MSK1 activity is controlled by multiple phosphorylation sites

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MSK1 (mitogen- and stress-activated protein kinase) is a kinase activated in cells downstream of both the ERK1/2 (extracellular-signal-regulated kinase) and p38 MAPK (mitogen-activated protein kinase) cascades. In the present study, we show that, in addition to being phosphorylated on Thr-581 and Ser-360 by ERK1/2 or p38, MSK1 can autophosphorylate on at least six sites: Ser-212, Ser-376, Ser-381, Ser-750, Ser-752 and Ser-758. Of these sites, the N-terminal T-loop residue Ser-212 and the 'hydrophobic motif' Ser-376 are phosphorylated by the C-terminal kinase domain of MSK1, and their phosphorylation is essential for the catalytic activity of the N-terminal kinase domain of MSK1 and therefore for the phosphorylation of MSK1 substrates *in vitro*. Ser-381 is also phosphorylated by the C-terminal kinase domain, and mutation of Ser-381 decreases MSK1 activity, probably

through the inhibition of Ser-376 phosphorylation. Ser-750, Ser-752 and Ser-758 are phosphorylated by the N-terminal kinase domain; however, their function is not known. The activation of MSK1 in cells therefore requires the activation of the ERK1/2 or p38 MAPK cascades and does not appear to require additional signalling inputs. This is in contrast with the closely related RSK (p90 ribosomal S6 kinase) proteins, whose activity requires phosphorylation by PDK1 (3-phosphoinositide-dependent protein kinase 1) in addition to phosphorylation by ERK1/2.

Key words: extracellular-signal-regulated kinase (ERK), mitogen- and stress-activated protein kinase (MSK1), MSK2, p38, 3-phosphoinositide-dependent protein kinase 1 (PDK1), p90 ribosomal S6 kinase (RSK).

INTRODUCTION

MAPK (mitogen-activated protein kinase) signalling networks are involved in many aspects of cellular function, including survival, differentiation and apoptosis as well as aspects of immune and neuronal function. Misregulation of these cascades is implicated in many human diseases ranging from cancer to inflammatory diseases. MAPKs regulate cell function by phosphorylating target proteins, including structural proteins, transcription factors and enzymes. In addition, some MAPKs are also capable of activating downstream kinases, thereby adding another level to these kinase cascades. For instance, ERK1/2 (extracellular-signal-regulated kinase) is capable of activating the RSK (p90 ribosomal S6 kinase) isoforms, whereas p38 α /SAPK2A (where SAPK stands for stress-activated protein kinase) activates MAPKAP-K2 and -K3 (where MAPKAP-K stands for MAPK-activated protein kinase). MSK1 (mitogen- and stress-activated protein kinase) and its closely related isoform, MSK2, are two more recently described downstream kinases and they are unusual in that they are activated by both the ERK1/2 and p38 MAP kinase cascades in cells [1–3]. MSKs appear to be constitutively localized to the nucleus of cells and, consistent with this localization, MSKs are involved in the regulation of transcription downstream of ERK1/2 and p38. Using mouse knockouts, MSK1 and MSK2 have been shown to be required for the phosphorylation of the transcription factors CREB (cAMP-response-element-binding protein) and ATF1 (activating transcription factor 1) [4,5] and the chromatin proteins histone H3 and HMG-14 (high-mobility group protein 14) [6,7], in response to mitogens and stress. MSKs have also been shown to be required for the full induction of several immediate early genes, including *c-fos*, *junB*, *mkip-1* (MAPK phosphatase-1) and *nurr1* in response to various stimuli [8,9]. In addition, MSKs

have been suggested to have several other targets including the transcription factors ER81 (ets-related protein) and nuclear factor κ B [10,11].

While it has been demonstrated that MSKs are activated as a result of phosphorylation by ERK1/2 or p38 in cells [3], it is probable that further phosphorylation sites in MSKs are also necessary for activation. The activation mechanism of MSK1 has not, however, been extensively studied. MSKs are homologous with the RSK family of kinases (see [12–14] for reviews) and, similarly to RSK, they contain two kinase domains, joined by a short linker region, in a single polypeptide [1–3,15,16]. The N-terminal kinase domains of both MSKs and RSKs are members of the AGC family of protein kinases [containing PKA (protein kinase A), PKG and PKC families], whereas the C-terminal kinase domains are members of the calmodulin-activated protein kinase family [17].

The activation of RSK has been studied in greater detail compared with the activation of MSKs. The activation of RSK occurs in response to mitogenic stimulation of cells and is dependent on the activation of the ERK1/2 MAP kinase cascade [18,19]. Consistent with this, ERK1/2 is capable of activating RSK *in vitro*. In cells, ERK1/2 is recruited to RSK through a C-terminal docking sequence, and mutation of this sequence prevents the activation of RSK by ERK1/2 [20–22]. Once recruited through this docking sequence, ERK1/2 phosphorylates RSK, leading to RSK activation through a complex phosphorylation-dependent mechanism. During its activation in cells, RSK has been shown to be phosphorylated on several residues, including Ser-222, Thr-360, Ser-364, Ser-381, Thr-574 and Ser-733 [23]. Of these sites, Thr-360, Ser-364 and Thr-574 are followed by a proline residue and are *in vitro* sites for ERK1/2, the upstream activator of RSK in cells. ERK1/2 is recruited to RSK through a C-terminal docking sequence, and mutation of this sequence prevents the activation

Abbreviations used: DMEM, Dulbecco's modified Eagle's medium; ERK, extracellular-signal-regulated kinase; GST, glutathione S-transferase; HEK-293 cells, human embryonic kidney 293 cells; MALDI-TOF, matrix-assisted laser-desorption/ionization-time of flight; MAPK, mitogen-activated protein kinase; MAPKAP-K, MAPK-activated protein kinase; MEF, mouse embryonic fibroblast; MSK, mitogen- and stress-activated protein kinase; PDK1, 3-phosphoinositide-dependent protein kinase 1; PEI, polyethyleneimine; PH domain, pleckstrin homology domain; PIP₃, phosphatidylinositol 3,4,5-trisphosphate; PKA, PKB, PKC and PKG, protein kinases A, B, C and G respectively; RSK, p90 ribosomal S6 kinase; TFA, trifluoroacetic acid.

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of RSK by ERK1/2 [20–22,24,25]. Mutation of Ser-381 revealed that phosphorylation of this residue was also involved in RSK activation. Ser-381 was found to be phosphorylated by the C-terminal domain of RSK and this phosphorylation was critical for the activation of the N-terminal kinase domain. Ser-222 phosphorylation was also found to be required for the activity of the N-terminal kinase domain; however, the mechanism of Ser-222 phosphorylation in RSK was not determined by these initial studies. It was proposed, based on these results, that activation of RSK required the phosphorylation of Ser-364 and Thr-574 by ERK1/2, which in turn activated the C-terminal kinase domain that phosphorylated Ser-381. The phosphorylation activated the N-terminal kinase domain, which could then phosphorylate the substrates [23,26].

This model has now been refined due to advances in the understanding of the activation of AGC kinase family members. Most AGC kinases require the phosphorylation of two sites for full activity, a serine or threonine residue in the T-loop sequence (Ser-222 in RSK) and a serine residue (Ser-381 in RSK) in a sequence termed the hydrophobic motif, which lies C-terminal to the AGC kinase domain [27,28]. Phosphorylation of the T-loop residue in AGC kinases is required for activity, and mutation of this residue to alanine in all the AGC kinase family members tested to date prevents activation of the kinase. For a subset of AGC kinases, including RSK, the T-loop residue is phosphorylated by PDK1 (3-phosphoinositide-dependent protein kinase 1) [29–31]. PDK1 appears to be active constitutively in cells, and T-loop phosphorylation of AGC kinases by PDK1 is controlled in two major ways depending on the AGC kinase involved [27]. PDK1 was first identified as the upstream activator of PKB. The phosphorylation of PKB by PDK1 is dependent on phosphoinositide 3-kinase activity. The activation of phosphoinositide 3-kinase at the membrane results in the production of PIP₃ (phosphatidylinositol 3,4,5-trisphosphate). Both PKB and PDK1 are recruited to the membranes by the binding of their PH (pleckstrin homology) domains to PIP₃. The binding of PIP₃ to the PH domains is necessary to allow PDK1 to phosphorylate the T loop of PKB. Consistent with this, mutation of the PH domain in PDK1 prevents the activation of PKB in cells. In contrast, for other AGC kinases, including RSK and p70S6K (p70 ribosomal S6 kinase), the phosphorylation of the T-loop residue by PDK1 is controlled by the 'hydrophobic motif' at the C-terminus of the AGC kinase domain [32,33]. Phosphorylation of the hydrophobic motif introduces a negative charge to this motif, which enables the recruitment of PDK1 through the phosphate binding in the 'PIF' (PDK1 interacting fragment) pocket of PDK1. Once bound, PDK1 is then capable of phosphorylating the T loop of the AGC kinase. The requirement of PDK1 for the phosphorylation of Ser-222 of RSK, and its subsequent activation, has been demonstrated in cells by the use of PDK1^{−/−} embryonic stem cells and a knock-in mutation that prevents PDK1 binding to the phosphorylated hydrophobic motif of AGC kinases [30,33].

It has been shown that MSKs are activated in cells by either the ERK1/2 or p38 pathways depending on the stimuli used, and that inhibition of these pathways blocks MSK activation in cells. In MSK2, the phosphorylation of the two probable MAPK sites, Thr-568 and Ser-343, by the upstream kinase (ERK or p38) has been shown to be required for activity. Furthermore, on the basis of mutational analysis [34], it has been found that phosphorylation of at least two further sites, Ser-196 and Ser-360 (the T loop and hydrophobic motif), is also required for MSK2 activity. Less is, however, known about the activation of MSK1. Mutation of either the C- or N-terminal kinase domains in MSK1 has been shown to be sufficient to block the phosphorylation of MSK substrates [3]. However, the mechanism of MSK1 activation has not been

studied further, but was initially believed to show some similarity to the activation of RSK. The study of embryonic stem cells lacking PDK1, however, revealed significant differences between RSK and MSK1. PDK1 phosphorylates the T loop of RSK and, because of this, PDK1^{−/−} embryonic stem cells have no RSK activity. In contrast, MSK1 has been shown to be activated normally in PDK1^{−/−} embryonic stem cells [30]. This raises the question of whether T-loop phosphorylation is necessary for MSK1 activation and, if so, which kinase is responsible. We have therefore re-examined the activation of MSK1 in cells. We find that MSK1 is capable of autophosphorylating its own T loop and also that MSK1 can autophosphorylate on several previously unidentified sites.

METHODS

Plasmids

Mammalian expression vectors for FLAG-MSK1 (where FLAG stands for Asp-Tyr-Lys-Asp-Asp-Asp-Lys) and GST (glutathione S-transferase)-MSK1 as well as N- and C-terminal kinase dead mutations have been described previously [3]. Further mutations of phosphorylation sites were generated using the Quik Change method (Stratagene). All constructs were confirmed by DNA sequencing by the Sequencing Service, School of Life Sciences (University of Dundee, Scotland; <http://www.dnaseq.co.uk>), using Applied Biosystems Big-Dye version 3.1 chemistry on an Applied Biosystems model 3730 automated capillary DNA sequencer.

Cell culture

HEK-293 cells (human embryonic kidney 293 cells) were cultured in DMEM (Dulbecco's modified Eagle's medium) containing 10% (v/v) foetal bovine serum (Sigma), 2 mM L-glutamine, 50 units/ml penicillin G and 50 µg/ml streptomycin (Invitrogen). MEF (mouse embryonic fibroblast) cells were isolated from E13.5 embryos and cultured in DMEM containing 10% foetal bovine serum (Sigma), 2 mM L-glutamine, 50 units/ml penicillin G and 50 µg/ml streptomycin (Invitrogen). MEF cells were immortalized by large T antigen transfection. HEK-293 cells were transfected using a modified CaPO₄-based method as described in [19]. MEF cells were transiently transfected with 25 kDa linear PEI (polyethyleneimine) obtained from Polysciences (Warrington, PA, U.S.A.) as described in [35], except that PEI was dissolved in 20 mM Hepes (pH 7.4) instead of water. MEF cells (7×10^5) were plated on to 10 cm plates 24 h before transfection. DNA (10 µg) was diluted in 1 ml of fresh serum-free DMEM. PEI was added at a rate of 1 µg of DNA in 6.25 µl of PEI, the mixture was vortex-mixed and incubated for 10 min at room temperature before adding to cells. After a 16 h incubation with DNA-PEI complexes at 37°C and 5% CO₂, cells were washed with PBS and the medium was refreshed with DMEM containing 10% serum, 2 mM L-glutamine, 50 units/ml penicillin G and 50 µg/ml streptomycin.

Before stimulation, cells were serum-starved in DMEM with L-glutamine, penicillin and streptomycin for 16 h. Cells were then stimulated with either PMA (also known as TPA; 400 ng/ml for 10 min) or UV-C (ultraviolet light with a commonly used range of wavelengths; 200 J/m², followed by incubation at 37°C for 30 min). Cells were then lysed in 50 mM Tris/HCl (pH 7.5), 1 mM EGTA, 1 mM EDTA, 1 mM sodium orthovanadate, 50 mM sodium fluoride, 1 mM sodium pyrophosphate, 0.27 M sucrose, 1% (v/v) Triton X-100, 0.1% (v/v) 2-mercaptoethanol and complete proteinase inhibitor cocktail (Roche). The lysates were centrifuged at 13 000 g for 5 min at 4°C and the supernatants were

removed, quick-frozen in liquid nitrogen and stored at -80°C until use.

Kinase assays

For kinase assays, FLAG-MSK1 was immunoprecipitated from 0.2 mg of precleared cell lysate using 2 μg of anti-FLAG coupled with Protein G-Sepharose, or GST-MSK1 pulled down with 10 μl of glutathione-Sepharose. Precipitates were washed twice with 0.5 M NaCl, 50 mM Tris/HCl (pH 7.5), 0.1 mM EGTA and 0.1 % 2-mercaptoethanol and once with 50 mM Tris/HCl (pH 7.5), 0.1 mM EGTA and 0.1 % 2-mercaptoethanol. Precipitates were then resuspended in 35 μl of reaction buffer [containing Tris/HCl (pH 7.5), EGTA, PKI (PKA inhibitor) and Crosstide peptide, GRPRTSSFAEG], and the reaction was started by the addition of 10 μl of 50 mM magnesium acetate, 0.5 mM [^{32}P]ATP and incubated at 30°C for 15 min. Final concentrations of reagents in the assay were 50 mM Tris/HCl (pH 7.5), 0.1 mM EGTA, 2.5 mM PKI, 0.1 % 2-mercaptoethanol, 30 μM Crosstide peptide, 10 mM magnesium acetate and 0.1 mM [^{32}P]ATP [5]. Reactions were stopped by transferring on to P81 paper and washing with 75 mM orthophosphoric acid. One unit was defined as the incorporation of 1 nmol of phosphate into the substrate peptide in 1 min.

Antibody production

Antibodies were raised in rabbits as described in [36] against the following phosphopeptides derived from the human MSK1 sequence: ERAY(phosphoS)FCGT (phospho-Ser-212); FVAP-(phospho-S)ILFKR (phospho-Ser-381); MKKT(phosphoS)-TSTET (phospho-Ser-750); and MKKT(phosphoS)T(phosphoS)TETRS (phospho-Ser-750/Ser-752). Antibodies from the serum were affinity-purified against the corresponding phosphopeptide. The purified antibody was used for immunoblotting at 1 $\mu\text{g}/\text{ml}$ in TBS-T (Tris-buffered saline with Tween20) in the presence of 10 $\mu\text{g}/\text{ml}$ dephosphopeptide. Specificity testing of the antibodies is provided in Supplementary Figure 1 (see <http://www.BiochemJ.org/bj/387/bj3870507add.htm>).

Immunoblotting

Soluble cell extract (25 μg) was run on 4–12 % Novex gels (Invitrogen) and transferred on to nitrocellulose membranes and blotted using standard procedures. For immunoprecipitation of endogenous MSK1, an anti-MSK1 peptide antibody was used as described in [3]. MSK1 antibodies against phospho-Ser-360, phospho-Ser-376 and phospho-Thr-581 were obtained from Cell Signalling, while the phospho-Ser-212, phospho-Ser-381, phospho-Ser-750 and dual phosphorylated Ser-750/Ser-752 were raised in house, as was the GST antibody. FLAG antibody was from Sigma. Horseradish peroxidase-conjugated secondary antibodies were obtained from Pierce and blots were developed using ECL[®] (enhanced chemiluminescence) reagent (Amersham Biosciences).

In vitro MSK1 phosphorylation

Human MSK1 was cloned with an N-terminal His tag into the pFastBAC vector and expressed in a Baculovirus expression system using Sf9 insect cells. MSK1 was purified by affinity chromatography on Ni^{2+} -nitrilotriacetate-agarose using standard procedures. Purified recombinant MSK1 (0.2 mg/ml) was activated by incubation with 2 units/ml GST-ERK2 in 50 mM Tris/HCl (pH 7.5), 0.1 mM EGTA, 0.01 % 2-mercaptoethanol, 10 mM magnesium acetate and 0.1 mM ATP at 30°C for 30 min. GST-

ERK2 was then removed by incubation with GSH-Sepharose, and the MSK1 was repurified by affinity chromatography on Ni^{2+} -nitrilotriacetate-agarose. MSK1 was then allowed to autophosphorylate in 50 mM Tris/HCl (pH 7.5), 0.1 mM EGTA, 0.01 % 2-mercaptoethanol, 10 mM magnesium acetate and 0.1 mM [^{32}P]ATP at 30°C for 20 min. The reaction was stopped by the addition of SDS to 1 % and the samples were processed for MS as described below.

Immobilized metal affinity chromatography

Peptides from in-gel tryptic digests were reconstituted in a solution of 70 μl of 0.25 M acetic acid and 30 % (v/v) acetonitrile to which had been added approx. 4 μl of PHOS-select gel (Sigma; catalogue no. P9740). After shaking for 30 min at room temperature, the unbound peptides were eluted with $2 \times 25 \mu\text{l}$ washes of 0.25 M acetic acid/30 % acetonitrile before the elution of bound peptides with 30 μl of 0.4 M NH_4OH . The flow-through from this process was remixed with a further 4 μl of PHOS-select gel and the binding/elution process was repeated. The eluates were combined, dried in a SpeedVac, taken up in 20 μl of water, re-dried and dissolved in 10 μl of 50 % acetonitrile/0.1 % TFA (trifluoroacetic acid) before an aliquot, usually 0.5 μl , was taken for MS analysis as described below.

Phosphorylation site identification

Samples were run on a 4–12 % (w/v) polyacrylamide gel, the substrate band was excised and the amount of ^{32}P incorporated was determined by Cerenkov counting. For phospho-site mapping, the protein was reduced with dithiothreitol, alkylated with iodoacetamide and digested with trypsin. The resulting peptides were applied to a Vydac 218TP5215 C18 column equilibrated in 0.1 % TFA and the column was developed with a linear gradient of acetonitrile/0.1 % TFA at a flow rate of 0.2 ml/min while collecting 0.1 ml fractions. ^{32}P radioactivity was recorded with an online monitor. Phosphorylation site mapping was performed essentially as described previously [37]. Identification of ^{32}P -labelled peptides was performed by MALDI-TOF (matrix-assisted laser-desorption ionization-time of flight) and MALDI-TOF-TOF MS on an Applied Biosystems 4700 using a matrix of 10 mg/ml α -cyanocinnamic acid in 50 % acetonitrile/0.1 % TFA/10 mM ammonium phosphate. Sites of phosphorylation within the peptides were determined by a combination of MALDI-TOF-TOF-MS and solid-phase Edman sequencing. Solid-phase sequencing was performed on an Applied Biosystems Procise 494C after coupling the peptide covalently with a Sequelon-arylamine membrane and measuring the ^{32}P radioactivity released after each cycle by Cerenkov counting.

RESULTS

MSK1 is autophosphorylated on multiple sites *in vitro*

Active MSK1 is capable of undergoing significant autophosphorylation *in vitro*; however, autophosphorylation sites have not previously been identified. To map these sites, His-tagged MSK1 was expressed in a baculovirus system and purified by Ni^{2+} -affinity chromatography. The MSK1 was then activated using GST-ERK2, and then the GST-ERK2 was removed from the MSK1 by using both GSH-agarose and Ni^{2+} -affinity chromatography. MSK1 was then allowed to autophosphorylate; subsequently, it was run on an SDS/polyacrylamide gel, then digested with trypsin and the peptides were resolved by reversed-phase HPLC. Since ERK2 was removed before MSK1 was allowed to autophosphorylate in the presence of [^{32}P]ATP, the ERK2

Figure 1 Autophosphorylation of MSK1 *in vitro*

Recombinant MSK1, which had been previously phosphorylated with ERK2, was autophosphorylated *in vitro* as described in the Methods section. The autophosphorylated MSK1 was then run on an SDS/polyacrylamide gel and the MSK1 band excised, digested with trypsin, and the resulting peptides were run on reversed-phase HPLC as described in the Methods section. The ³²P elution trace is shown in (A). Phosphopeptides eluted in the ³²P-labelled fractions were identified by MS, and the positions of phosphate in the peptides were determined by solid-phase sequencing for each of the peaks (B–F).

Table 1 Analysis of *in vitro* MSK1 autophosphorylation

Peak	Residue	Peptide sequence	Phospho amino acid analysis	Solid-phase cycle no.	Phosphorylation site
1	748–756	KTSTSTETR	Phospho-Ser	3, 5	Ser-750, Ser-752
2	748–756	KTSTSTETR	Phospho-Ser	2, 3	Ser-750
	757–773	SSSESSHSSSHSHGK			Ser-758
3	210–226	AYFCGTIEYMAPDIVR	Phospho-Ser	3	Ser-212
4	210–226	AYFCGTIEYMAPDIVR	Phospho-Ser	3	Ser-212
5	371–385	LFQGYSFVAPSIILFK	Phospho-Ser	6, 11	Ser-376, Ser-381

phosphorylation sites in MSK1 (Ser-360 and Thr-581) would not be identified in the analysis of ³²P-labelled peptides. Five major peaks of radioactivity were obtained and the peptides in these

peaks were identified by MS. Phospho-amino acid analysis demonstrated that only phosphoserine was present in these five peaks (Figure 1, Table 1).

The first peak contained a peptide corresponding to residues 748–756 of MSK1 (Figure 1B). MS analysis of this peptide showed that it contained two phosphate groups at Ser-750 and Ser-752, and solid-phase sequencing confirmed that these positions were phosphorylated. The second peak contained two peptides; one peptide corresponded to residues 748–756 of MSK1, and both MS and solid-phase sequencing showed that it was phosphorylated on Ser-750 (Figure 1C). The second peptide corresponded to residues 757–773 of MSK1. Solid-phase sequencing suggested that this peptide was phosphorylated on Ser-758. The third peak contained a peptide corresponding to residues 210–226 of MSK1 and MS and solid-phase sequencing identified the phosphorylated residue as Ser-212, the threonine residue of the N-terminal kinase domain (Figure 1D). The fourth peak also corresponded to residues 210–226 phosphorylated on Ser-212; however, this peptide had become N-acetylated, which may explain its delayed elution on the HPLC gradient (Figure 1E). The yield of ^{32}P in the solid-phase sequencing at cycle 3 was only 7 % (Figure 1E), compared with a 50 % yield for the non-acetylated peptide (Figure 1D). If the peptide in peak 4 was acetylated at the N-terminus, this would explain the low yield, and the signal seen may be due to some carry over of the non-acetylated peptide from peak 3. The final peak contained a peptide corresponding to residues 371–385 (Figure 1F). Interestingly, MS analysis showed that, whereas this peptide contained only one phosphate, both MS/MS (tandem MS) and solid-phase sequencing showed that either Ser-376 or Ser-381 could be phosphorylated. No evidence for the dual-phosphorylated peptide was seen in the MS analysis; however, this may be due to low levels of ionization of the diphosphopeptide and does not indicate that the dual phosphorylation of Ser-376 and Ser-381 is not possible in MSK1.

MSK1 is phosphorylated on multiple sites in cells in response to mitogenic stimulation or cellular stress

By analogy with RSK, MSK1 should require phosphorylation of Ser-360 and Thr-581 by either ERK1/2 or p38 and autophosphorylation on Ser-376 for activation by mitogens or cellular stress. However, phosphorylation of the other *in vitro* sites in MSK1, namely Ser-212, Ser-381, Ser-750 and Ser-752 of MSK1, has not been previously shown in cells. To determine which residues were phosphorylated in MSK1, GST-tagged MSK1 was transfected into HEK-293 cells, which were then stimulated with either UV-C (a cellular stress that activates MSK1 through the p38 MAPK cascade) or PMA (a mitogen that activates MSK1 through the ERK1/2 cascade) [3]. Levels of MSK1 phosphorylated on Ser-212, Ser-360, Ser-376, Ser-381, Thr-581 or Ser-750 or dual phosphorylation of both Ser-750 and Ser-752 were determined by Western blotting of the lysates with phosphospecific antibodies to these sites. An antibody to the final site, Ser-758, is not currently available.

In unstimulated cells, no signal was seen for Ser-360, Ser-376, Ser-381 or Thr-581, and only weak signals were detected for Ser-212, Ser-750 and Ser-750/Ser-752. The phosphorylation of all these sites was stimulated by both UV-C and PMA (Figure 2A). The UV-C-induced phosphorylation of all these sites was blocked by the p38 MAPK inhibitor SB203580, consistent with a role of p38 in the phosphorylation of these sites, whereas the PMA-induced phosphorylation was blocked by PD184352, which blocks the activation of the ERK1/2 cascade. In HEK-293 cells, PMA was consistently found to give a greater activation of transfected MSK1 compared with UV-C. Since previous studies have suggested that both ERK1/2 and p38 are equally effective activators of recombinant MSK1 *in vitro* [3], the difference in PMA- and UV-C-induced activation of MSK1 in HEK-293 cells may reflect

Figure 2 Phosphorylation of MSK1 in HEK-293 cells

(A) Wild-type (WT), C-terminal kinase dead (CT-KD) or N-terminal kinase dead (NT-KD) GST-tagged MSK1 was expressed in HEK-293 cells by transient transfection. Cells were serum-starved for 16 h and incubated with PD184352 (2 μM) or SB203580 (5 μM) where indicated. Cells were then stimulated with UV-C (200 J/m 2 , followed by 30 min at 37°C) or PMA (TPA; 400 ng/ml, 10 min) as indicated and lysed as described in the Methods section. Lysates were immunoblotted using antibodies against GST, or MSK1 phosphorylated on Ser-212, Ser-360, Ser-376, Ser-381, Thr-581, Ser-750 or dual phosphorylated Ser-750/Ser-752. (B) Endogenous MSK1 was immunoprecipitated (IP) from HEK-293 cells and treated as indicated. Immunoprecipitates were run on SDS gels and blotted for total MSK1, phospho-Ser-212, Ser-360, Ser-376, Ser-381, Thr-581, Ser-750 and dual phosphorylated Ser-750/Ser-752.

differences in the levels of activity of ERK1/2 and p38 induced by these stimuli.

Ser-360 and Thr-581 are proline-directed phosphorylation sites that are expected to be direct phosphorylation targets of p38 or ERK1/2 rather than MSK autophosphorylation sites. Consistent with this, both sites were still phosphorylated in the MSK in which either the N- or C-terminal kinase domain had been inactivated by point mutation (Figure 2A, second and bottom panels). Mutation of the C-terminal kinase domain abolished the phosphorylation of MSK1 on Ser-212, Ser-376, Ser-381, Ser-750 and Ser-750/Ser-752, whereas inactivation of the N-terminal kinase domain only blocked phosphorylation of MSK1 on Ser-750 and Ser-750/Ser-752. This would be consistent with a mechanism by which the C-terminal kinase domain of MSK1 autophosphorylates MSK1 on Ser-212, Ser-376 and Ser-381, with at least one of these

phosphorylation sites being required for the activation of the N-terminal kinase domain. Once activated, the N-terminal kinase domain autophosphorylates Ser-750 and Ser-752. It is not possible to exclude the possibility, especially for Ser-212 as discussed below, that another kinase may be recruited to MSK1 in cells and is required for the phosphorylation of some of these sites.

It is possible that the phosphorylation of some of these sites in MSK1 is a result of overexpression and does not occur in endogenous protein. To confirm that all these sites are true physiological sites, endogenous MSK1 was immunoprecipitated from untransfected HEK-293 cells and the immunoprecipitate blotted using the phosphospecific antibodies. Similar to the transfected protein, UV-C and PMA were capable of stimulating the phosphorylation of endogenous MSK1 on Ser-212, Ser-360, Ser-376, Ser-381, Thr-581, Ser-750 and Ser-750/Ser-752 (Figure 2B).

MSK1 activity requires phosphorylation of Ser-376 and Thr-581

The Ser-360, Ser-376 and Thr-581 phosphorylation sites of MSK1 are conserved in RSK, and mutation of these sites in RSK inhibits RSK activity. To determine the requirement of each of these phosphorylations for MSK1 activity, each residue was mutated to an alanine residue. The protein was expressed in HEK-293 cells and the ability of PMA and UV-C to activate these mutants in cells was determined (Figure 3). As expected, mutation of Thr-581 to an alanine residue prevented the activation of MSK1 in cells in response to either PMA or UV-C. Interestingly, mutation of this site blocked the phosphorylation of Ser-212, Ser-376 and Ser-381 and significantly decreased the phosphorylation of Ser-360. The loss of Ser-212, Ser-376 and Ser-381 autophosphorylation in the Thr⁵⁸¹ → Ala mutation can be explained by the inactivity of this protein. Ser-360, however, lies in a consensus ERK/p38 sequence and is expected to be phosphorylated in the Thr⁵⁸¹ → Ala mutant. This suggests that phosphorylation on Thr-581 might induce a conformational change in MSK1 that increases the accessibility of Ser-360 to ERK1/2 or p38.

Mutation of Ser-360 decreased the activation of MSK1 by PMA or UV-C by approx. 60 %, but did not completely inactivate the enzyme (Figure 3). The Ser³⁶⁰ → Ala mutation was phosphorylated on Ser-212, Ser-376, Ser-381 and Thr-581, but at lower levels when compared with the wild-type protein. These decreases in phosphorylation of Ser-212, Ser-376 and Ser-381 may be due to the decrease in activity of the Ser³⁶⁰ → Ala protein. The decrease in phosphorylation of the second MAPK site, Thr-581, again raised the possibility that the phosphorylation of one MAPK site promotes the phosphorylation of the other site.

Mutation of Ser-376 to an alanine residue prevented the activation of MSK1 and, although it did not block the phosphorylation of the MAPK sites Ser-360 or Thr-581, the phosphorylation of these sites was decreased (Figure 3).

MSK1 can phosphorylate its T-loop in cells

Phosphorylation of the T-loop serine in RSK by PDK1 is required for activity. Mutation of Ser-212, the equivalent T-loop residue in MSK1, to an alanine residue also inactivates the N-terminal kinase domain (Figure 4A). However, Ser²¹² → Ala MSK1 was still phosphorylated by ERK or p38 on Ser-360 and Thr-581, and the C-terminal kinase domain was still able to phosphorylate Ser-376 and Ser-381, although decreased levels of phosphorylation were seen compared with the wild-type protein. Several lines of evidence suggest that MSK1 has the ability to autophosphorylate the N-terminal T-loop. Expression of MSK1 in *Escherichia coli* results in an MSK1 with a specific activity similar to the MSK1 purified from PMA-stimulated HEK-293 cells

Figure 3 Ser-376 and Thr-581 phosphorylation is required for MSK1 activity

Wild-type, Ser³⁶⁰ → Ala, Ser³⁷⁶ → Ala or Thr⁵⁸¹ → Ala FLAG-tagged MSK1 was expressed in HEK-293 cells by transient transfection. Cells were serum-starved for 16 h and then stimulated with UV-C (200 J/m², followed by 30 min at 37 °C) or PMA (TPA; 400 ng/ml, 10 min) as indicated and lysed as described in the Methods section. MSK1 was immunoprecipitated using an anti-FLAG antibody and assayed against a peptide substrate as described in the Methods section. The kinase activity from unstimulated cells is shown by open bars, UV-C stimulation as hatched bars and PMA stimulation as grey bars. One unit (U) of activity is defined as the amount of kinase required to incorporate 1 nmol of phosphate in 1 min. Error bars represent the S.D. for three assays. The same lysates were also immunoblotted with antibodies against FLAG or MSK1 phosphorylated on Ser-212, Ser-360, Ser-376, Ser-381 or Thr-581.

(results not shown). MSK1 purified from a baculovirus system is capable of phosphorylating its T-loop *in vitro* (Figure 1). Neither of these, however, exclude that another kinase could phosphorylate this residue in cells. To examine further the mechanism of Ser-212 phosphorylation in cells, both MS and phosphoblotting approaches were used. For MS analysis, GST-MSK1 or C-terminal kinase dead GST-MSK1 were transfected into MSK1/MSK2 double-knockout fibroblasts. The GST-MSK1 protein was purified from both unstimulated and PMA-stimulated cells, digested with trypsin and phosphopeptides were isolated using a Sigma PHOS-select metal chelate affinity matrix. MS analysis was then used to look for the presence of the peptide 210–226 of MSK1 phosphorylated on Ser-212. For the wild-type protein (Figure 5A), a peptide of the correct mass for a methionine-oxidized form of the expected phosphopeptide was seen (molecular mass, 2088.96 Da), and small peaks (molecular mass, 2072.9 Da) were also observed for the non-oxidized form of the peptide. The peptide of mass 2088 Da was also analysed by MS/MS and the fragment ions obtained corresponded to those expected from the peptide 210–226 with phospho-Ser-212, Met-Ox220 and Cys-214 as carbamidomethylcysteine (results not shown).

Figure 4 MSK1 autophosphorylates on Ser-212 in HEK-293 cells

(A) Wild-type MSK1 or Ser²¹² → Ala FLAG-tagged MSK1 was expressed in HEK-293 cells by transient transfection. Cells were serum-starved for 16 h and then stimulated with UV-C (200 J/m², followed by 30 min at 37°C) or with PMA (400 ng/ml, 10 min) as indicated and lysed as described in the Methods section. MSK1 was immunoprecipitated using an anti-FLAG antibody and assayed against a peptide substrate as described in the Methods section. One unit (U) of activity is defined as the amount of kinase required to incorporate 1 nmol of phosphate in 1 min. The kinase activity from unstimulated cells is shown by open bars, UV-C stimulation as hatched bars and PMA stimulation as grey bars. Error bars represent the S.D. for three assays. The same lysates were also immunoblotted with antibodies against FLAG or MSK1 phosphorylated on Ser-212, Ser-360, Ser-376, Ser-381 or Thr-581. (B) Same as (A) except that GST wild-type (WT), C-terminal kinase dead (CT-KD), Ser³⁷⁶ → Asp or C-terminal kinase dead/Ser³⁷⁶ → Asp were expressed in HEK-293 cells. MSK1 was pulled down from 0.2 mg of lysate with glutathione-Sepharose and assayed against a peptide substrate as described in the Methods section. The same lysates were also immunoblotted with antibodies against FLAG or MSK1 phosphorylated on either Ser-212 or Ser-381.

In the PMA-stimulated sample, both oxidized and non-oxidized forms of the peptide 210–226 were clearly seen in the wild-type protein (Figure 5E). Although this provides clear evidence that Ser-212 is phosphorylated in cells in wild-type MSK1, it is not possible to establish from the MS data the degree of

Figure 5 Phosphorylation of Ser-212 of MSK1 in HEK-293 cells

MS of the tryptic phosphopeptide (210–226 of MSK1) containing Ser-212. MSK1 [GST wild-type (A, E), C-terminal kinase dead (B, F), Ser³⁷⁶ → Asp (C, G) or C-terminal kinase dead/Ser³⁷⁶ → Asp (D, H)] was expressed in HEK-293 cells (control samples, A–D respectively) and from a similar set of PMA-stimulated cells (+PMA samples, E–H respectively). MSK1 was pulled down from 15 mg of lysate with glutathione-Sepharose and run on a 4–12% acrylamide/SDS gel. Bands corresponding to MSK1 were excised, reduced, alkylated with iodoacetamide and then digested or not with trypsin [37]. The resultant tryptic phosphopeptides were subjected to immobilized metal affinity chromatography as described in the Methods section, followed by MS on an Applied Biosystems 4700 MALDI-TOF-TOF with 0.25 µl of α-cyanocinnamic acid + 10 mM NH₄PO₃ as the matrix. The theoretical monoisotopic mass for the peptide 210–226 (AYFCGTIEYMAPDIVR) with one phosphoserine is 2072.89 Da, and with one phosphoserine and one oxidized methionine residue is 2088.88 Da (the cysteine residue having been converted into carbamidomethylcysteine).

stimulation of the phosphorylation by PMA. In contrast with wild-type protein, when C-terminal kinase dead MSK1 was used, no evidence for peptides of the correct mass for the peptide 210–226 with phospho-Ser-212 could be seen either before or after PMA stimulation (Figures 5B and 5F). Peptides of mass 2086 and 2068 were observed (Figures 5F and 5H); however, they gave MS/MS fragmentation data that indicated that they were similar to each other but not related to the Ser-212 peptides (results not shown). Although this might indicate that the C-terminal kinase domain of MSK1 directly phosphorylates Ser-212, it could also indicate that the phosphorylation of the 'hydrophobic motif' Ser-376 by the C-terminal domain is required to recruit a T-loop kinase to phosphorylate Ser-212, as it occurs with RSK and PDK1. To investigate this, Ser-376 was mutated to aspartic acid. This mutation did not prevent the activation of MSK1 by PMA or UV-C, demonstrating that the aspartic acid mutation successfully mimicked phosphorylation at this site (Figure 4B). GST Ser³⁷⁶ → Asp MSK1 and GST Ser³⁷⁶ → Asp C-terminal kinase dead were transfected into MSK1/MSK2 double-knockout fibroblasts and the phosphorylation of Ser-212 was examined. Analysis of the Ser³⁷⁶ → Asp MSK1 MS data clearly showed the expected peaks for the phosphorylated Ser-212 peptide both before and after PMA stimulation (Figures 5C and 5G). In contrast, no evidence was seen for these peptides in the C-terminal kinase

dead/Ser³⁷⁶ → Asp MSK1 (Figures 5D and 5F). When the spectra were analysed for the N-acetylated form of this peptide (as observed in Figure 1E during *in vitro* mapping), the N-acetylated form was only detected in the Ser³⁷⁶ → Asp PMA-stimulated sample and not in any of the other spectra.

Consistent with the MS results, when MSK1 was expressed in HEK-293 cells, C-terminal kinase dead MSK1 was not capable of phosphorylating Ser-212 as judged by phosphoblotting, in contrast with the wild-type protein where PMA strongly stimulated Ser-212 phosphorylation (Figure 4B). UV-C also stimulated Ser-212 phosphorylation in wild-type (but not C-terminal kinase dead) MSK1; however, in line with the lower activity of MSK1 induced by UV-C, the phosphorylation of Ser-212 induced by UV-C was lower when compared with PMA. Mutation of Ser-376 to aspartic acid did not affect Ser-212 phosphorylation or MSK activity, but the Ser³⁷⁶ → Asp mutation was unable to restore Ser-212 phosphorylation in the C-terminal kinase dead MSK1.

Phosphorylation of Ser-381

Phosphorylation of Ser-381 in MSK1 has not been shown previously, and its function is not clear. The site is conserved in MSK2 and RSK3; however, it is absent from RSK1 and RSK2. Mutation of Ser-381 to an alanine residue resulted in a decreased activity of MSK1 in the cells after PMA or UV-C stimulation (Figure 6). Mutation of Ser-381 did not significantly affect the phosphorylation of Ser-360 or Thr-581; however, it did result in some decrease in the phosphorylation of Ser-376 and to a lesser extent of Ser-212, as judged by immunoblotting with phosphospecific antibodies. This effect seemed more pronounced with UV-C stimulation compared with PMA stimulation.

Phosphorylation of C-terminal autophosphorylation sites

Of the three C-terminal phosphorylation sites, Ser-750 is conserved in MSK2 and RSK, whereas Ser-758 is conserved in MSK2. These C-terminal sites lie next to a potential ERK1/2 or p38 docking motif. Mutation of the two conserved arginine residues in this docking motif abolished or greatly decreased the activation and phosphorylation of MSK in cells (Figure 7A), indicating that the MAPK docking sequence is essential for MSK activation by either ERK1/2 or p38 (Figure 6A). In contrast, mutation of the conserved leucine residue in the docking sequence only partly inhibited the activation of MSK1 by PMA, but almost completely blocked the activation of MSK1 by UV-C. This was consistent with the analysis of phosphorylation of the MAPK sites, Ser-360 and Thr-581, in this mutant, since these residues showed some phosphorylation in response to PMA, but little or no phosphorylation in response to UV-C. However, mutation of both Ser-750 and Ser-752 to an alanine residue or Ser-758 to an alanine residue (Figure 7B) did not significantly affect the activation of MSK1 by either PMA or UV-C or the phosphorylation of other sites in MSK1 (Figure 6B). In addition, mutation of Ser-750 and Ser-752 to aspartic acid, which might be expected to interfere with the interaction of the positively charged MAPK docking sequence with the upstream kinase, did not affect MSK1 activation (Supplementary Figure 2; see <http://www.BiochemJ.org/bj/387/bj3870507add.htm>). In co-immunoprecipitation experiments, a weak interaction could be seen between FLAG-MSK1 and ERK1/2 and p38 α . Mutation of the MAPK docking site abolished this interaction; however, mutation of the phosphorylation of Ser-750, Ser-752 or Ser-758 to an alanine residue did not appear to affect MAPK binding to MSK1 (Figure 8).

Figure 6 Phosphorylation of Ser-381 is not essential for activity

Wild-type or Ser³⁸¹ → Ala FLAG-tagged MSK1 was expressed in HEK-293 cells by transient transfection. Cells were serum-starved for 16 h and then stimulated with UV-C (200 J/m², followed by 30 min at 37 °C) or PMA (400 ng/ml, 10 min) as indicated and lysed as described in the Methods section. MSK1 was immunoprecipitated using an anti-FLAG antibody and assayed against a peptide substrate as described in the Methods section. One unit (U) of activity was defined as the amount of kinase required to incorporate 1 nmol of phosphate in 1 min. The kinase activity from unstimulated cells is shown by open bars, UV-C stimulation as hatched bars and PMA stimulation as grey bars. Error bars represent the S.D. for three assays. The same lysates were also immunoblotted with antibodies against FLAG, phospho-Ser-360 MSK1, phospho-Ser-376 MSK1 or phospho-Thr-581 MSK1.

DISCUSSION

We provide evidence that MSK is regulated by multiple phosphorylation sites in cells and, on the basis of this, propose a model of MSK activation. Activation of MSK1 requires a MAPK docking sequence at the C-terminus of MSK1. Recruitment of active ERK1/2 or p38 allows the MAPK to phosphorylate MSK1 on two sites, Ser-360 and Thr-581. Of these, Thr-581 is essential for the activation of the C-terminal kinase domain. This domain then phosphorylates two further sites in the linker region of MSK1, Ser-376 and Ser-381, as well as the T-loop residue of the N-terminal kinase domain, Ser-212. Interestingly, mutation of either of the MAPK sites in MSK1 resulted in decreased phosphorylation of the second MAPK site. This may suggest that Thr-581 or Ser-360 phosphorylation may cause a conformational change in MSK1 that makes the other site more accessible to phosphorylation. This apparent co-operativity between phosphorylation sites was also suggested by other mutations, especially of other

Figure 7 MSK1 requires a MAPK docking site for activation

(A) Wild-type, Leu⁷⁴⁰ → Ala or Arg⁷⁴³ → Ala/Arg⁷⁴⁴ → Ala FLAG-tagged MSK1 was expressed in HEK-293 cells by transient transfection. Cells were serum-starved for 16 h and then stimulated with UV-C (200 J/m², followed by 30 min at 37 °C) or PMA (400 ng/ml, 10 min) as indicated and lysed as described in the Methods section. MSK1 was immunoprecipitated using an anti-FLAG antibody and assayed against a peptide substrate as described in the Methods section. One unit (U) of activity was defined as the amount of kinase required to incorporate 1 nmol of phosphate in 1 min. The kinase activity from unstimulated cells is shown by open bars, UV-C stimulation as hatched bars and PMA stimulation as grey bars. Error bars represent the S.D. for three data points. The same lysates were also immunoblotted with antibodies against FLAG, or MSK1 phosphorylated on Ser-212, Ser-360, Ser-376, Ser-381, Thr-581, Ser-750 or dual phosphorylated Ser-750/Ser-752. (B) Same as (A) except that wild-type, Ser⁷⁵⁰ → Ala/Ser⁷⁵² → Ala or Ser⁷⁵⁸ → Ala was expressed in HEK-293 cells.

Figure 8 C-terminal phosphorylation of MSK1 does not affect its interaction with MAPKs

FLAG-MSK1 (wild-type, Leu⁷⁴⁰ → Ala, Arg⁷⁴² → Ala/Arg⁷⁴³ → Ala, Ser⁷⁵⁰ → Ala/Ser⁷⁵² → Ala or Ser⁷⁵⁸ → Ala) was transfected into HEK-293 cells. Cells were serum-starved for 16 h and then stimulated with UV-C (200 J/m², followed by 30 min at 37 °C) or PMA (400 ng/ml, 10 min) as indicated and lysed as described in the Methods section. MSK1 was immunoprecipitated using an anti-FLAG antibody and immunoprecipitates were run on SDS gels. Gels were immunoblotted for FLAG, ERK1, ERK2 or p38α.

sites essential for MSK1 activity. Mutation of either Ser-212 or Ser-376 also decreased the phosphorylation of other regulatory sites in MSK1. One explanation of this is that each of these phosphorylations helps to stabilize the active conformation of MSK1, making the other phosphorylation sites either more accessible

for phosphorylation or less accessible to phosphatases in cells. In this respect, it is worth noting that MSK1 activation in cells is transient and that the mechanisms and kinetics of MSK1 dephosphorylation and inactivation are not well understood. Although a structure for the N-terminal kinase domain has been reported [38], the structure of the full-length MSK1 has not been solved. Knowledge of the three-dimensional structure of active and inactive MSK1 would, however, aid the interpretation of these results.

Phosphorylations of Ser-212 and Ser-376 are essential for the activation of the N-terminal kinase domain, whereas Ser-381 phosphorylation promotes but is not essential for this activation. Mutation of Ser-381 resulted in a decrease in Ser-376 phosphorylation, suggesting that a negative charge at Ser-381 may promote or stabilize the phosphorylation of Ser-376. Once activated, the N-terminal kinase domain phosphorylates Ser-750, Ser-752 and Ser-758 and is probably also responsible for the phosphorylation of MSK substrates. Phosphorylation of the C-terminal sites is not required for MSK1 activation.

MSKs are closely related to the RSK kinases and, similar to RSK, they contain two kinase domains in a single polypeptide. Although the regulation of RSK activation and that of MSK activation are similar and many of the regulatory phosphorylation sites are conserved (Figure 9), there are two significant differences in the mechanisms of activation for RSK and MSK. Phosphorylation of the T-loop residue in RSK is performed by PDK1 in cells; however, in MSK1, the T-loop appears to be a site of autophosphorylation by the C-terminal kinase domain. PDK1

Figure 9 Sequence alignment of MSK and RSK around phosphorylation sites

The sequences around the T-loop, linker and C-terminal phosphorylation sites of MSK were aligned with RSK. Phosphorylation sites are highlighted in black and conserved residues are highlighted in grey. The hydrophobic motif in RSK is underlined and the MAPK docking site is indicated by asterisks. Black 'lollipops' are phosphorylation sites that are conserved between MSK and RSK isoforms. White 'lollipops' are phosphorylation sites that are unique to MSK1.

does not phosphorylate this site in cells, even though it lies in a PDK1 consensus motif [28] and can be phosphorylated by PDK1 *in vitro*. One possible reason for this is that, in cells, PDK1 is recruited to RSK by binding to the phosphorylated hydrophobic motif in RSK. Although the equivalent residue is present and phosphorylated in MSK1, the sequence surrounding this site is not conserved (Figure 9), suggesting that it may not be able to function as a PDK1 docking motif. In addition, we found that mutation of this residue to aspartic acid to mimic phosphorylation did not affect MSK activation, this mutation was not able to rescue Ser-212 phosphorylation in a C-terminal kinase dead MSK1. This suggests that Ser-212 phosphorylation in MSK1 is due to autophosphorylation and not due to recruitment of another kinase.

The second major difference is in how MSK and RSK associate with their upstream kinases. When inactive, RSK is reported to be associated with ERK1/2 in cells through a docking motif at the C-terminus of RSK. On activation, RSK autophosphorylates sites in its C-terminus adjacent to the docking motif, and these phosphorylations promote the dissociation of RSK and ERK1/2. MSK also contains a potential docking motif, similar to both the docking motif of RSK and the p38-activated kinase, MAPKAP-K2. The sequence of this docking motif probably determines which MAPK kinases can bind and activate RSK, MSK and MAPKAP-K2 in cells, since replacing the docking site in RSK with that of MAPKAP-K2 converts RSK from an ERK1/2-activated kinase into a p38-activated kinase [39]. Interestingly, whereas both p38 and ERK1/2 require the RR sequence in the MSK1 docking site to activate MSK1, p38 seems to have a greater dependence on the leucine residue in the docking site compared with ERK1/2, as shown by the lack of UV-C-induced MSK1 activity of the leucine mutant. In contrast with RSK, MSK1 appears to be only weakly associated with its upstream kinase in cells, although we show that the MAPK docking motif is required for the phosphorylation of MSK by ERK1/2 or p38. Mutation of the conserved basic residues in this motif inhibits MSK activation and phosphorylation by ERK1/2 or p38, suggesting that this region serves to recruit transiently ERK1/2 or p38 to MSK1. Like RSK, MSK1 phosphorylates residues next to its ERK/p38 docking motif; however, unlike RSK, mutation of these sites does not seem to regulate the dissociation of MSK from its upstream kinase. This mechanism has potential implications for the use of inactive mutants of these kinases as dominant-negative mutants to study their function. Overexpression of a dominant-negative inactive RSK would give protein that bound to ERK1/2. On stimulation of the ERK1/2 cascade, ERK1/2 would phosphorylate RSK, but the inactive RSK would be incapable of phosphorylating the C-terminal sites next to the ERK1/2 docking site. This would mean that the active ERK1/2 would be held in a complex

with the dominant-negative RSK, instead of dissociating from RSK as would happen with the wild-type RSK protein. Since the dominant-negative RSK may sequester much of the active ERK1/2 in the cells, there may not be sufficient free active ERK to be recruited to and activate MSK1. The recent observation that dominant-negative RSK can block MSK activation is most probably explained by this mechanism [8].

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REFERENCES

- Pierrat, B., Correia, J. d. S., Mary, J.-L., Tomas-Zuber, M. and Lesslauer, W. (1998) RSK-B, a novel ribosomal S6 kinase family member, is a CREB kinase under dominant control of p38alpha mitogen-activated protein kinase (p38alpha MAPK). *J. Biol. Chem.* **273**, 29661–29671.
- New, L., Zhao, M., Li, Y., Bassett, W. W., Feng, Y., Ludwig, S., Padova, F. D., Gram, H. and Han, J. (1999) Cloning and characterization of RLKP, a novel RSK-related protein kinase. *J. Biol. Chem.* **274**, 1026–1032.
- Deak, M., Clifton, A. D., Lucocq, J. M. and Alessi, D. R. (1998) Mitogen- and stress-activated protein kinase-1 (MSK1) is directly activated by MAPK and SAPK/p38, and may mediate activation of CREB. *EMBO J.* **17**, 4426–4441.
- Arthur, J. S. C. and Cohen, P. (2000) MSK1 is required for CREB phosphorylation in response to mitogens in mouse embryonic stem cells. *FEBS Lett.* **482**, 44–48.
- Wiggin, G. R., Soloaga, A., Foster, J. M., Murray-Tait, V., Cohen, P. and Arthur, J. S. (2002) MSK1 and MSK2 are required for the mitogen- and stress-induced phosphorylation of CREB and ATF1 in fibroblasts. *Mol. Cell. Biol.* **22**, 2871–2881.
- Thomson, S., Clayton, A. L., Hazzalin, C. A., Rose, S., Barratt, M. J. and Mahadevan, L. C. (1999) The nucleosomal response associated with immediate-early gene induction is mediated via alternative MAP kinase cascades: MSK1 as a potential histone H3/HMG-14 kinase. *EMBO J.* **18**, 4779–4793.
- Soloaga, A., Thomson, S., Wiggin, G. R., Rampersaud, N., Dyson, M. H., Hazzalin, C. A., Mahadevan, L. C. and Arthur, J. S. (2003) MSK2 and MSK1 mediate the mitogen- and stress-induced phosphorylation of histone H3 and HMG-14. *EMBO J.* **22**, 2788–2797.
- Arthur, J. S. C., Fong, A. L., Dwyer, J. M., Davare, M., Reese, E., Obrietan, K. and Impey, S. (2004) Mitogen- and stress-activated protein kinase 1 mediates cAMP response element-binding protein phosphorylation and activation by neurotrophins. *J. Neurosci.* **24**, 4324–4332.
- Schuck, S., Soloaga, A., Schmitt, G., Arthur, J. S. and Nordheim, A. (2003) The kinase MSK1 is required for induction of c-fos by lysophosphatidic acid in mouse embryonic stem cells. *BMC Mol. Biol.* **4**, 6.
- Janknecht, R. (2003) Regulation of the ERB1 transcription factor and its coactivators by mitogen- and stress-activated protein kinase 1 (MSK1). *Oncogene* **22**, 746–755.
- Vermeulen, L., De Wilde, G., Van Damme, P., Vanden Berghe, W. and Haegeman, G. (2003) Transcriptional activation of the NF-kappaB p65 subunit by mitogen- and stress-activated protein kinase-1 (MSK1). *EMBO J.* **22**, 1313–1324.

- 12 Blenis, J. (1993) Signal transduction via the MAP kinases: proceed at your own RSK. *Proc. Natl. Acad. Sci. U.S.A.* **90**, 5889–5892
- 13 Frodin, M. and Gammeltoft, S. (1999) Role and regulation of 90 kDa ribosomal S6 kinase (RSK) in signal transduction. *Mol. Cell. Endocrinol.* **151**, 65–77
- 14 Roux, P. P. and Blenis, J. (2004) ERK and p38 MAPK-activated protein kinases: a family of protein kinases with diverse biological functions. *Microbiol. Mol. Biol. Rev.* **68**, 320–344
- 15 Bjorbaek, C., Zhao, Y. and Moller, D. E. (1995) Divergent functional roles for p90Rsk kinase domains. *J. Biol. Chem.* **270**, 18848–18852
- 16 Moller, D. E., Xia, C. H., Tang, W., Zhu, A. X. and Jakubowski, M. (1994) Human rsk isoforms: cloning and characterization of tissue-specific expression. *Am. J. Physiol.* **266**, C351–C359
- 17 Manning, G., Whyte, D. B., Martinez, R., Hunter, T. and Sudarsanam, S. (2002) The protein kinase complement of the human genome. *Science* **298**, 1912–1934
- 18 Sturgill, T. W., Ray, L. B., Erikson, E. and Maller, J. L. (1988) Insulin-stimulated MAP-2 kinase phosphorylates and activates ribosomal protein S6 kinase II. *Nature (London)* **334**, 715–718
- 19 Alessi, D. R., Cuenda, A., Cohen, P., Dudley, D. T. and Saltiel, A. R. (1995) PD 098059 is a specific inhibitor of the activation of mitogen-activated protein kinase kinase *in vitro* and *in vivo*. *J. Biol. Chem.* **270**, 27489–27494
- 20 Smith, J. A., Poteet-Smith, C. E., Malarkey, K. and Sturgill, T. W. (1999) Identification of an extracellular signal-regulated kinase (ERK) docking site in ribosomal S6 kinase, a sequence critical for activation by ERK *in vivo*. *J. Biol. Chem.* **274**, 2893–2898
- 21 Roux, P. P., Richards, S. A. and Blenis, J. (2003) Phosphorylation of p90 ribosomal S6 kinase (RSK) regulates extracellular signal-regulated kinase docking and RSK activity. *Mol. Cell. Biol.* **23**, 4796–4804
- 22 Gavin, A. C. and Nebreda, A. R. (1999) A MAP kinase docking site is required for phosphorylation and activation of p90(rsk)/MAPKAP kinase-1. *Curr. Biol.* **9**, 281–284
- 23 Dalby, K. N., Morrice, N., Caudwell, F. B., Avruch, J. and Cohen, P. (1998) Identification of regulatory phosphorylation sites in mitogen-activated protein kinase (MAPK)-activated protein kinase-1a/p90rsk that are inducible by MAPK. *J. Biol. Chem.* **273**, 1496–1505
- 24 Zhao, Y., Bjorbaek, C. and Moller, D. E. (1996) Regulation and interaction of pp90rsk isoforms with mitogen-activated protein kinases. *J. Biol. Chem.* **271**, 29773–29779
- 25 Tanoue, T., Adachi, M., Moriguchi, T. and Nishida, E. (2000) A conserved docking motif in MAP kinases common to substrates, activators and regulators. *Nat. Cell Biol.* **2**, 110–116
- 26 Chrestensen, C. A. and Sturgill, T. W. (2002) Characterization of the p90 ribosomal S6 kinase 2 carboxyl-terminal domain as a protein kinase. *J. Biol. Chem.* **277**, 27733–27741
- 27 Mora, A., Komander, D., van Aalten, D. M. and Alessi, D. R. (2004) PDK1, the master regulator of AGC kinase signal transduction. *Semin. Cell Dev. Biol.* **15**, 161–170
- 28 Frodin, M., Antal, T. L., Dummier, B. A., Jensen, C. J., Deak, M., Gammeltoft, S. and Biondi, R. M. (2002) A phosphoserine/threonine-binding pocket in AGC kinases and PDK1 mediates activation by hydrophobic motif phosphorylation. *EMBO J.* **21**, 5396–5407
- 29 Jensen, C. J., Buch, M.-B., Krag, T. O., Hemmings, B. A., Gammeltoft, S. and Frodin, M. (1999) 90-kDa ribosomal S6 kinase is phosphorylated and activated by 3-phosphoinositide-dependent protein kinase-1. *J. Biol. Chem.* **274**, 27168–27176
- 30 Williams, M. R., Arthur, J. S., Balendran, A., van der Kaay, J., Poli, V., Cohen, P. and Alessi, D. R. (2000) The role of 3-phosphoinositide-dependent protein kinase 1 in activating AGC kinases defined in embryonic stem cells. *Curr. Biol.* **10**, 439–448
- 31 Frodin, M., Jensen, C. J., Merienne, K. and Gammeltoft, S. (2000) A phosphoserine-regulated docking site in the protein kinase RSK2 that recruits and activates PDK1. *EMBO J.* **19**, 2924–2934
- 32 McManus, E. J., Collins, B. J., Ashby, P. R., Prescott, A. R., Murray-Tait, V., Armit, L. J., Arthur, J. S. and Alessi, D. R. (2004) The *in vivo* role of PtdIns(3,4,5)P(3) binding to PDK1 PH domain defined by knockin mutation. *EMBO J.* **23**, 2071–2082
- 33 Collins, B. J., Deak, M., Arthur, J. S., Armit, L. J. and Alessi, D. R. (2003) *In vivo* role of the PIF-binding docking site of PDK1 defined by knock-in mutation. *EMBO J.* **22**, 4202–4211
- 34 Tomas-Zuber, M., Mary, J.-L. and Lesslauer, W. (2000) Control sites of ribosomal S6 kinase B and persistent activation through tumor necrosis factor. *J. Biol. Chem.* **275**, 23549–23558
- 35 Durocher, Y., Perret, S. and Kamen, A. (2002) High-level and high-throughput recombinant protein production by transient transfection of suspension-growing human 293-EBNA1 cells. *Nucleic Acids Res.* **30**, e9
- 36 Harlow, E. and Lane, D. (1998) *Antibodies: A Laboratory Manual*, Cold Spring Laboratory Press, Plainview, NY
- 37 Campbell, D. G. and Morrice, N. (2002) Identification of protein phosphorylation sites by a combination of mass spectrometry and solid phase Edman sequencing. *J. Biomol. Tech.* **13**, 119–130
- 38 Smith, K. J., Carter, P. S., Bridges, A., Horrocks, P., Lewis, C., Pettman, G., Clarke, A., Brown, M., Hughes, J., Wilkinson, M. et al. (2004) The structure of MSK1 reveals a novel autoinhibitory conformation for a dual kinase protein. *Structure (Camb.)* **12**, 1067–1077
- 39 Smith, J. A., Poteet-Smith, C. E., Lannigan, D. A., Freed, T. A., Zoltoski, A. J. and Sturgill, T. W. (2000) Creation of a stress-activated p90 ribosomal S6 kinase. The carboxyl-terminal tail of the MAPK-activated protein kinases dictates the signal transduction pathway in which they function. *J. Biol. Chem.* **275**, 31588–31593

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