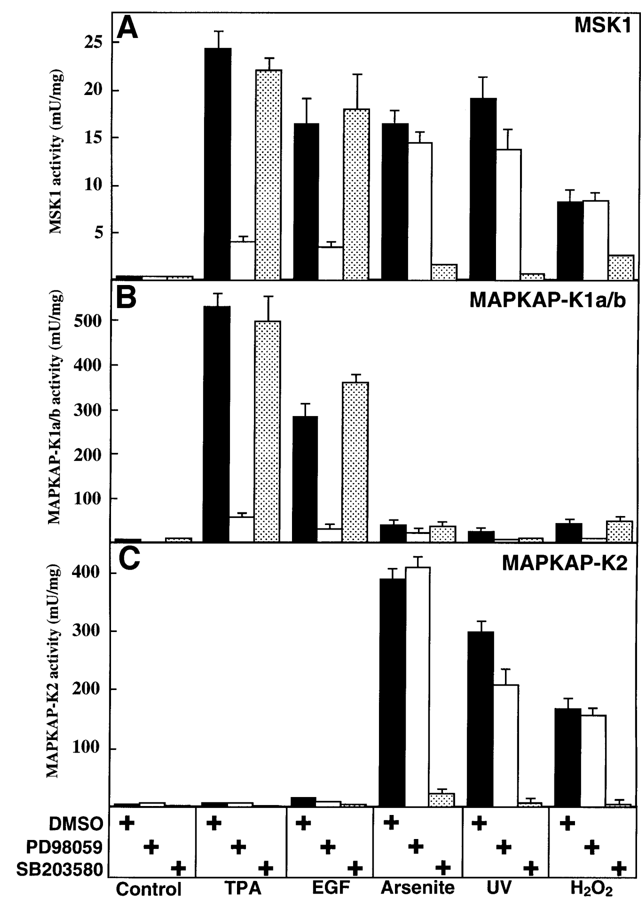


Fig. 5. Activation of endogenous MSK1 by TPA, EGF and cellular stresses. 293 cells were serum-starved for 16 h, and then incubated for 1 h in the presence of 50 µM PD 98059 (open bars), 10 µM SB 203580 (speckled bars) or in the absence of either compound (solid bars). The cells were either left unstimulated (control) or stimulated with TPA (200 ng/ml, 10 min), EGF (100 ng/ml, 10 min), sodium arsenite (0.5 mM, 30 min) or exposed to UV radiation (200 J/m^2 followed by incubation for 30 min at 37°C) or H_2O_2 (2 mM, 30 min) in the continued presence or absence of inhibitors. The cells were lysed and MSK1 (**A**), MAPKAP-K1a/b (**B**) or MAPKAP-K2 (**C**) was immunoprecipitated from the same lysate and assayed. The data are presented as the mean ± SEM for two separate experiments with each determination carried out in triplicate.

type MSK1 (WT-MSK1), a mutant MSK1 in which the N-terminal kinase domain should be inactivated by a point mutation (NT-KD MSK1), and a further mutant in which the C-terminal kinase domain should be inactivated (CT-KD MSK1). All the constructs possessed an N-terminal 'Flag' tag (see Materials and methods) to enable their immunoprecipitation and assay from cell lysates. Stimulation of the cells with TPA or exposure to UV radiation induced 150- and 30-fold activation of the wild-type MSK1, respectively. Similar to the results with the endogenous MSK1, the activation of transfected MSK1 by TPA was prevented by PD 98059 but not by SB 203580, while UV-induced activation was prevented by SB 203580 but unaffected by PD 98059 (Figure 6A). The transfected, wild-type enzyme was also activated 50- to 100-fold by EGF, basic FGF and serum, and activation was prevented by PD 98059 but not SB 203580 (data not shown). These observations confirm the results obtained by immunoprecipitation of the endogenous protein kinase; i.e. that MSK1 can be activated in cells by either the classical MAP kinase cascade or via the SAPK2a/p38 pathway.

In contrast, neither the N-terminal 'kinase-dead' mutant nor the C-terminal kinase-dead mutant of MSK1 possessed detectable activity either before or after cell stimulation with TPA or exposure to UV radiation (Figure 6A). Both of the kinase-dead MSK1 mutants were expressed at the same level as the wild-type MSK1 protein (Figure 6B). These observations also establish that the MSK1 activity measured in Figure 6 is due to MSK1 itself and not a contaminant kinase that is co-immunoprecipitated with MSK1.

MSK1 is localized in nuclei and MAPKAP-K1a in the cytoplasm

The subcellular location of wild-type MSK1 that was overexpressed in 293 cells was investigated by quantitative immunoelectron microscopy (Figure 7). MSK1 was largely

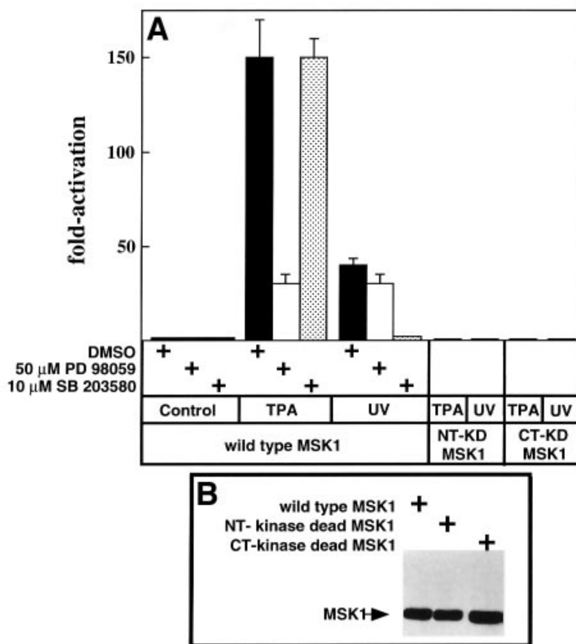


Fig. 6. Effect of mutations on the activation of MSK1 by TPA and UV. 293 cells were transiently transfected with DNA constructs expressing Flag-epitope-tagged wild-type MSK1, N-terminal kinase dead (NT-KD) MSK1 and C-terminal kinase dead (CT-KD) MSK1. The cells were incubated for 1 h with 50 μM PD 98059, 10 μM SB 203580 or in the absence of either compound. They were then stimulated for 10 min with TPA (200 ng/ml) or exposed to UV radiation (200 J/m^2 followed by incubation for 30 min at 37°C) in the continued presence or absence of inhibitors. The cells were lysed and MSK1 immunoprecipitated from the lysates and assayed with Crosstide. The data are presented as the mean ± SEM for three separate experiments with each determination carried out in triplicate. (**B**) 2 μg of protein from each lysate was subjected to electrophoresis on a 10% SDS–PAGE and immunoblotted using monoclonal Flag-antibody. No immunoreactive MSK1 protein was observed in untransfected cells (data not shown). The Flag-epitope-tagged MSK1 comigrates with glycogen phosphorylase (apparent molecular mass 97 kDa).

located in the nucleus of unstimulated cells. The density of MSK1 in the nuclear compartment was 12- to 30-fold higher than in the cytoplasm (Figure 7A and C). Activation of MSK1 by stimulation with TPA (Figure 7A and D) or by exposure to UV radiation (data not shown) did not induce any change in the subcellular location of MSK1. MSK1 possesses a putative bipartate nuclear localization signal (Robins *et al*., 1991) between residues 726 and 748 (Figure 1) that is not present in MAPKAP-K1a/b. Consistent with this observation, MAPKAP-K1a when overexpressed in cells was largely localized in the cytoplasm (Figure 7B). Morover, no significant translocation of MAPKAP-K1a to the nucleus was observed following TPA stimulation of cells (Figure 7B and E) which induces 100-fold activation of this kinase (data not shown).

MSK1 is a highly efficient CREB kinase in vitro

The substrate specificities of MSK1 and MAPKAP-K1a were compared after expression of the GST fusion proteins in 293 cells, followed by TPA stimulation and purification from the cell lysates (Figure 3A). The specific activity of GST–MSK1 at a saturating concentration of Crosstide (GRPRTSSFAEG, peptide 1; Figure 8A) was similar to that of MAPKAP-K1a (see Figure 8 legend). The K_m for Crosstide was 2–3 μM for MSK1, MAPKAP-K1a or MAPKAP-K1b. Crosstide is efficiently phosphorylated by MAPKAP-K1 because it contains two arginines located three and five residues N-terminal to the serine that is phosphorylated. For this reason, the peptide KKRNRTLSVA (peptide 2; Figure 8A) is phosphorylated by MAPKAP-K1a/b with almost identical K_m and V_{max} values as Crosstide. This peptide was found to be phosphorylated even more efficiently by MSK1, the K_m value being 0.2 μM. Changing the arginine at position N-3 in KKRNRTLSVA to lysine (peptide 3; Figure 8A) increased the K_m ~100-fold for MSK1, as well as for MAPKAP-K1a/b. However, changing the arginine at position N-5 to leucine (peptide 4; Figure 8A) did not increase the K_m for MSK1 significantly, although the K_m for MAPKAP-K1a/b was increased 5-fold. These experiments indicate that MSK1 requires an arginine at position N-3, but not a basic residue at N-5.

It has been reported that MAPKAP-K1b (Xing *et al*., 1996) and MAPKAP-K2 (Tan *et al*., 1996) mediate the activation of the transcription factor CREB by growth factors and stressful stimuli, respectively. In view of the predominantly nuclear localization of MSK1, it was of interest to investigate the efficiency with which it phosphorylated CREB compared with these other protein kinases. These experiments led to the surprising finding that CREB is an extremely good substrate for MSK1, being phosphorylated with a K_m of 2 μM and with a V_{max} similar to Crosstide or KKRNRTLSVA. In contrast, CREB was phosphorylated by PKA with a K_m of 17 μM under the same conditions (data not shown), while the K_m was too high to be measured when phosphorylation was catalysed by MAPKAPK1a/b (Figure 8A) or MAPKAP-K2 (data not shown). As a result, when the activities of MSK1, MAPKAP-K1a/b and MAPKAP-K2 were compared with Crosstide and/or the peptide KKLNRTLSVA, the rate of phosphorylation of 5 μM CREB by MSK1 was 30-fold higher than MAPKAP-K1a, 12-fold higher than MAPKAP-K1b and 60-fold higher than MAPKAP-K2 (Figure 8B). In contrast, MAPKAP-K2 was more active towards heat-shock protein 27 (one of its physiological substrates; Cuenda *et al*., 1995) than MSK1 or MAPKAP-K1a/b (Figure 8B).

The residue on CREB phosphorylated by MSK1 was established by tryptic digestion, followed by chromatography of the digest on a C_{18} column. One major ^{32}P-labelled peptide was observed to elute at 15% acetonitrile (Figure 8C). This peptide contained phosphoserine, and when subjected to solid-phase sequencing ^{32}P-radioactivity was released after the third cycle of Edman degradation (data not shown). Its identity was established by MALDI-TOF mass spectrometry which revealed that the molecular weight of the peptide (758.33) was identical to that of the expected tryptic phosphopeptide, comprising residues 131–135, and phosphorylated at Ser133 (calculated mass 758.36). MAPKAP-K1b, which is known to phosphorylate the same residue (Xing *et al*., 1996), labelled the same tryptic peptide as MSK1 (data not shown).

Consistent with the results obtained with the CREB protein, a synthetic peptide (termed CREBtide) corresponding to residues 126–136 of CREB (EILSRRPSYRK, peptide 5; Figure 8A) was phosphorylated by MSK1 with a K_m value too low to be measured ($<$0.1 μM). In contrast,

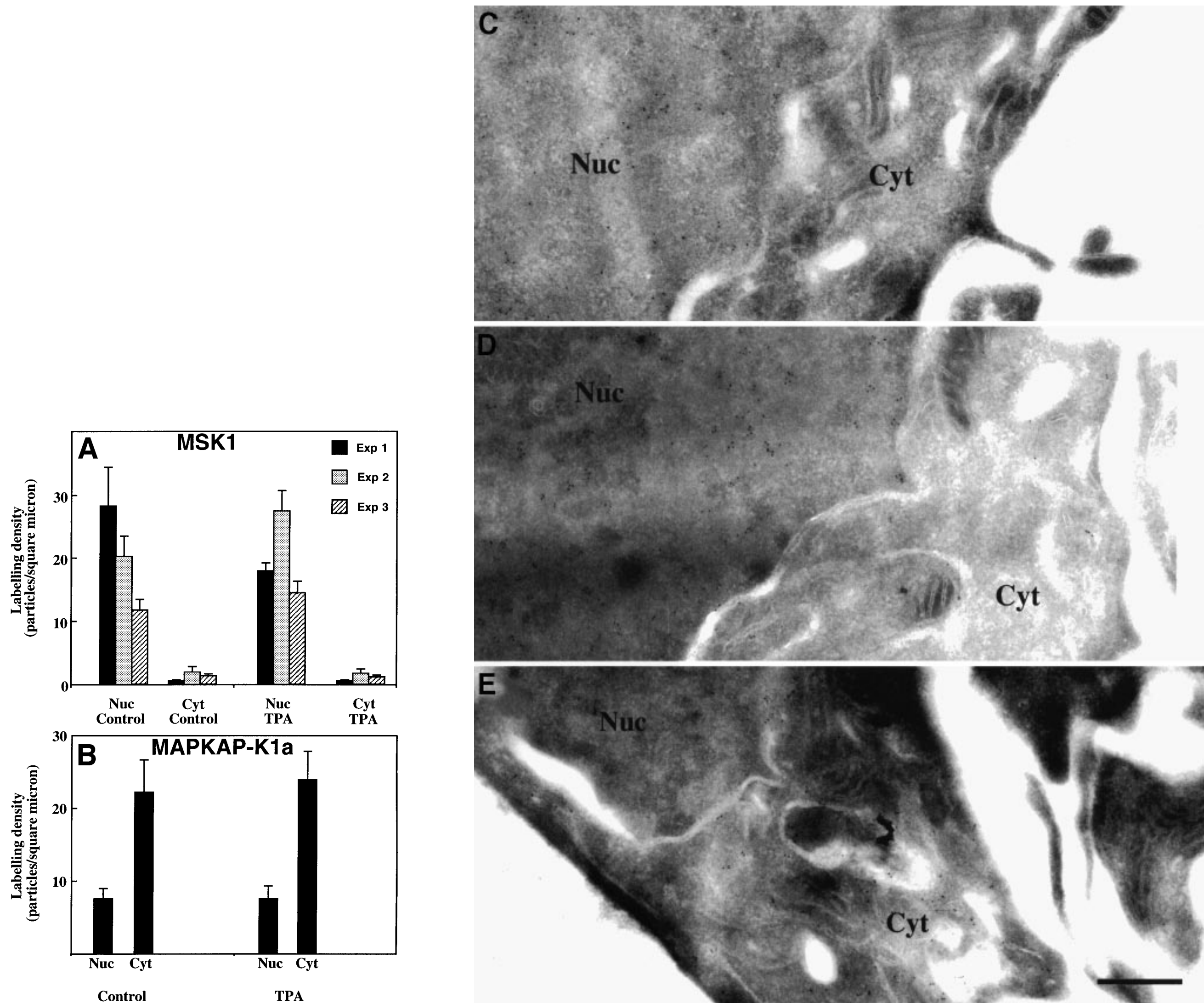


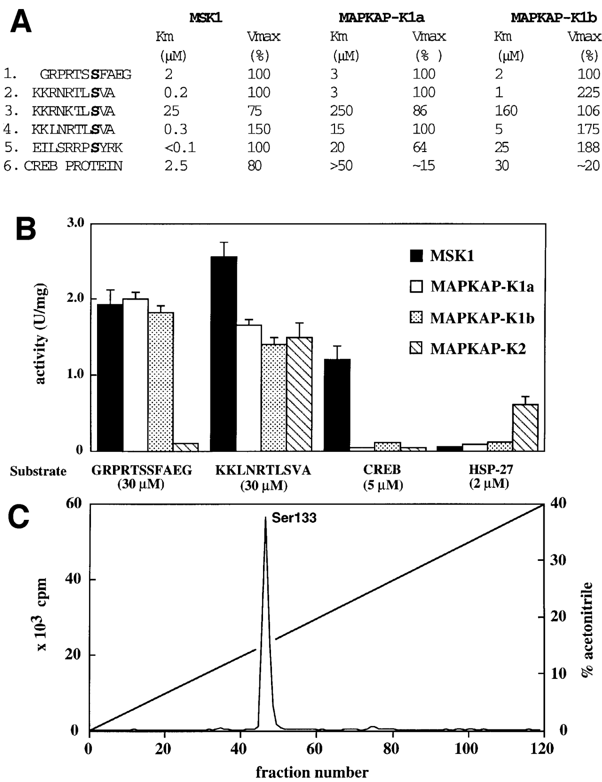
Fig. 7. MSK1 is mainly localized in the nucleus of cells and MAPKAP-K1a in the cytosol. 293 cells expressing N-terminally Flag-epitope-tagged wild-type MSK1 or N-terminally HA-epitope-tagged MAPKAP-K1a were incubated in serum-free medium for 16 h and then either left unstimulated, or stimulated with TPA (100 ng/ml, 10 min) before formaldehyde fixation, sectioning and immunogold labelling for the appropriate tag [Flag for MSK1 (**A**) and HA for MAPKAP-K1a (**B**)]. Quantitation of the concentration of MSK1 and MAPKAP-K1a in the nucleus (Nuc) and cytoplasm (Cyt) of cells was carried out as described in Materials and methods. The results show that TPA stimulation has no detectable effect on MSK1 or MAPKAP-K1a localization. Data is from three independent experiments (for MSK1 controls $n = 9$, 9 and 14; for TPA $n = 10$, 11 and 15). For MAPKAP-K1a controls total $n = 22$; for TPA total $n = 9$ (excluding cells with extensive clumps of immunoreactive material in the cytoplasm). Bars represent standard error of the mean. (**C**) Selected examples for MSK1 (C, unstimulated and **D**, TPA) and for MAPKAP-K1a (**E**, unstimulated) are shown to illustrate structures and labelling distributions.
Bar is 500 nm.

MAPKAP-K1a and MAPKAP-K1b phosphorylated this peptide rather poorly with K_m values that were at least 200-fold higher (Figure 8A).

Evidence that MAPKAP-K2 does not mediate the phosphorylation of CREB and ATF1 by UV radiation or EGF in 293 cells

The staurosporine analogue Ro 318220 inhibits all the PKC isoforms (Davis *et al.*, 1989) as well as MAPKAP-K1a/b, with IC_{50} values ranging from 10 to 30 nM (Alessi, 1997; Figure 9A). In the present work, Ro 318220 was found to be an equally potent inhibitor of MSK1 activity *in vitro* (Figure 9A). Ro 318220 does not inhibit MAPKAP-K2 and MAPKAP-K3 *in vitro*, even at 10 μM (Figure 9A), nor does it affect the activation of MAPKAP-K2 *in vivo* in response to UV radiation (Figure 9B). Furthermore, incubation of cells with Ro 318220 did not significantly affect the UV-induced phosphorylation of HSP27, a physiological substrate for MAPKAP-K2 (Cuenda *et al.*, 1995; Figure 9C). In parallel experiments, the phosphorylation of HSP27 by UV radiation was blocked by SB 203580. The cyclic AMP-dependent protein kinase, which mediates phosphorylation of CREB and ATF1 by cyclic AMP-elevating agents such as forskolin (Gonzalez *et al.*, 1989), is also not significantly inhibited by 10 μM Ro 318220 (Davis *et al.*, 1989).

The results presented in Figure 9 offered an opportunity to evaluate the role of MAPKAP-K2 in mediating the phosphorylation of CREB at Ser133 (and its close relative ATF1 at the equivalent residue, Ser63). We observed that CREB and ATF1 became rapidly phosphorylated following exposure of 293 cells to UV radiation or following



A	MSK1		MAPKAP-K1a		MAPKAP-K1b	
	Km (μM)	Vmax (%)	Km (μM)	Vmax (%)	Km (μM)	Vmax (%)
1. GRPRTS**S**FAEG	2	100	3	100	2	100
2. KKRNRTL**S**VA	0.2	100	3	100	1	225
3. KKRNKTL**S**VA	25	75	250	86	160	106
4. KKLNRTL**S**VA	0.3	150	15	100	5	175
5. EILSRRP**S**YRK	<0.1	100	20	64	25	188
6. CREB PROTEIN	2.5	80	>50	~15	30	~20

Fig. 8. MSK1 phosphorylates CREB at Ser133. (**A**) Comparison of the substrate specificities of MSK1, MAPKAP-K1a and MAPKAP-K1b towards the indicated peptides and CREB. Phosphorylation and analysis was carried out as described in Materials and methods. The standard errors for all reported kinetic constants were within less than ±20% (SEM), and the data are reported as mean values for two independent experiments. The V_{max} values are reported as a percentage of the value obtained using Crosstide (GRPRTSSFAEG, peptide 1) as a substrate. The peptide EILSRRPSYRK corresponding to residues 126–136 of CREB is termed CREBtide. The serine residue shown in bold corresponds to the phosphorylated serine residues on the peptides. The V_{max} values of MSK1, MAPKAP-K1a and MAPKAP-K1b towards Crosstide are 200, 350 and 800 U/mg, respectively. (**B**) GST–MSK1 (solid bars), GST–MAPKAP-K1a (open bars), MAPKAP-K1b (speckled bars) or GST–MAPKAP-K2 (diagonal bars) were assayed with the indicated substrates. Under the conditions used, GST–MAPKAP-K2 phosphorylated Ser98 of the 341 residue splice variant of CREB (Gonzalez *et al.*, 1989) to a 10-fold higher level than Ser133 (data not shown). The phosphorylation of CREB by MAPKAP-K2 shown in the figure is corrected to show only the contribution of Ser133 phosphorylation to the total incorporation of phosphate into CREB. The data are presented as the mean ± SEM for three separate experiments with each determination carried out in triplicate. (**C**) CREB that had been phosphorylated with MSK1 was digested with trypsin and chromatographed on a Vydac 218TP54 C_{18} column (Separations Group, Hesperia, CA) equilibrated in 0.1% (v/v) trifluoroacetic acid (TFA) in water. The column was developed with a linear acetonitrile gradient (diagonal line) at a flow rate of 0.8 ml/min and fractions of 0.4 ml were collected. 75% of the radioactivity applied to the column was recovered from the major ^{32}P-containing peptide at 13% acetonitrile. The peptide map of CREB phosphorylated with MAPKAP-K1b was identical to that of CREB phosphorylated with MSK1 (data not shown).

treatment with EGF, and that phosphorylation was largely inhibited by 5 μM Ro 318220 (Figure 10A and B). In contrast, phosphorylation of CREB and ATF1 induced by forskolin, which is mediated by the cyclic-AMP-dependent protein kinase (Gonzalez *et al.*, 1989), was unaffected by 5 μM Ro 318220 (Figure 10C). Like the activation of MSK1, the UV-radiation-induced phosphorylation of

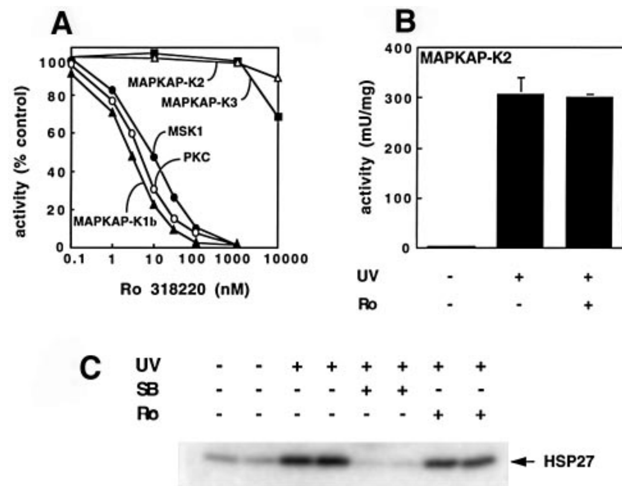


Fig. 9. Effect of Ro 318220 on the activity of MSK1. (**A**) Effect of Ro 318220 on GST–MSK1 (closed circles), mixed PKC isoforms (open circles), GST–MAPKAP-K1b (closed triangles), MAPKAP-K2 (open triangles) and MAPKAP-K3 (closed squares) *in vitro*. The results are presented relative to control incubations in which the inhibitor was omitted and are shown as the average of two experiments with each determination carried out in triplicate. The error for each point is ± 10%. (**B**) 293 cells were pre-treated for 1 h with or without 5 μM Ro 318220 (Ro) before being exposed to UV radiation (200 J/m^2 followed by incubation at 37°C for 30 min) or left untreated and then incubated for a further 30 min in the continued presence of Ro 318220. The cells were lysed and MAPKAP-K2 immunoprecipitated and assayed. The data are presented as the mean ± SEM for two separate experiments with each determination carried out in triplicate. (**C**) Same as (B) except that 293 cells were incubated for 2 h in ^{32}phosphate (0.1 mCi/ml) before treatment with inhibitors. Cells were then exposed to either UV (200 J/m^2 followed by incubation at 37°C for 30 min) or left untreated, and incubated for a further 20 min in the continued presence of inhibitors. Following cell lysis, HSP27 was immunoprecipitated from cell extracts (Cuenda *et al.*, 1995) and run on a 15% polyacrylamide gel and autoradiographed.

CREB and ATF1 was inhibited by SB 203580, but not PD 98059 (Figure 10D), while EGF-induced CREB phosphorylation was prevented by PD 98059 and not SB 203580 (Figure 10E).

Evidence that neither MAPKAP-K2 or MAPKAP-K1 mediate the phosphorylation of CREB and ATF1 by TNF in HeLa cells

Endogenous MSK1 was rapidly (but transiently) activated by tumour necrosis factor (TNF) in HeLa cells. Interestingly, the effect of SB 203580 and PD 98059 on the activation of MSK1 varied with the time of stimulation. After 5 min, activation was completely inhibited by SB 203580, but unaffected by PD 98059. However, after 15 min the activation of MSK1 was partially blocked by either SB 203580 or PD 98059, and almost completely suppressed if both drugs were added together (Figure 11A). These observations are explained by the different rates of activation of the SAPK2/p38 and MAPK/ERK cascades. Thus, after 5 min of TNF stimulation, the SAPK2 pathway is activated as judged by the activation of MAPKAP-K2 (which is inhibited by SB 203580 but not by PD 98059). In contrast, the MAPK/ERK cascade is not activated after 5 min, as judged by lack of activation of MAPKAP-K1a/b (Figure 11B and C). However, both the MAPK/ERK and SAPK2/p38 cascades are activated

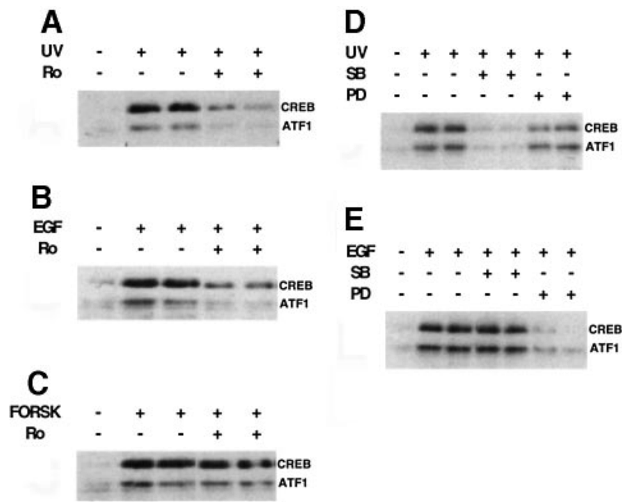


Fig. 10. Ro 318220 inhibits EGF- and UV-induced phosphorylation of CREB and ATF1 in 293 cells. Cells were pre-treated either in the presence or absence of 5 μM Ro 318220 (Ro), 10 μM SB 203580 (SB), 50 μM PD 98059 (PD), and then exposed to UV radiation (200 J/m^2 followed by incubation for 30 min at 37°C; **A** and **D**) EGF (100 ng/ml, 15 min; **B** and **E**), or forskolin (20 μM, 15 min; **C**). The cells were lysed and proteins were separated on 10% polyacrylamide gels and immunoblotted using a phospho-specific antibody that recognizes both CREB and ATF1 when phosphorylated on Ser133 and Ser63, respectively. The position of CREB and ATF1 on the blots is indicated.

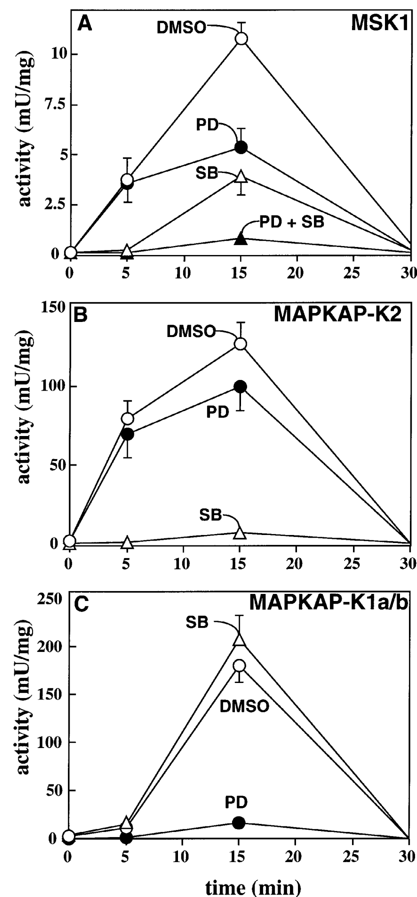


Fig. 11. Activation of MSK1 by TNF. HeLa cells were incubated for 2 h in serum-free medium in the presence of 50 μM PD 98059 (solid circles), 10 μM SB 203580 (open triangles), 50 μM PD 98059 plus 10 μM SB 203580 (solid triangles), or in the absence of both compounds (open circles). The cells were then stimulated with TNF (10 ng/ml) for the times indicated, in the continued presence or absence of inhibitors. After cell lysis, MSK1 (**A**), MAPKAP-K2 (**B**), and MAPKAP-K1a/b (**C**) were immunoprecipitated from the same lysates and assayed. The data are presented as the mean ± SEM for two separate experiments with each determination carried out in triplicate.

after 15 min of TNF stimulation, as judged by the activation of both MAPKAP-K1a/b and MAPKAP-K2. The activation of MAPKAP-K1a/b after 15 min is suppressed by PD 98059, but not by SB 203580 (Figure 11B and C).

Stimulation of HeLa cells with TNF rapidly induced the phosphorylation of CREB and ATF1. After 5 min, the phosphorylation of CREB and ATF1 was prevented by SB 203580 but unaffected by PD 98059 (Figure 12A). After 15 min, TNF-induced phosphorylation of CREB was partially suppressed by SB 203580, not significantly affected by PD 98059 and completely prevented in the presence of both compounds (Figure 12B). The effects of SB 203580 and PD 98059 on the activation of CREB were therefore similar to their effects on TNF-induced MSK1 activation (Figure 11). TNF-induced CREB phosphorylation was completely blocked by 5 μM Ro 318220 (Figure 12C), but the activation of MAPKAP-K2 was unaffected by this drug (data not shown).

Evidence that CREB phosphorylation at Ser133 in PC12 cells correlates with activation of MSK1 rather than MAPKAP-K1 or MAPKAP-K2

NGF stimulation of a PC12 cell-line induced the phosphorylation of CREB (Figure 13A) and the activation of MSK1 (Figure 13B). The phosphorylation of CREB and the activation of MSK1 were not significantly affected by incubation of the cells with either PD 98059 or SB 203580, but strongly inhibited in the presence of both drugs (Figure 13). These results indicate that inhibition of both the MAPK/ERK and SAPK2/p38 pathways is needed to prevent CREB phosphorylation at Ser133 and MSK1 activation in these cells.

Like MSK1, MAPKAP-K1 was also activated 5-fold by NGF, but in contrast to MSK1, activation of MAPKAP-K1 was unaffected by SB 203580, only partially inhibited by PD 98059 and not further inhibited by a combination of both drugs. Thus MAPKAP-K1 activity did not correlate with CREB phosphorylation. As reported previously (Rouse *et al.*, 1994), NGF did not induce any significant activation of MAPKAP-K2 activity in PC12 cells.

Evidence that CREB phosphorylation at Ser133 in SK-N-MC cells correlates with activation of MSK1 rather than MAPKAP-K1 or MAPKAP-K2

Earlier work from this laboratory showed that the phosphorylation of CREB at Ser133 in SK-N-MC cells, induced by either sodium arsenite or FGF, is suppressed by SB 203580 (Tan *et al.,* 1996). In the present work we confirmed that the sodium arsenite-induced activation of CREB and MSK1 in these cells is prevented by SB 203580, but not by PD 98059. However, the FGF-induced phosphorylation of CREB was only inhibited slightly by either SB 20380 or PD 98059 and the presence of both drugs

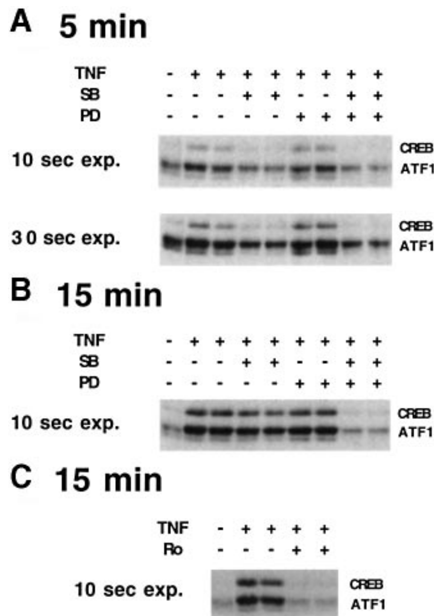


Fig. 12. Effect of PD 98059, SB 203580 and Ro 318220 on TNF-induced CREB and ATF1 phosphorylation. HeLa cells were incubated for 2 h in serum-free medium in the presence or absence of 50 μM PD 98059 (PD), 10 μM SB 203580 (SB) or 5 μM Ro 318220 (Ro) as indicated. The cells were then stimulated with TNF (10 ng/ml) for the times indicated in the continued presence or absence of inhibitors. After cell lysis, aliquots were immunoblotted for phosphorylation of CREB and ATF1 as described in the legend to Figure 11C. The positions of CREB and ATF1 on the blots is indicated. The time of exposure (exp.) of the blots before development is indicated.

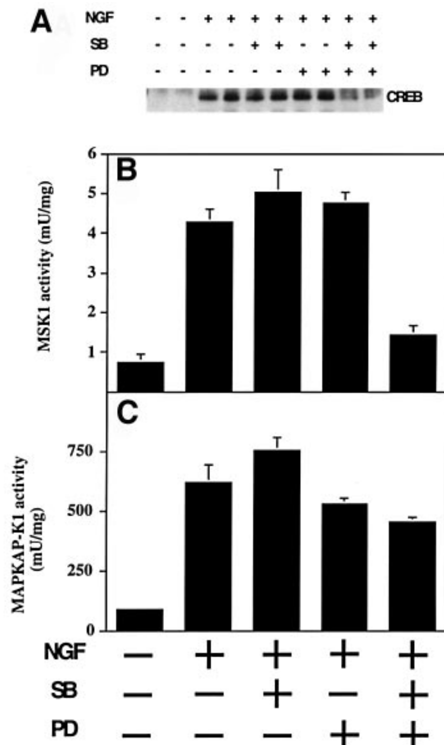


Fig. 13. Effect of PD 98059, SB 203580 and Ro 318220 on NGF-induced activation of CREB and MSK1 in PC12 cells. PC12 cells were incubated for 2 h in serum-free medium in the presence or absence of 50 μM PD 98059 (PD) or 10 μM SB 203580 (SB). The cells were then stimulated with NGF (30 ng/ml) for 15 min in the continued presence or absence of inhibitors. After cell lysis aliquots were immunoblotted for phosphorylation of CREB (**A**), or used to assay MSK1 (**B**) or MAPKAP-K1 (**C**) after their immunoprecipitation from the lysates.

was needed to completely suppress CREB phosphorylation (Figure 14D). Similarly, the FGF-induced activation of MSK1 in these cells is also only partially suppressed in the presence of either SB 203580 or PD 98059, and prevented in the presence of both compounds (Figure 14A).

In contrast to MSK1, the activation of MAPKAP-K2 by sodium arsenite and FGF was completely prevented by SB 203580 but not by PD 98059 (Figure 14C), and Ro 318220 (which does not affect the activation or activity of MAPKAP-K2) largely suppressed phosphorylation of CREB at Ser133. These findings indicate that MAPKAP-K2 activity is not rate-limiting for CREB phosphorylation at Ser133. To avoid any confusion it should be noted that Ro 318220 is a reversible inhibitor of MSK1 activity and does not affect MSK1 activation in cells (Figure 14A). Once the cells have been lysed and MSK1 immunoprecipitated from the lysates, the Ro 318220 inhibitor has been removed and MSK1 is no longer inhibited.

In contrast to MSK1, the activation of MAPKAP-K1 by FGF was abolished by PD 98059 but not by SB 203580 (Figure 14B). However, PD 98059 (in the absence of SB 203580) did not significantly suppress the phosphorylation of CREB at Ser133.

Discussion

In this paper we present the sequence of a novel, widely expressed protein kinase, termed MSK1 (Figure 1), and the nearly complete sequence of a close relative, MSK2 (Figures 1 and 2; Table I). MSK1 and MSK2 are most similar (40% overall amino acid sequence identity) to the isoforms of MAPKAP-K1, which they also resemble in possessing two protein kinase domains within a single polypeptide (Figure 2). The N-terminal kinase domain of MAPKAP-K1 phosphorylates exogenous substrates, while the only known role of the C-terminal domain is to activate the N-terminal domain. The importance of the C-terminal kinase domain of MAPKAP-K1 is indicated by the finding that an inactivating mutation suppresses the activation of the N-terminal kinase domain by 85–90% (Bjorbaek *et al.*, 1995; Leighton *et al.*, 1996). In contrast, an inactivating mutation in the C-terminal kinase domain of MSK1 (like an inactivating mutation in the N-terminal kinase domain) completely abolishes MSK1 activation (Figure 6). If it is assumed that the mechanism of activation of MSK1 is analogous to that of MAPKAP-K1, as suggested by the conservation of the four key phosphorylation sites (Figure 2), then this indicates that the C-terminal kinase domain of MSK1 is essential for activation of the N-terminal domain.

MSK1 is activated *in vivo* by either the MAPK/ERK cascade or the SAPK2/p38 pathway. This has been established by the finding that PD 98059 largely suppresses the activation of endogenous or transfected MSK1 by growth factors and phorbol esters, while SB 203580 suppresses activation induced by exposure to UV radiation or oxidative stress (Figures 5 and 6). Consistent with these findings, MSK1 can be activated *in vitro* by either MAPK2/ERK2 or by SAPK2/p38 (Figure 3). Several signals, such as TNF in HeLa cells (Figure 11), FGF in SK-N-MC cells

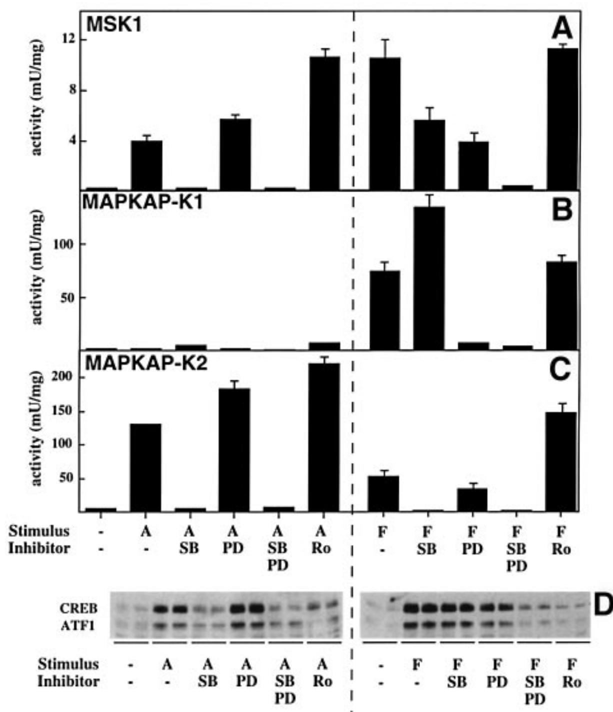


Fig. 14. Effect of PD 98059, SB 203580 and Ro 318220 on arsenite- and FGF-induced activation of CREB and MSK1 in SK-N-MC cells. SK-N-MC cells were incubated for 1 h in serum-free medium in the presence or absence of 50 μM PD 98059 (PD), 10 μM SB 203580 (SB) and 5 μM Ro 318220 (Ro). The cells were then stimulated for 15 min with sodium arsenite (0.5 mM) or FGF (50 ng/ml) in the continued presence or absence of inhibitors. After cell lysis aliquots were assayed for either MSK1 (**A**), MAPKAP-K1 (**B**) or MAPKAP-K2 (**C**) after their immunoprecipitation from the lysates, or immunoblotted for phosphorylation of CREB and ATF1 (**D**).

(Figure 14), and NGF in PC12 cells (Figure 13) activate both the MAPK2/ERK2 and SAPK2/p38 cascades, and both signalling pathways contribute to the activation of MSK1 in these cells. Why MSK1 and MNK1 (Fukunaga and Hunter, 1997; Waskiewicz *et al*., 1997) can be activated *in vitro* by both MAP kinase family members while MAPKAP-K1 can only be activated by MAPK/ERK is unclear. Presumably, MAPKAP-K1 either lacks a motif required for recognition by SAPK2/p38 or contains a motif that prevents recognition by these enzymes.

The finding that MSK1 is activated *in vivo* by signals that trigger activation of the MAPK/ERK cascade or the SAPK2/p38 pathway implies that, like MNK1, it plays a role in integrating the effects of different extracellular signals. The substrates of MSK1 are therefore likely to be proteins that become phosphorylated in response to either mitogenic or stress signals. Two such proteins are the transcription factors CREB and ATF1, and we have found that CREB is a remarkably good substrate for MSK1 *in vitro* (Figure 8). MSK1 phosphorylates CREB only at Ser133, the activating site that becomes phosphorylated *in vivo* in response to mitogens or stress signals. The K_m for phosphorylation of CREB by MSK1 is much lower than for phosphorylation by PKA, MAPKAP-K1 or MAPKAP-K2 (Figure 8). MSK1 phosphorylates CREBtide at the equivalent residue and with a remarkably low K_m value, estimated to be <0.1 μM (Figure 8A). To our knowledge, this is the lowest K_m of any peptide substrate of any protein kinase that has so far been identified. These observations suggest that MSK1 (and/or MSK2) may mediate the activation of CREB by mitogenic- and stress-stimuli, and the nuclear location of MSK1 (Figure 7) is consistent with such a role.

Earlier work from this unit suggested that MAPKAP-K2 may mediate the activation of CREB by sodium arsenite in the neuronal cell line SK-N-MC (Tan *et al*., 1996). However, in the present work, neither the activation of MAPKAP-K2 *in vivo* nor its activity *in vitro* (Figures 9 and 14, respectively) were affected by Ro 318220 up to 10 μM; yet 5 μM Ro 318220 suppressed CREB phosphorylation at Ser133 in response to all the signals that activate MAPKAP-K2 in SK-N-MC cells and other cell lines (Figures 10, 12 and 14). In addition, we have found that MAPKAP-K2 phosphorylates the alternatively spliced CREB2 variant (Gonzalez *et al*., 1989) much more rapidly at Ser98 than at Ser133, but no phosphorylation of Ser98 is observed after stimulation by agonists that strongly activate MAPKAP-K2 (A.Clifton, unpublished results). These results demonstrate that MAPKAP-K2/K3 activity is not rate-limiting for stress-induced activation of CREB in SK-N-MC cells. The only other protein kinase known to be activated by stressful stimuli, and which phosphorylates CREB at Ser133, is MSK1. MSK1 phosphorylates CREB far more efficiently than MAPKAP-K2 *in vitro* (Figure 8) and is potently inhibited by Ro 318220 (Figure 9). For these reasons MSK1 (and/or MSK2) is currently the best candidate to mediate stress-induced CREB phosphorylation at Ser133. MSK1 (and/or MSK2) is possibly the UV-activated CREB kinase detected by other investigators using an 'in gel' kinase assay that migrated with a slightly higher molecular mass (~108 kDa) than MAPKAP-K1 (Iordanov *et al*., 1997). The UV-induced activation of this kinase was prevented by SB 203580.

MSK1 and MAPKAP-K1 are both activated by MAPK/ERKs after cell stimulation by growth factors or phorbol esters, both phosphorylate CREB at Ser133 *in vitro* and both are potently inhibited by Ro 318220. This raises the question of whether MSK1 or a MAPKAP-K1 isoform mediates CREB phosphorylation at Ser133 in response to growth factors or phorbol esters. It has been reported that MAPKAP-K1b is a much more efficient CREB kinase than MAPKAP-K1a (Xing *et al*., 1996) and that MAPKAP-K1b is the major CREBtide kinase detectable in lysates prepared from NGF-stimulated PC12 cells (Ginty *et al*., 1994; Xing *et al*., 1996). However, in our hands, MAPKAP-K1a and MAPKAP-K1b phosphorylate CREB or CREBtide with similar kinetics and neither is remotely as efficient as MSK1 (Figure 8). Furthermore, there are numerous examples where the major protein kinase detected biochemically has subsequently been shown not to be the relevant enzyme *in vivo* towards a particular substrate. For example, MAPKAP-K1 is the major insulin-stimulated protein kinase in extracts prepared from L6 myotubes that phosphorylates and inactivates glycogen synthase kinase-3 (Cross *et al*., 1994), and yet subsequent work using PD 98059 excluded its involvement in this process (Cross *et al*., 1995). The reason why MSK1 was not detected previously by biochemical analysis (Tan *et al*., 1996; Xing *et al*., 1996) may be due to the much lower abundance

of MSK1 compared with the MAPKAP-K1 and MAPKAP-K2 in the cells examined.

Recently, Xing *et al*. (1998) have reported that both SB 203580 and PD 98059 are required to prevent the NGF-induced phosphorylation of CREB in PC12 cells. We have confirmed this observation (Figure 13A) and also demonstrated that NGF activates MSK1 in these cells (Figure 13B). Like the phosphorylation of CREB, the activation of MSK1 by NGF is only significantly inhibited in these cells in the presence of both SB 203580 and PD 98059. These observations indicate that activation of either the MAPK/ERK cascade or the SAPK2/p38 pathway is sufficient to produce maximal activation of MSK1 and CREB phosphorylation at Ser 133. In contrast, the NGF-induced activation of MAPKAP-K1 isoforms is unaffected by SB 203580 and only partially inhibited by PD 98059 (in the absence or presence of SB 203580). Thus, MAPKAP-K1 isoforms alone cannot account for the NGF-induced phosphorylation of CREB at Ser133.

The phosphorylation of CREB at Ser133 also correlated much better with the activation of MSK1 in TNF-stimulated HeLa cells (compare Figure 11 with 12) and FGF-treated SK-N-MC cells (Figure 14). In both situations, the MAPK and SAPK2/p38 pathways were activated and suppression of CREB phosphorylation at Ser133, as well as the activation of MSK1, required the presence of both SB 203580 and PD 98059. In SK-N-MC and HeLa cells, PD 98059 completely suppressed the activation of MAPKAP-K1 by FGF and TNF, respectively, but only had a small effect on CREB phosphorylation induced by these agonists.

In summary, while a role for one or more MAPKAP-K1 isoforms (or an as yet unidentified protein kinase) in the activation of CREB by growth factors or phorbol esters cannot be entirely excluded, our results suggest that MSK1 (and/or MSK2) mediates the activation of CREB by these stimuli. However, the development of specific inhibitors of MSK1/MSK2 and/or ways to prevent their expression will be needed to establish that MSK1/MSK2 mediate the activation of CREB *in vivo*.

Materials and methods

Materials

Peptides for protein kinase assays were synthesized at The University of Dundee, Scotland by Mr F.B.Caudwell (MRC unit), and those used to used to raise antibodies were synthesized by Dr G.Blomberg at The University of Bristol, UK. Protein G–Sepharose and glutathione–Sepharose were purchased from Pharmacia (Milton Keynes, UK), alkylated trypsin from Promega (Southampton, UK), tissue culture reagents, microcystin-LR, FGF, and EGF from Life Technologies Inc. (Paisley, UK), 12-*O*-tetradecanoylphorbol 13-acetate (TPA) from Sigma-Aldrich (Poole, Dorset, UK), sodium arsenite and hydrogen peroxide (H_2O_2, Aristar grade) from E.Merck (Lutterworth, UK), SB 203580 and PD 98059 from Calbiochem (Nottingham, UK) and the pCR 2.1-TOPO cloning vector from Invitrogen (Leek, The Netherlands). Activated GST–MAPK2/ERK2 (Alessi *et al*., 1994), GST–SAPK1/JNK1γ (Lawler *et al*., 1997), GST–SAPK2a/p38a, GST–SAPK2b/p38β and GST–SAPK3/p38γ (Cuenda *et al*., 1997), GST–SAPK4/p38δ (Goedert *et al*., 1997), GST–MAPKAP-K2 (BenLevy *et al*., 1995) and GST–MAPKAP-K3 (Clifton *et al*., 1996) were expressed in bacteria and maximally activated *in vitro* using the appropriate upstream kinase kinase as described previously. MAPKAP-K1b was purified from rabbit skeletal muscle by Dr N.Morrice in the MRC Unit as previously described (Sutherland *et al*., 1993). GST–MAPKAP-K2 was expressed in bacteria and activated *in vitro* using GST–MAPK as previously described (Ben-Levy *et al*., 1995). PKA was prepared from bovine heart by Dr C.MacKintosh in the MRC Unit, Dundee, Scotland.

Antibodies

The MSK1 A, B and C antibodies were raised in sheep against the peptides LTVKHELRTANLTGHAEKV (corresponding to residues 26–44 of MSK1), FKRNAAVIDPLQFHMGVER (corresponding to residues 384–402 of MSK1) and KATFHAFNKYKREGFCLQN (corresponding to residues 716–734 of MSK1), respectively. Antibodies that immunoprecipitate MAPKAP-K2 specifically (Clifton *et al*., 1996), or both MAPKAP-K1a and MAPKAP-K1b (Alessi *et al*., 1995), were raised in sheep against the peptides KEDKERWEDVKEEMTS (residues 343–358 of human MAPKAP-K2) and RNQSPVLEPVGRSTLAQRRGIKK (residues 712–734 of human MAPKAP-K1b). All of the antibodies used in this study were affinity-purified on CH-Sepharose columns to which the appropriate peptides were covalently coupled, and are available commercially from UBI (Lake Placid, USA). A monoclonal antibody recognising the Flag epitope was purchased from Anachem (Luton, UK). Rabbit polyclonal antibody recognizing HSP27 was purchased from Stressgen (York, UK).

Buffer solutions

Buffer A comprised 50 mM Tris–HCl pH 7.5, 1 mM EGTA, 1 mM EDTA, 1% Triton-X 100 (w/v), 1 mM sodium orthovanadate, 50 mM sodium fluoride, 5 mM sodium pyrophosphate, 0.27 M sucrose, 1 μM microcystin-LR, 0.1% (v/v) β-mercaptoethanol and 'complete' proteinase inhibitor cocktail (one tablet per 50 ml; Boehringer Mannheim, Lewes, UK).

Buffer B comprised of 50 mM Tris–HCl pH 7.5, 0.1 mM EGTA and 10 mM β-mercaptoethanol.

Cloning of MSK1 and MSK2

The sequence of MSK1 was obtained by sequencing the human EST cDNA (AA15 8571) obtained from the I.M.A.G.E. consortium (Lennon *et al*., 1996). The mouse and human MSK2 sequences were obtained from sequencing some of the EST cDNA clones shown in Table I. DNA sequencing was carried out on an Applied Biosystems 373A automatic DNA sequencer using the *Taq* dye terminator cycle sequencing kit.

Preparation of DNA expression constructs encoding GST–MSK1, Flag-MSK1 and GST–MAPKAP-K1a

A DNA construct expressing human MSK1 with the FLAG DYKDDDDK epitope tag at the N-terminus (Flag-MSK1) was prepared as follows: A PCR was carried out using as a template the MSK1 cDNA and the oligonucleotides 5′-GAGATCTGCCACCATGGACTACAAGGACG-ACGATGACAAGGAGGAGGAGGGTGGCAGCAGCGGCG-3′ (incorporating the underlined *Bgl*II site) and 5′-GGATCCATTTCT-GTGAACTCTTCTG-3′. The resulting PCR product was ligated into pTopo vector. A triple ligation was then set up to generate a full-length Flag-MSK1 construct in the pCMV5 mammalian expression vector (Anderson *et al*., 1989) by excision of the N-terminal MSK1 PCR product from the pTOPO vector as an *Eco*R1–*Eco*RV fragment and ligating this together with the C-terminal *Eco*RV–*Kpn*1 fragment derived from the EST cDNA AA15 8571 into the *Eco*R1–*Kpn*1 sites of the pCMV5 vector. A GST–MSK1 expression construct was prepared by subcloning the Flag-MSK1 cDNA from the pCMV5 vector as a *Bgl*II–*Kpn*I fragment into the *Bam*HI and *Kpn*I sites of the pEBG2T (Sanchez *et al*., 1994) expression vector. Full-length Flag-MSK1 mutants (in the pCMV5 vector) in which either the N-terminal or C-terminal kinase domains have been inactivated were prepared by changing the conserved Asp195 and Asp565 residues in subdomain VII of the kinase domain to Ala. This was achieved using the PCR-based megaprimer strategy. A GST–MAPKAP-K1a expression construct was prepared by subcloning of HA-MAPKAP-K1a from the pGEX4T.1 vector described in Dalby *et al*. (1998) as a *Not*I–*Not*I fragment into the *Not*I site of the pEBG2T expression vector. The structures of all of the expression constructs were verified by DNA sequencing, after purification from bacteria using Qiagen plasmid Mega kit according to the manufacturer's instructions.

Expression of GST–MSK1 and GST–MAPKAP-K1a

Twenty 10 cm diameter dishes of human embryonic kidney 293-cells were cultured and each dish transfected with 20 μg of DNA encoding either GST–MSK1 or GST–MAPKAP-K1a using a modified calcium phosphate method (Alessi *et al*., 1996b). After transfection (24 h), the cells were serum starved for 16 h and either left unstimulated, or stimulated with TPA (200 ng/ml for 15 min) and each dish of cells lysed in 1 ml of ice-cold Buffer A. The 20 lysates were pooled, centrifuged

at 4°C for 10 min at 13 000 *g* and the supernatant incubated for 60 min on a rotating platform with 1 ml of glutathione–Sepharose, previously equilibrated in Buffer A. The suspension was centrifuged for 1 min at 3000 *g*, the beads washed three times with 10 ml of Buffer A containing 0.5 M NaCl and then a further three times with 10 ml of Buffer B containing 0.27 M sucrose. GST–MSK1 or GST–MAPKAP-K1a were eluted from the resin at ambient temperature with 3× 1 ml portions of Buffer B containing 20 mM glutathione and 0.27 M sucrose. The combined eluates (0.5 mg/ml protein for GST–MSK1 and 0.1 mg/ml GST–MAPKAP-K1a) were divided into aliquots, snap frozen in liquid nitrogen and stored at –80°C.

Cell culture, stimulation and cell lysis

Human embryonic kidney 293 cells were cultured to confluence on 10 cm diameter dishes and incubated for 16 h in Dulbecco's Modified Eagle's medium (DMEM) from which fetal calf serum was omitted. HeLa and SK-N-MC cells were cultured to confluence on 10 cm diameter dishes and incubated for 2 h in DMEM from which fetal calf serum was omitted. PC12 cells expressing high levels of the NGF receptor, which start to differentiate within a few hours of the addition of NGF, were cultured and incubated for 2 h in DMEM from which fetal calf serum was omitted (Rouse *et al*., 1994). Due to the high levels of the NGF receptor in these cells the PD 98059 inhibitor only partially suppresses the NGF-induced activation of MAPK (Alessi *et al*., 1995). The cells were then incubated for the times indicated in the figure legends with 50 μM PD 98059, 10 μM SB 203580, 5 μM Ro 318220 or the equivalent volume of DMSO as a control, then stimulated as indicated in the figure legends and the cells lysed in 1.0 ml of ice cold Buffer A. The lysates were immediately frozen in liquid nitrogen and stored at –80°C until use. Protein concentrations were determined (Bradford *et al*., 1976) using bovine serum albumin (BSA) as a standard.

Immunoprecipitation and assay of MSK1, MAPKAP-K2 and MAPKAP-K1

The amount of cell lysate used for each immunoprecipitation was as follows: 500 μg protein (for MSK1), 50 μg protein (for MAPKAP-K2) and 50 μg protein (for MAPKAP-K1a/b). The lysates were incubated at 4°C for 30 min on a shaking platform with 5 μg of each antibody coupled to 5 μl of protein G–Sepharose. The immunoprecipitates were washed twice with 1 ml of Buffer A containing 0.5 M NaCl, and once with 1 ml of Buffer B. The standard MSK1 or MAPKAP-K1a/b assay (50 μl) contained washed protein G–Sepharose immunoprecipitate, 50 mM Tris–HCl pH 7.5, 0.1 mM EGTA, 0.1% (v/v) 2-mercaptoethanol, 2.5 μM PKI (peptide inhibitor of cyclic-AMP-dependent protein kinase), Crosstide (30 μM), 10 mM $Mg(Ac)_2$ and 0.1 mM [γ-^{32}P]ATP (100–200 c.p.m./pmol). The assays were carried out for 10 min at 30°C, the assay tubes being agitated continuously to keep the immunoprecipitate in suspension, then terminated and analysed as described (Alessi *et al*., 1994). MAPKAP-K2 was assayed in the same way except that the peptide KKLNRTLSVA (30 μM) was used as substrate. One unit of activity was the amount of enzyme that catalysed the phosphorylation of 1 nmol of peptide substrate in 1 min.

Expression of CREB

The *Escherichia coli* strain BL21, transformed with a DNA construct encoding the 341 amino acid splice variant of CREB (Gonzalez *et al*., 1989), kindly provided to us by M.J.Comb (New England Biolabs, Boston, USA), was induced with 0.1 mM isopropyl-β-D-thiogalactoside for 5 h at 37°C. The GST–CREB was purified on glutathione–Sepharose as described previously for GST–MAPK2/ERK2 (Alessi *et al*., 1994), and dialysed against Bufffer B containing 50% glycerol and stored at –20°C. The preparation showed two major Coomassie Blue staining bands at 62 and 43 kDa as well as a number of minor bands. Only the 62 and 43 kDa bands were phosphorylated by MAPKAP-K1 and MSK1. The protein concentration of CREB was estimated by comparing the intensity of the 62 and 43 kDa bands relative to a BSA standard.

Immunoblotting for phosphorylated CREB and ATF1

Cell extracts were prepared and immunoblotting of these were carried out as described in Tan *et al*. (1996) using a phospho-specific antibody recognizing CREB phosphorylated on Ser 133, and ATF1 phosphorylated on Ser63, purchased from UBI (Lake Placid, USA). Detection of phosphorylated CREB and ATF1 proteins was performed using the enhanced chemiluminescence reagent (Amersham).

Phosphorylation of CREB and HSP27 by MSK1 and MAPKAP-K1a/b

MSK1 and MAPKAP-K1a expressed as GST fusion proteins were purified from TPA-stimulated 293 cells (Figure 3), and MAPKAP-K1b was purified from rabbit skeletal muscle (Sutherland *et al*., 1993). The peptides indicated in Figure 8, as well as CREB and HSP27, were incubated at 30°C with 2 U/ml GST–MSK1, GST–MAPKAP-K1a or GST–MAPKAP-K1b in Buffer B containing 10 mM $Mg(Ac)_2$, 100 μM [γ-^{32}P]ATP (1×10^6 c.p.m./nmol), 10 μM PKI and 1 μM microcystin-LR. After incubation for 10 min, incorporation of phosphate into peptides was determined using P81 phosphocellulose paper (Alessi *et al*., 1994), and the incorporation of phosphate into CREB and HSP27 was measured by addition of trichloroacetic acid (0.2 vol of 100%), and the sample was then incubated for 1 h on ice. The suspension was centrifuged for 10 min at 13 000 *g*, the supernatant discarded and the pellet washed five times with 0.2 ml of ice-cold water. The incorporated ^{32}P-radioactivity was then determined by Cherenkov counting. In order to map the site in CREB phosphorylated by MSK1 and GST–MAPKAP-K1b, the pellet was resuspended in 0.3 ml of 50 mM Tris–HCl pH 8.0, 0.1% (v/v) reduced Triton X-100 containing 2 μg of alkylated trypsin, and after incubation for 16 h at 30°C, the digest was centrifuged for 5 min at 13 000 *g*. The supernatant, containing 95% of the ^{32}P-radioactivity, was chromatographed on a Vydac C_{18} column as described in the legend to Figure 8. The K_m and V_{max} values were determined from double reciprocal plots of 1/V against 1/S, where V is the initial rate of phosphorylation, and S is the substrate concentration.

Transfection of MSK1 into 293 cells

293 cells were cultured on 10 cm-diameter dishes and transfected with the pCMV5 vector encoding the Flag-epitope-tagged MSK1 constructs using a modified calcium phosphate method (Alessi *et al*., 1996b). Twenty four hours post-transfection, the cells were deprived of serum for 16 h, stimulated with TPA or exposed to UV radiation, then lysed in 1 ml of ice cold Buffer A, centrifuged at 13 000 *g* for 5 min. The Flag-tagged MSK1 protein immunoprecipitated from aliquots of lysate (containing 25 μg protein) using 2 μg of Flag antibody coupled to 5 μl of protein G–Sepharose. Immunoprecipitates were incubated, washed and assayed for MSK1 activity as described above.

Immunoelectron microscopy

Cells were fixed in 8% paraformaldehyde in 0.2 M PIPES pH 7.2 for at least one day, scraped using a rubber policeman and embedded as a pellet in 10% pig-skin gelatin. Blocks were soaked in 2.1 M sucrose/phosphate-buffered saline (PBS) for at least 15 min before mounting on iron stubs and freezing in liquid nitrogen. Ultra-thin cryosections were then prepared on an Reichert Ultracut E cryomicrotome at –100°C and mounted on carbon-/formvar-coated grids. Grids were incubated first on 0.5% fish skin gelatin/PBS (5 min), followed by anti-Flag mouse monoclonal antibodies (15 μg/ml), then rabbit antimouse antibodies (2 μg/ml Southern Biotechnology Associates Inc. Birmingham, AL, USA) and lastly on protein A–8 nm gold complex prepared as previously described (Lucocq, 1994). PBS washes followed each of the affinity reagents which were themselves diluted in 0.5% fish-skin gelatin/PBS. Finally, after washing in distilled water the sections were contrasted in methyl cellulose/uranyl acetate.

Labelling was quantified as follows. At a magnification of ×15 000, micrographs were taken of labelled cell profiles possessing both cytoplasm and nucleus (these nuclear weighted sections allowed data from these compartments in individual cells to be compared). Cytoplasm and nuclear areas were estimated using point counting with a square lattice grid of 1 cm line spacing and gold labelling counted (Lucocq, 1994). Coefficients of error were calculated according to Cochran (1953).

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