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Identification of novel phosphorylation sites in MSK1 by precursor ion scanning mass spectrometry.

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Regulation of MSK1 activation

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Abstract

MSK1 is a dual kinase domain protein that acts downstream of the ERK1/2 and p38 MAPK signalling pathways in cells. MSK1, and its related isoform MSK2, phosphorylate the transcription factors CREB and ATF1, and the chromatin proteins histone H3 and HMGN1 in response to either mitogenic stimulation or cellular stress. MSK1 activity is tightly regulated in cells and activation requires the phosphorylation of MSK1 by either ERK1/2 or p38 α . This results in activation of the C-terminal kinase domain, which then phosphorylates further sites in MSK1 leading to the activation of the N-terminal kinase domain and phosphorylation of substrates. Here we use precursor ion scanning mass spectrometry to identify 5 previously unknown sites in MSK1, Thr630, Ser647, Ser657, Ser695 and Thr700. One of these sites, Thr700, was found to be a third site in MSK1 phosphorylated by the upstream kinases ERK1/2 and p38 α . Mutation of Thr700 resulted in an increased basal activity of MSK1, but this could be further increased by stimulation with PMA or UV-C. Surprisingly however mutation of Thr700 resulted in a dramatic loss of Thr581 phosphorylation, a site essential for activity. Mutation of Thr700 and Thr581 to alanine resulted in an inactive kinase, while mutation of both sites to aspartic acid resulted in a kinase with a significant basal activity but which could not be further stimulated. Together these results are consistent with a mechanism by which Thr700 phosphorylation relieves the inhibition of MSK1 and a C-terminal autoinhibitory helix and helps induce a conformational shift that protects Thr581 from dephosphorylation.

Introduction

MSK1 and MSK2 are closely related kinases involved in the regulation of gene transcription downstream of mitogenic signalling, proinflammatory cytokines and cellular stress. MSKs are able to regulate transcription by the phosphorylation of both transcription factors, including CREB and ATF1 [1-6], and the chromatin associated proteins HMG-14 and histone H3 [7-10]. Genes reported to be regulated by MSK downstream of CREB or ATF1 include, c-fos [11], junB [4], MUC5A [12] and the NR4A group of nuclear orphan receptors [13].

The molecular mechanism of MSK activation is complex and requires multisite phosphorylation by an upstream kinases (either ERK1/2 or p38 α) and subsequent autophosphorylation for its activity. MSKs are most closely related to the RSK family of kinases and, like RSK, MSKs contain two distinct kinase domains within a single polypeptide [14-16]. The N-terminal domain, which is thought to phosphorylate MSK substrates, is a member of the AGC family of kinases, while the C-terminal kinase domain is related to the calmodulin dependent protein kinase family [17]. MSK1 was originally identified [14] through a search for novel AGC kinases, which might be regulated by PDK1 – a ‘master’ kinase required for the activation of a subset of AGC kinases including RSK. PDK1 acts by phosphorylating a specific residue in the T loop of its substrates, and the phosphorylation of this site is essential for kinase activation [18-20]. Work using PDK1 ES^{-/-} cells confirmed that PDK1 was required for the activation of RSK1, 2 and 3 [20]. Surprisingly however the use of these knockout cells has also demonstrated that the activation of MSK1 [20] and RSK4 [21] is independent of PDK1, and that T loop phosphorylation for these kinases occurs via a different mechanism.

In cells it has been shown that the activation of MSK by mitogens could be blocked by inhibitors of the ERK1/2 cascade, while the activation of MSK by cellular stress was blocked by inhibitors of p38 α / β [Pierrat, 1998 #49][14, 15]. While both p38 α and β can phosphorylate MSK1 *in vitro* [14], p38 α appears to be the isoform responsible *in vivo* as MSK1 activation is greatly reduced in cells from p38 α , but not p38 β , knockout mice [13, 22]. In response to agonists capable of activating both ERK1/2 and p38, such

as NGF or TNF, it was found that inhibitors of both pathways were required to block MSK activation [14-16, 23].

Based on the similarity of MSKs with the ERK1/2 regulated kinase RSK it was suggested that MSK was activated by phosphorylation on two sites by the upstream MAP Kinase [14]. One site was in the activation loop of the C-terminal kinase domain (Thr581 in human MSK1) while the other (Ser360) was in the linker region between the two domains. Subsequent work has shown that mutagenesis of these sites in MSK1 and MSK2 prevents the full activation of MSK, and these sites have been shown to be phosphorylated in cells by ERK1/2 or p38 [24, 25]. Phosphorylation of MSK1 by ERK1/2 or p38 was found to activate the C-terminal kinase domain, which then autophosphorylates two sites in the linker region (Ser376 and Ser381) and a site in the T loop of the N-terminal kinase domain (Ser212). Of these 3 sites, Ser212 and Ser376 appear to be essential for MSK activation as their mutation to alanine prevents MSK1 activation [24]. These autophosphorylation events activate the N-terminal kinase domain, allowing the phosphorylation of 3 sites in the C-terminus of MSK1 (Ser750, Ser752 and Ser758), as well as the phosphorylation of MSK substrates such as CREB and ATF1.

Here we report the use of a 4000 Q TRAP mass spectrometry system to identify further cellular phosphorylation sites in MSK1 and examine the role of these sites in the activation of MSK1.

Methods

Plasmids

Mammalian expression vectors for FLAG and GST tagged MSK1 have been described previously [14]. Mutagenesis was performed by PCR using the quickchange mutagenesis kit (Stratagene). The MSK1 encoding region of each expression vector was fully sequenced to confirm the presence of the desired mutation. DNA sequencing was carried out by the sequencing service (School of Life Sciences, University of Dundee, Scotland, www.dnaseq.co.uk) using Applied Biosystems Big-Dye Ver3.1 chemistry on a capillary sequencer.

Antibodies

MSK1 antibodies against phospho Ser360, phospho Ser376 and phospho Thr581 were from Cell Signalling while the phospho Ser212, phospho Ser381 phospho Ser750 and dual phosphorylated Ser750/752 and GST antibodies have been described previously [24]. FLAG antibody was from Sigma. The phospho Thr700 antibody was raised in a rabbit as described [26] against the phospho peptide SNPLM(phosphoT)PDIL (695-704 of MSK1). The resulting antibody was affinity purified from serum against the immunogenising phospho peptide.

Cell culture

HEK-293 cells were cultured in DMEM containing 10% FBS (Sigma), 2 mM L-glutamine, 50 units/ml penicillin G and 50µg/ml streptomycin (Invitrogen). HEK-293 cells were transfected using a modified calcium phosphate protocol as described [14]. Purification of GST-MSK1 from transfected HEK-293 cell lysates was carried out on glutathione Sepharose as described previously [14].

Q-Trap mass spectrometer phosphorylation site analysis.

GST-MSK1 samples were separated by SDS-PAGE, stained with colloidal Coomassie Blue (Invitrogen) and the gel bands were excised and digested in 50mM Triethyl ammonium bicarbonate with 5 µg/ml trypsin (Promega) at 30°C for 18hrs. The supernatant was removed, the gel pieces were extracted with 2.5% formic acid/50% acetonitrile and the combined extracts were dried under vacuum. Digests were reconstituted in 0.1ml of 1% formic acid in water and analysed by LC-MS on an LC-Packings Ultimate HPLC system interfaced to an Applied Biosystems 4000 Q TRAP system.

Peptides were separated on a 150 x 0.075mm PepMapC₁₈ column equilibrated in 0.1% formic acid in water at a flow rate of 350nl/min and eluted with a discontinuous acetonitrile gradient at the same flow rate. The column eluate was mixed with a sheath liquid of 40% isopropanol/water at 300 nl/min using a capillary mixing Tee (Upchurch) and the combined flow plumbed into the microionspray head of the 4000 Q TRAP system mass spectrometer fitted with a New Objectives Picotip emitter (FS-360 75 15 N).

Electrospray mass spectrometry was performed in an automated precursor of 79 duty cycle (6 sec total) in negative ion mode (-2300V), with Q1 masses scanned between 500-2000 m/z (3sec), collided with a variable collision energy of -65 to -110V and a daughter ions detected in Q3 after trapping and expelling from the linear ion trap (50ms fill time). If a daughter ion of PO_3^- was detected, the polarity at the microionspray head was automatically switched to positive ion mode (+2400V after 700ms dwell) and an enhanced resolution scan followed by an enhanced product ion scan (ms/ms) of the precursors was performed. The polarity was then switched back to -2300V and the duty cycle repeated. All the ms/ms spectra were searched against local databases using the Mascot search engine (MatrixScience) run on a local server and sites of phosphorylation were manually assigned from individual ms/ms spectra viewed using Bioanalyst software (MDS-Sciex).

A list of phosphopeptides to be analysed by Multiple Reaction Monitoring were generated using the MRM Builder Script supplied by MDS-Sciex as described [27].

MSK1 kinase assays

For kinase assays, FLAG-MSK1 was immuno-precipitated from 0.2 mg of pre-cleared cell lysate using 2 μg of anti-FLAG coupled to protein G sepharose, or GST-MSK1 pulled down with 10 μl of glutathione sepharose. Precipitates were washed twice in 0.5M NaCl, 50 mM Tris-HCl pH 7.5, 0.1 mM EGTA and 0.1% (v/v) 2-mercaptoethanol and once in 50 mM Tris-HCl pH 7.5, 0.1 mM EGTA and 0.1% (v/v) 2-mercaptoethanol. Precipitates were then resuspended in 35 μl of reaction buffer (containing Tris/HCl pH7.5, EGTA, PKI and Crosstide peptide, GRPRTSSFAEG), and the reaction was started by the addition of 10 μl 50 mM MgAc, 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 pH7.5, 0.1mM EGTA, 0.1% 2-mercaptoethanol 2.5 μM PKI (an inhibitor of PKA), 30 μM Crosstide peptide, 10 mM MgAc and 0.1 mM ^{32}P -ATP. Reactions were stopped by transfer onto P81 paper and washing in 75 mM orthophosphoric acid. One unit was defined as the incorporation of 1 nmole of phosphate into the substrate peptide in 1 min. To account for minor differences in the expression level of different constructs, the value for each sample was normalised relative to MSK expression. MSK1 expression was

quantified using immunoblotting for the FLAG tag on the MSK1 constructs. Blots were visualised using the Licor Odyssey system and densitometry of bands carried out using Li-cor software.

In vitro phosphorylation of MSK1

A GST- MSK1 C-terminal kinase domain -His tag construct, which comprised of amino acids 398 to 802 of the human MSK1 sequence was expressed in *E. coli* and purified by affinity chromatography on glutathione Sepharose and then NiNTA Sepharose using standard techniques. 2 µg of MSK1 or myelin basic protein was incubated with 40 mUnits of active GST-ERK2 or GST-p38α in 50 mM Tris/HCl pH7.5, 0.1mM EGTA, 0.1% 2-mercaptoethanol 2.5 µM PKI, 10 mM MgAc and 0.1 mM 32P-ATP. After 20 min at 30°C, the reaction was stopped by the addition of SDS sample buffer.

Immunoblotting

Soluble cell extract (25 µg) was run on 4-12% NuPAGE gels (Invitrogen) and transferred onto nitro-cellulose membranes and blotted according to the manufactures protocols. For total antibodies, membranes were blocked in 5% w/v dried milk in 1X TBS-T (50mM Tris/HCl pH 7.6, 150 mM NaCl, 0.1% v/v Tween 20) and the primary antibody incubation carried out in the same buffer. For phospho antibodies blocking and primary antibody incubations were carried out in Odyssey blocking reagent (Li-cor). Commercial antibodies were used according to manufactures recommendations and in house antibodies were used at 1 µg/ml in the presence of 10 µg/ml of the appropriate dephospho peptide.

Detection was achieved using secondary antibodies coupled to an appropriate fluorophore and the membranes scanned using a Li-cor Odyssey Scanner. Densitometry of bands carried out using Li-cor software according to manufactures protocols.

Results

Analysis of MSK1 phosphorylation by precursor ion scanning mass spectrometry

GST-MSK1 was expressed in HEK-293 cells by transient transfection, and the GST-MSK1 was then purified by affinity chromatography from the lysates of cells which had either been serum starved or stimulated with either PMA or UV-C (Fig 1 A-C). Purified MSK1 was run on SDS polyacrylamide gels and the MSK1 band excised and digested with trypsin. The tryptic peptides were analysed on a 4000 Q TRAP system using precursor ion scanning to identify phosphorylated peptides. Potential phosphorylated peptides were then identified by ms/ms. For MSK purified from PMA stimulated cells, this resulted in the identification of 7 peaks, and for 6 of these ms/ms analysis was able to establish that the sequence corresponded to an MSK1 fragment (Fig 1C). The 7th peak corresponded to a peptide from the linker region between GST and MSK1. Two of the peaks corresponded to residues 371 to 385 of MSK1 that had been phosphorylated on Ser376 or on both Ser376 and Ser381 (Fig 1, peaks 1 and 2). These sites had been previously identified as sites phosphorylated by the C-terminal kinase domain [24]. A peptide corresponding to 210 to 226 of MSK1 that was phosphorylated on Ser212, another site phosphorylated by the C-terminal kinase domain, was also identified in this analysis (Fig 1, peak 3). The remaining three peptides all corresponded to previously unidentified sites in MSK1. Two of these peptides corresponded to residues 683 to 716 of MSK1 with either one or two phosphates. The mono phosphorylated peptide contained a phosphate at Thr700 while the dual phosphorylated peptide contained phosphate at both Ser695 and Thr700 (Fig 1, peaks 5 and 6). The final peptide corresponded to 644 to 661 of MSK1 and contained phosphates on residues Ser647 and Ser657 (Fig 1 peak4). Similar peptides were also identified in MSK1 purified from UV-C stimulated cells (Fig 1B), although at reduced levels compared to PMA. This reduction is consistent with the weaker activation of MSK1 by UV-C than PMA in this system (see Fig 2). Peak 1 (S376) was not detected above the background in the UV-C sample. It is likely however that this reflects a problem of sensitivity rather than a lack of S376 phosphorylation for two reasons. UV-C stimulation did result in the detection of peak 2, which corresponds to Ser376 and Ser381 phosphorylation. In addition UV-C has previously been shown to induce phosphorylation of MSK1 on S376, as judged by immunoblotting [24]. The levels of the phospho MSK1 peaks from unstimulated cells were not detected above background (Fig 1A). The two known

MAPK sites, Ser360 and Thr581, were not identified in this analysis. The Thr581 tryptic peptide (residues 571-627 + PO₄ = 6653.19 Da) would generate an ion outside the useable mass range of the 4000 Q TRAP system mass spectrometer. The reason Ser360 phosphorylation was not detected was not clear, however it could be a result of a trypsin miscleavage resulting a peptide outside the mass range of the spectrometer.

Ser647, Ser657 and Ser695 phosphorylation is not required for MSK activation.

The requirement for phosphorylation on Ser212, Ser376 or Ser381 for MSK1 activity has been investigated previously by site directed mutagenesis [24]. To examine if the novel sites, Ser647, Ser657, Ser695 and Thr700, identified in the mass spectrometric analysis were required for MSK1 activation, each site was mutated to alanine in a FLAG tagged MSK1 construct. Mutated constructs were transfected into HEK-293 cells which were then starved for 16 hours. The cells were then stimulated by PMA (400 ng/ml for 10 min) or UV-C (200 J/m² followed by 30 min at 37°C) and MSK1 activity measured by immunoprecipitation kinase assays. Mutation of any of these sites did not greatly affect the level of MSK1 expression, as judged by immunoblotting for the FLAG tag.

Mutation of Ser647 to alanine did not affect the ability of PMA or UV-C to activate MSK1 when compared to the wild type protein (Fig 2A). Consistent with this, the mutation of Ser647 did not affect the phosphorylation Ser376, Ser360 and Ser212, sites shown previously to be involved in the activation of MSK1 in response to UV-C or PMA (Fig 2B-D). Surprisingly the PMA, and to a lesser extent UV-C, induced phosphorylation of Thr581 was reduced by this mutation.

Mutation of Ser657 to alanine resulted in a modest increase MSK1 activity after stimulation with either UV-C for 30 min or PMA for 10 min compared to wild type MSK1 (Fig 2A). These increases were however less pronounced at later time points, suggesting that this mutation is affecting the rate of activation rather than the specific activity of the activated protein (data not shown). The increased activation at early time points correlated with increases in the phosphorylation of the N-terminal T loop site, Ser212, in the Ser657Ala mutant (Fig 2 B-D). Mutation of Ser657 to alanine did not

greatly affect the phosphorylation of Ser360, Ser376 or Thr581 induced by either PMA or UV-C.

Mutation of Ser695 to alanine also resulted in small increases in the UV-C or PMA induced activation of MSK1 (Fig 2A), and again this correlated to increases Ser212 phosphorylation, as well as slight increases in the PMA induced level of Ser376 and Thr581, but not Ser360, phosphorylation compared to wild type protein (Fig2 B-D). Mutation of Ser647, Ser657 or Ser695 to an aspartic acid had similar effects as mutation to alanine on the activation of MSK1 in cells (data not shown).

Thr700 is a new MAPK site involved in the regulation of MSK1 activity.

Mutation of Thr700 resulted in a moderate increase in the PMA and UV-C stimulated level of MSK1 activity, however for this mutant an increase in the basal level of activity was also observed (Fig 2A). This correlated with an increase in the phosphorylation of Ser212. Surprisingly however phosphorylation of Thr581 was greatly reduced in the Thr700 mutant compared to wild type MSK1 after either UV-C or PMA stimulation (Fig 2B-D). This was unexpected as the mutation of Thr581 to alanine prevents the activation of MSK1 [24].

Interestingly Thr700 is followed by a proline suggesting that it may be a new MAPK site. A phosphospecific antibody was therefore raised against Thr700. This antibody was specific for phospho Thr700 as judged by both dot blotting Thr700 phospho and dephosphopeptides, and immunoblotting of UV-C stimulated HEK-293 cells expressing wild type or Thr700Ala MSK1 (Fig 3A). To confirm that this site was phosphorylated in endogenous MSK1, MSK1 was immunoprecipitated from serum starved, UV-C or PMA stimulated HEK-293 cells and immunoblotted. Both UV-C and PMA were found to stimulate the phosphorylation of Thr700 in endogenous MSK1 (Fig 3B).

To determine if ERK2 or p38 α could phosphorylate this site *in vitro*, the C-terminal kinase domain (residues 398-804) of MSK1 was expressed in *E. coli* and used as a substrate for these kinases. Both ERK2 and p38 α were able to phosphorylate this domain on the Thr581 and Thr700 MSK1 sites, although p38 α appeared to be slightly more effective than ERK2 at the phosphorylation of Thr700 *in vitro*, as judged by

immunoblotting using antibodies against these sites (Fig 3C). The presence of Thr700 phosphorylation in the ERK2 phosphorylated MSK1 domain was further confirmed using precursor ion scanning mass spectrometry (data not shown). Surprisingly, this also identified a further site (equivalent to Thr630 in full length MSK1) as being phosphorylated. Reanalysis of the mass spectrometry data on HEK-293 cell expressed MSK1 using Multiple Reaction Monitoring (MRM) techniques suggested that this site may also be phosphorylated in cells, however mutation of this site to alanine or aspartic acid in full length MSK1 did not affect the phosphorylation of the other sites (Fig 3D) or the activation of MSK1 (Fig 3E).

In HEK-293 cells both UV-C and PMA were able to stimulate the phosphorylation of Thr700 in wild type MSK1. The phosphorylation of Thr700 downstream of UV-C stimulation was blocked by the p38 α / β inhibitor SB203580, but unaffected by the MEK1/2 inhibitor PD184352, suggesting that p38 can directly phosphorylate this site in cells (Fig 3F). In response to PMA the MEK1/2 inhibitor greatly decreased the phosphorylation of Thr700, suggesting that in response to PMA ERK1/2 are the major kinases for this site (Fig 3F). Surprisingly, the p38 inhibitor SB203580 was also able to decrease the phosphorylation of Thr700 in response to PMA, although the effect was not as great as for PD184352. As PMA did not significantly activate p38 α , as judged by immunoblotting for phospho p38 (Fig 3F), this may be due to a role for basal p38 activity in this phosphorylation, or represent a non specific effect of the inhibitor. Similar results were obtained for Ser360 and Thr581, the other MAPK sites in MSK1 (Fig 3F). To confirm that Thr700 was not an autophosphorylation site, its phosphorylation was examined after UV-C treatment of cells expressing either N- or C-terminal kinase dead forms of MSK1. As expected Thr700 was phosphorylated after UV-C treatment in both the N- and C-terminal kinase dead MSK1, although phosphorylation of the appropriate autophosphorylation sites (Ser212 for the C-terminal kinase domain and Ser750/752 for the N-terminal kinase domain) was blocked in the kinase dead mutants (Fig 3G).

To further examine the role of Thr700 in MSK activation, Thr700 was also mutated to an aspartic acid in an attempt to mimic phosphorylation. Wild type, Thr700Ala or Thr700Asp MSK1 were expressed in HEK-293 cells and their activation

measured over a time course of PMA and UV-C stimulation. As seen in Fig 4, mutation of Thr700 to alanine resulted in a two fold increase in the basal level of MSK1 activity in the transfected cells. The basal MSK1 activity was even higher (approx 7 fold increase compared to wild type) for the Thr700Asp mutant. UV-C and PMA were however able to further stimulate the activities of both these mutants, as well as the wild type protein (Fig 4A and B). At 10 and 40 min of PMA treatment or 30 and 60min after UV-C treatment the mutant proteins were still more active than their wild type controls, however this was less pronounced at longer time points (120min for PMA or 180 min for UV-C). The level of Ser212, Ser360 or Ser376 phosphorylation was either unchanged or slightly increased in the Thr700 to Ala or Asp mutants after PMA or UV-C stimulation compared to wild type protein. In contrast, phosphorylation of Thr581, a critical site for MSK1 activation, was greatly reduced in both Thr700 mutants compared to wild type protein in response to UV-C treatment. In response to PMA stimulation, phosphorylation of Thr581 was also significantly reduced in the Thr700Ala mutant in response to PMA, however this phosphorylation was only slightly reduced in the Thr700Asp MSK1 compared to wild type protein.

The reduction in Thr581 phosphorylation that occurs when Ser700 was mutated is surprising given that the Thr700 mutations were active, as Thr581 phosphorylation has previously been shown to be critical for activity. We therefore analysed the effects of combinations of Thr581 and Thr700 mutations (Fig 5). Mutation of both Thr581 and 700 to alanine resulted in an inactive kinase that was not stimulated by either PMA (Fig 5A) or UV-C (not shown) and was not phosphorylated on either of the Ser212 and Ser376 MSK1 autophosphorylation sites (Fig 5B), consistent with what had previously been reported for the single Thr581Ala mutation [24]. The Thr581/700Ala double mutation did not however prevent the interaction of the mutant with its upstream kinases ERK1/2 or p38, as the MAPK site Ser360 was phosphorylated normally in response to PMA or UV-C in this mutant (Fig 5B). Mutation of Thr581 to Ala and Thr700 to Asp also resulted in an inactive kinase, reinforcing the critical role of Thr581 (Fig 5A). To further investigate the interaction between Thr581 and 700, Thr581 was mutated to an aspartic acid to try and mimic phosphorylation. A single Thr581Asp mutation was found to have extremely low activity, indicating that this mutation was not completely successful at

replicating the effects of phosphorylation (Fig 5A). The Thr581Asp mutant was however phosphorylated on the other MAPK sites, Ser360 and Thr700, in response to PMA stimulation and a small increase in Ser212 phosphorylation was also observed (Fig 5B). Mutation of both Thr581 and 700 to Asp resulted in a kinase with an elevated basal activity in unstimulated cells, similar to what was seen for the single Thr700Asp mutant. However in contrast to the Thr700Asp single mutant the activity of the double mutant could not be further stimulated by PMA (Fig 5A) or UV-C (data not shown). Consistent with the elevated basal activity of the Thr581/700Asp mutant, an elevated level of Ser212 phosphorylation, that was not significantly enhanced by PMA treatment, was also observed (Fig 5B).

Previous studies have suggested that mutation of Ser376 to Asp successfully mimics phosphorylation of this residue [24]. We therefore tested if mutation of Ser376 to Asp could further increase the activity of the Thr581Ala/Thr700Asp mutant. The triple mutation of Ser376, Thr581 and Thr700 to aspartic acid however had similar activity and phosphorylation pattern to that of the double mutant (Fig 5). Mutation of all the critical phosphorylation sites in MSK1, Ser212, Ser276, Thr581 and Thr700, to an aspartic acid resulted in an inactive kinase. This is consistent with the previous finding that mutation of Ser212 to an acidic residue the N-terminal kinase domain T-loop residue, was unable to activate this domain [28].

Discussion

We have previously studied the activation mechanism of MSK1 and demonstrated that ERK1/2 or p38 α phosphorylate two sites in MSK1, Ser360 and Thr581, that are required for its activation [24]. These phosphorylation events activate the C-terminal kinase domain of MSK1 which then autophosphorylates Ser212, Ser 376 and Ser381 resulting in the activation of the N-terminal kinase domain. Here we describe the identification of five new phosphorylation sites in MSK1, Thr630, Ser647, Ser657, Ser695 and Thr700. One of these sites, Thr700 lies in a consensus MAPK phosphorylation sequence (Ψ XS/TP where Ψ is a proline or aliphatic residue), and was found to be phosphorylated by both ERK and p38 α both *in vitro* and in cells. The

remaining sites were not direct targets of MAPKs, and represent autophosphorylation sites, or sites for an as yet undetermined kinase. All of these sites lie in the C-terminal domain of MSK1, which is most closely related to MAPKAP-K2/3, MNK1/2 and the C-terminal domain of RSK [29]. The equivalent site to Ser647 in MSK1 is a Thr in RSK1 however it is replaced by a glutamic acid in MNK and MAPKAP-K2, while the Ser657 site in MSK1 is conserved in RSK, MSK and MAPKAP-K2. In contrast, the Thr630 and Ser695 sites are not conserved in the other kinases. The MAPK site Thr700 is not conserved in RSK, however it is conserved in MNK1 and MNK2. A threonine is present at this position in MAPKAP-K2 however it is not followed by a proline making it an unlikely MAPK target. Interestingly MAPKAP-K2 does contain a potential p38 phosphorylation site 4 residues N-terminal to this position (Fig 6).

Mutation of Ser647, Ser657, Ser695 or Thr700 to alanine (Fig 1) or aspartic acid (data not shown) did not prevent the activation of MSK1 in response to UV-C or PMA. Mutation of Thr700 to alanine slightly increased the basal activity of MSK1, while mutation to aspartic acid increased the basal activity still further. A similar result has been shown for mutagenesis of the equivalent site of MSK2 [30], although this study did not directly establish that this residue could be phosphorylated. Mutation of the equivalent site in MNK1 or 2 also has also been reported to give similar results [31, 32], however while this site is phosphorylated *in vitro* by ERK [33], it was unclear from these studies if this was an *in vivo* phosphorylation site. Interestingly the equivalent site (Thr338) is phosphorylated in MAPKAP-K2, although this probably occurs via autophosphorylation rather than by p38 or ERK1/2 [34]. A MAPK site (Thr 334) does exist in MAPKAP-K2 4 residues N-terminal to the Thr348 autophosphorylation site. Mutation of the Ser334 in MAPKAP-K2 to alanine does not prevent the activation of MAPKAP-K2, while mutation to an acidic residue increased the basal activity. Mutation of both Ser344 and the T loop phosphorylation site in MAPKAP-K2 to acidic residues resulted in a kinase which is constitutively active [34, 35]. In combination with structural information from crystallographic studies [36, 37] this has suggested a model for MAPKAP-K2 activation in which phosphorylation of Ser334 promotes the dissociation of an autoinhibitory C-terminal helix from the substrate binding groove of the kinase

domain, while phosphorylation of the activation loop on T222 stabilises its structure and promotes substrate binding [35, 37].

Although the C-terminal regions of MSK1 and MAPKAP-K2 are not well conserved, activation of the MSK1 C-terminal kinase domain may occur via a similar mechanism, with phosphorylation of Thr700 in MSK1 promoting the dissociation of an autoinhibitory sequence from the kinase domain (Fig 7). Consistent with this, C-terminal truncation of MSK2 has been reported to increase its basal activity [30], although this truncation also deletes nuclear localisation and MAPK docking sequences. Such a mechanism would explain the increased basal activity of the Thr700 MSK1 mutations, however it would not explain why Thr581 phosphorylation is reduced in these mutants. Thr581 phosphorylation is still required for MSK1 activation, as mutation of Thr700 to Asp does not rescue the activity of a Thr581Ala mutation. One possibility is that mutation of Thr700 does not prevent Thr581 phosphorylation but instead reduces the stability of the Thr581 phosphorylation. In such a model, Thr581 would be phosphorylated, allowing the activation of MSK1, however its prolonged phosphorylation would require interaction of the phospho-Thr581 with another part of MSK1 in a way that protects it from dephosphorylation. Mutation of Thr 700 would interrupt this interaction and therefore result in accelerated dephosphorylation of Thr581. Interestingly reduced Thr581 phosphorylation has also been observed in other MSK1 mutants, such as those that inactivate the C-terminal or N-terminal kinase domains [24], in line with the suggestion that this site can be easily dephosphorylated if the correct active conformation of MSK1 cannot be formed. The idea that the active conformation of a kinase can protect it from dephosphorylation has been suggested before for other kinases. For instance binding of the phosphorylated hydrophobic motif of PKB, RSK and S6K to a phosphate binding pocket in the kinase has been suggested to protect the hydrophobic motif from dephosphorylation [18, 38, 39].

In total 13 phosphorylation sites have now been identified in MSK1, of which at least 6 have significant roles in MSK1 activation. The role of the remaining sites is unclear, but may influence other processes such as localisation or protein/protein interactions *in vivo*. MSK1 is sequentially activated through phosphorylation by ERK1/2 or p38 to activate the C-terminal kinase domain which then activates the N-terminal

kinase domain, however the work presented here suggests that this may also be influenced by phosphorylation of a C-terminal autoinhibitory domain. To fully understand the mechanism by which phosphorylation regulates MSK1 will however require three dimensional structural studies on the phosphorylated and dephosphorylated enzymes.

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Figure legends

Figure 1 Precursor ion scan analysis of MSK1.

GST tagged MSK1 was expressed in HEK-293 cells by transient transfection. Cells were serum then serum starved for 16 h and then left unstimulated (control, A) or stimulated with UV-C (200 J/m² followed by 30min at 30°C, B) or TPA (400 ng/ml, 10 min, C). MSK1 was purified on glutathione Sepharose, samples were run on polyacrylamide gels and the MSK1 band excised and digested with trypsin. The tryptic digests were analysed by LC-MS with precursor ion scanning on a 4000 Q TRAP system as described in the methods. The sum of the peptide masses detected by precursor ion scanning in the negative ion mode across the 45 min HPLC separation are shown for the control (A) and TPA treated (B) MSK-1. Stimulation with UV-C gave similar results to TPA, however the peak heights were smaller compared to the TPA sample. Peptides 1-6 were identified from the MS/MS fragmentation spectra by database searching and manual inspection of the spectra as:-

1= LFQGY(pS)FVAPSILFK (phospho Ser376), 2= LFQGY(pS)FVAP(pS)ILFK (phospho Ser376 / Ser381), 3= AY(pS)FCGTIEYMAPDIVR (phospho Ser212), 4= GDF(pS)FEGEAWKNV(pS)QEAK (phospho Ser647 / Ser657), 5= YNEWLQDGSQLSSNPLM(pT)PDILGSSGAAHTCVK (phospho Thr700), 6= YNEWLQDG(pS)QLSSNPLM(pT)PDILGSSGAAHTCVK (phospho Ser695 / Thr700). The peptide ion denoted with an asterisk (*) was found to be a phosphopeptide derived from the GST-FLAG tag used to generate this fusion protein.

Figure 2 Effect of phosphorylation site mutagenesis on MSK1 activation.

Ser647, Ser657, Ser695 or Thr700 were changed to Alanine by site directed mutagenesis of a FLAG-MSK1 expression vector. Wild type or mutant protein was then overexpressed in HEK-293 cells by transient transfection. Once transfected, cells were serum starved for 16 h and then left unstimulated (clear bars) or stimulated with UV-C (200 J/m² followed by incubation at 30°C for 30 min, grey bars) or PMA (400 ng/ml, 10 min, black bars). Cells were then lysed and MSK1 activity determined by immunoprecipitation kinase assays as described in the methods (A). One unit was defined as the incorporation of 1 nmole of phosphate into the substrate peptide in 1 min. The

results of the different mutations were compared to the wild type protein from either control, UV-C or PMA stimulations by t-tests. A P value of less than 0.05 is indicated by *. Soluble protein (25 μ g) was run on SDS polyacrylamide gels and immunoblotted using antibodies against FLAG, phospho Ser360, phospho Ser376 and phospho Thr581 (B). To analyse Ser212 phosphorylation, MSK1 was first immunoprecipitated using an anti FLAG antibody and the immunoprecipitates were blotted for Ser212 phosphorylation (B). Immunoblots were quantified using an Odyssey Licor scanner and the phospho signal calculated relative to the FLAG loading control (C). Error bars represent the standard error of three separate stimulations.

Figure 3 Thr700 of MSK1 is phosphorylated by ERK1/2 and p38.

A) To allow analysis of Thr700 phosphorylation in MSK1 a phospho Thr700 antibody was raised in rabbits. To analyse the specificity of this antibody, it was tested in dot blots against peptides SNPLMTPDIL (D, dephospho) and SNPLM(phosphoT)PDIL (P, phospho), which correspond to the sequence around Thr700 in MSK1 (A, left panel). The antibody was tested for its ability to recognise wild type, Thr700Ala or Thr700Asp MSK1 expressed in HEK-293 cells which had been PMA stimulated before lysis (A, right panel).

B) Endogenous MSK1 was immunoprecipitated from 1 mg of soluble lysate from serum starved HEK-293 cells or cells stimulated with UV-C (200 J/m² followed by 30 min at 37 °C) or PMA (400 ng/ml, 15 min). Immunoprecipitates were then immunoblotted for total and phospho Thr700 MSK1.

C) 2 μ g of recombinant C-terminal kinase domain of MSK1 (CT-MSK1), purified from *E. coli*, was phosphorylated using by 40 mUnits ERK2 (grey bars) or p38 α (black bars) for 20 min at 30°C as described in the methods. As controls, MSK was incubated without ERK or p38 (control), and ERK or p38 α was used to phosphorylate the known MAPK substrate myelin basic protein (MBP). The protein was then run on SDS polyacrylamide gels and after Coomassie staining the MSK1 bands were excised and the ³²P incorporation was determined by Cerenkov counting (B, upper panel). Samples from the same reactions were also immunoblotted using phospho Thr581 and phospho Thr700 antibodies (B, lower panel).

D) FLAG tagged wild type, Thr630Ala or Thr630Asp MSK1 was expressed in HEK-293 cells by transient transfection. Following transfection cells were serum starved for 16 h and then either left unstimulated or stimulated with UV-C (200 J/m² followed by 30 min at 37 °C) or PMA (400 ng/ml, 15 min). Lysates were immunoblotted for phospho Ser212, Ser360, Ser376, Thr581 and Thr700 MSK1 and for GST as a loading control.

E) As D except anti-FLAG antibodies were used to immunoprecipitate MSK1 and kinase activity was measured as described in the methods. The kinase activity from unstimulated cells is shown by white bars, UV-C stimulated light grey bars and PMA stimulated cells by dark grey bars. Error bars represent the SEM of three independent stimulations. One unit was defined as the incorporation of 1 nmole of phosphate into the substrate peptide in 1 min. The results of the different mutations were compared to the wild type protein from either control, UV-C or PMA stimulations by t-tests. A P value of less than 0.05 is indicated by *.

F) Wild type GST tagged MSK1 was expressed in HEK-293 cells by transient transfection. Following transfection cells were serum starved for 16 h and then incubated for a further hour with either no inhibitor, 2 μM PD 184352 (PD), 5 μM SB 203580 (SB) or both PD 184352 and SB203580 (PD+SB). Cells were then left unstimulated or stimulated with UV-C (200 J/m² followed by incubation at 30°C for 30 min) or PMA (400 ng/ml, 10 min). Soluble protein (30 μg) was run on SDS polyacrylamide gels and immunoblotted using antibodies against GST, phospho Ser360, phospho Ser376, phospho Thr581, phospho Thr700 or phospho Ser750/752. To analyse Ser212 phosphorylation, MSK1 was first pulled down using GSH-Sepharose and the pull downs blotted for Ser212 phosphorylation.

G) As F except cells were transfected with either C-terminal (CT-KD, top panel) or N-terminal kinase dead (NT-KB, lower panel) GST-tagged MSK1, and cells were stimulated with UV-C only.

Figure 4 Effect of Thr700 mutagenesis on MSK1 activation.

Wild type (open squares), Thr700Ala (closed triangles) or Thr700Asp (closed circles) MSK1 was overexpressed in HEK-293 cells by transient transfection. Once transfected, cells were serum starved for 16 h and then left unstimulated or stimulated with 400 ng/ml

PMA (A) or 200 J/m² UV-C followed by incubation at 37°C (B) for the times indicated. Cells were then lysed and MSK1 activity determined by immunoprecipitation kinase assays as described in the methods (upper panels). One unit was defined as the incorporation of 1 nmole of phosphate into the substrate peptide in 1 min. Soluble protein (25 µg) was run on SDS polyacrylamide gels and immunoblotted using antibodies against FLAG, phospho Ser360, phospho Ser376, phospho Thr581 and phospho700. To analyse Ser212 phosphorylation, MSK1 was first immunoprecipitated using an anti FLAG antibody and the immunoprecipitates were blotted for Ser212 phosphorylation (lower panels).

Figure 5 Analysis of MSK1 Thr581/Thr700 mutants.

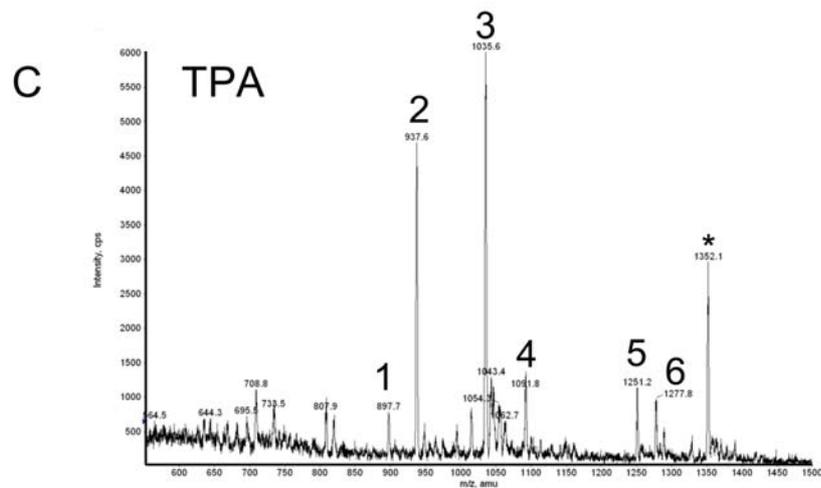
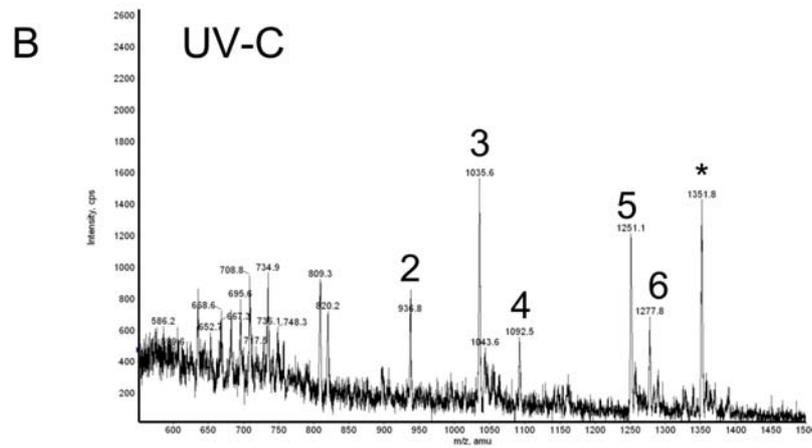
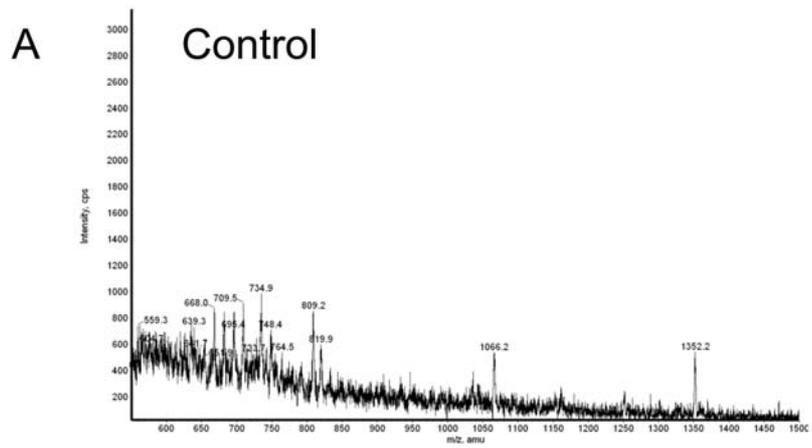
Wild type or mutant MSK1 was expressed in HEK-293 cells by transient transfection. After transfection cells were serum starved for 16 h and then left unstimulated (clear bars) or stimulated PMA (400 ng/ml, 10 min, grey bars). Cells were then lysed and MSK1 activity determined by immunoprecipitation kinase assays as described in the methods (A). Activity was normalised to expression based on anti-FLAG blots. One unit was defined as the incorporation of 1 nmole of phosphate into the substrate peptide in 1 min. Error bars represent the standard error of three stimulations. One unit was defined as the incorporation of 1 nmole of phosphate into the substrate peptide in 1 min. The results of the different mutations were compared to the wild type protein from either control, UV-C or PMA stimulations by t-tests. A P value of less than 0.05 is indicated by *, and a value of less than 0.01 by **. Soluble protein (30 µg) was run on SDS polyacrylamide gels and immunoblotted using antibodies against FLAG, phospho Ser360, phospho Ser376, phospho Ser381, phospho Thr581, phospho Thr700. To analyse Ser212 phosphorylation, MSK1 was first immunoprecipitated using an anti FLAG antibody and the immunoprecipitates were blotted for Ser212 phosphorylation (B). Analysis of wild type (WT) as well as Thr581Asp (581D), Thr700Asp (700D), Thr581Asp/Thr700Asp (DD), Ser376Asp/Thr581Asp/Thr700Asp (DDD), Ser212Asp/Ser376Asp/Thr581Asp/Thr700Asp (DDDD), Thr581Ala/Thr700Ala (AA) and Thr581Ala/Thr700Asp (AD) mutants are shown.

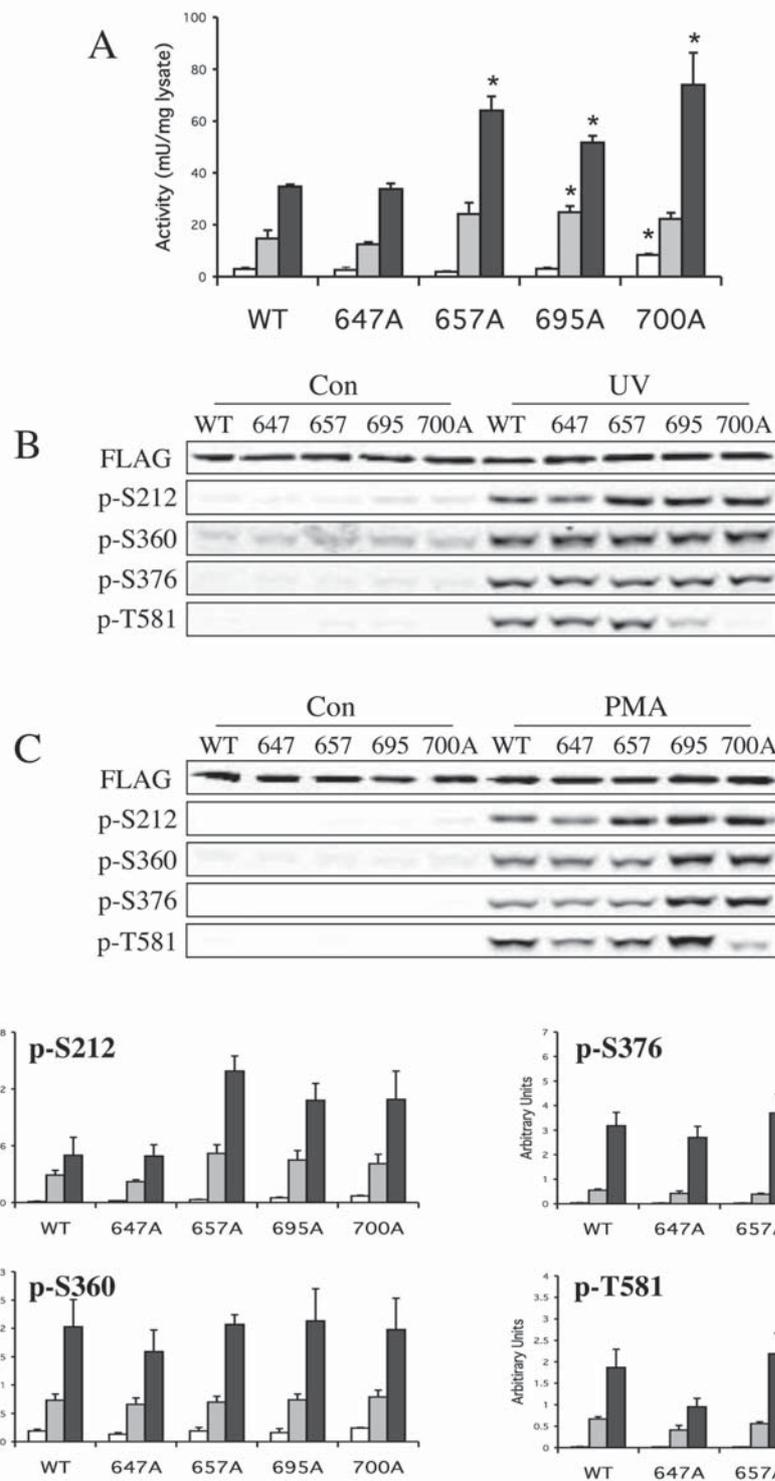
Figure 6 Sequence alignment of kinase domains.

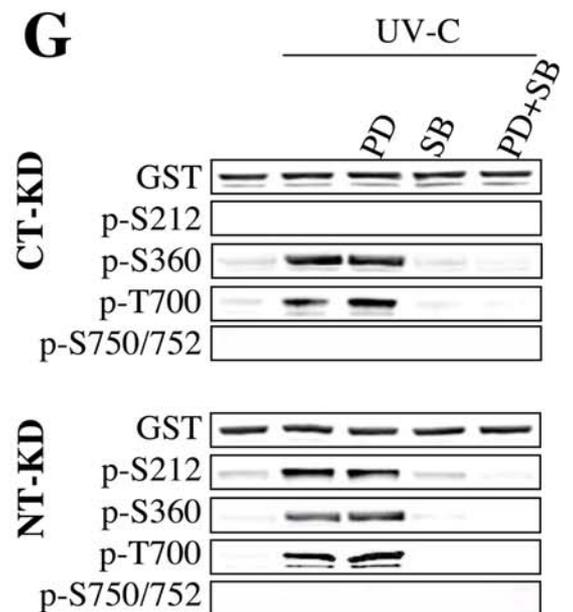
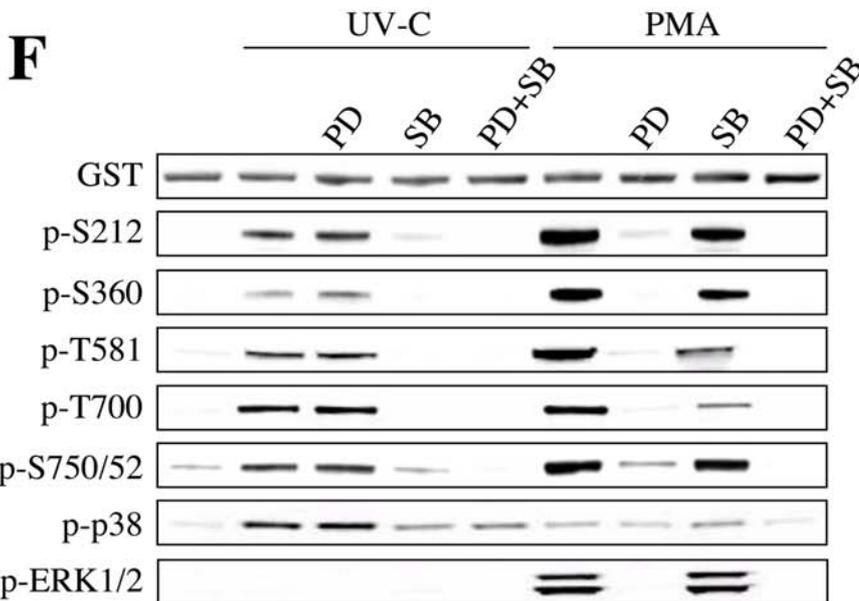
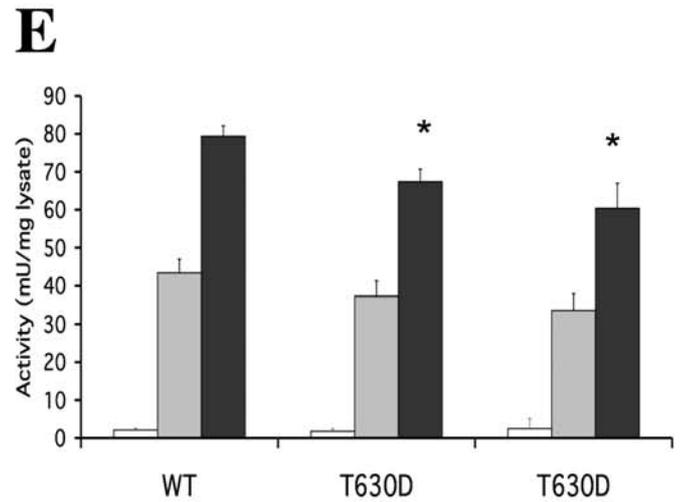
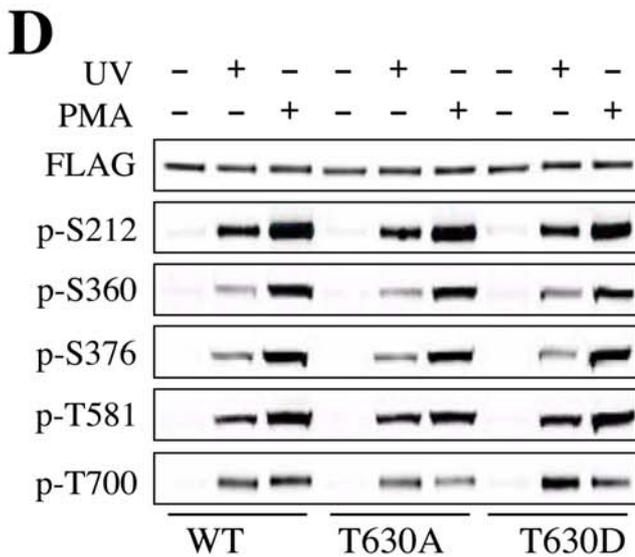
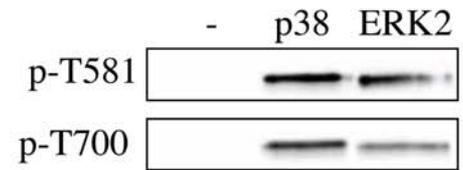
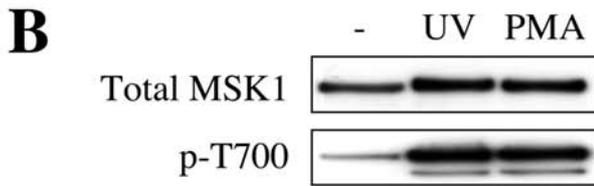
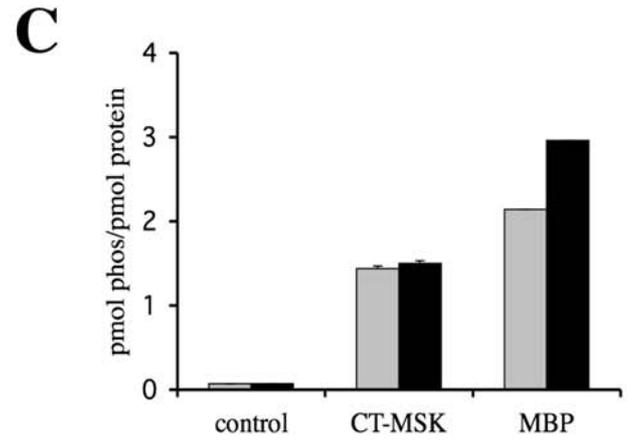
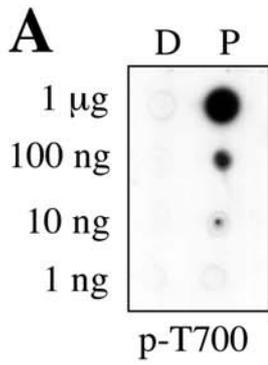
The C-terminal kinase domains human MSK1, MSK2 and RSK1 as well as the kinase domains of MAPKAP-K2, MNK1 and MNK2 were aligned. Phosphorylation sites in MSK1 are indicated by asterisks

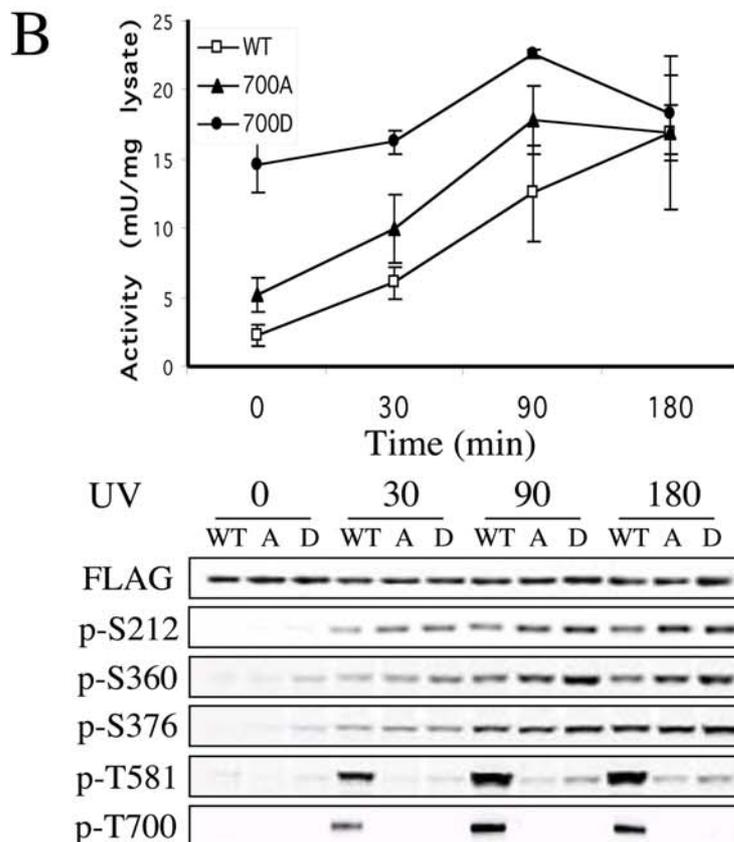
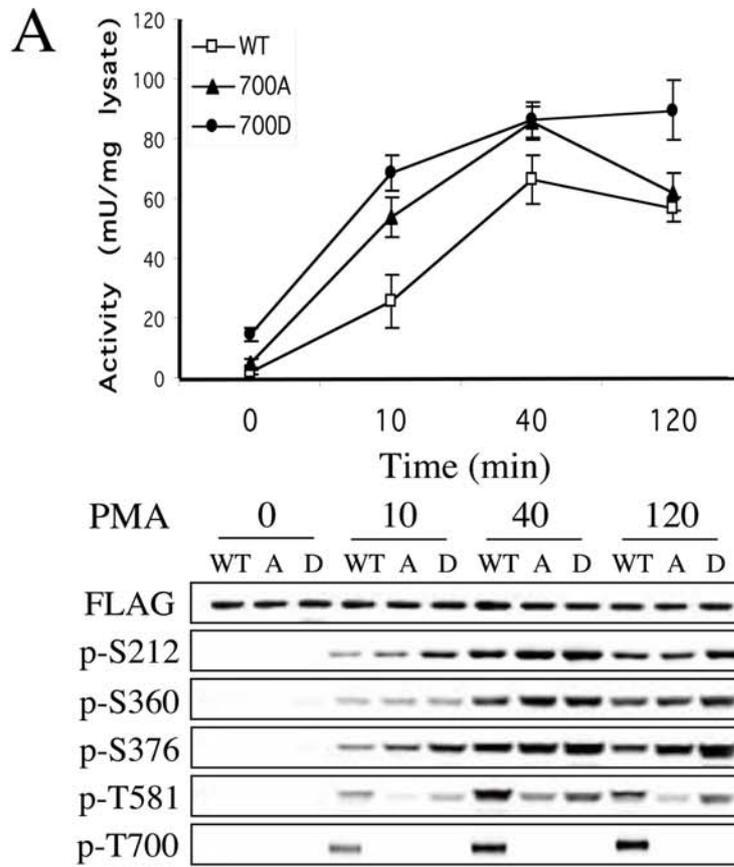
Figure 7 Activation model for MSK1.

MSK1 consists of two kinase domains, joined by a short linker region, and a C-terminal extension containing a MAPK kinase docking domain after the 2nd kinase domain. In the inactive state (A) MSK1 is unphosphorylated and the C-terminus of the protein may fold back and act as an inhibitor of the C-terminal kinase domain, as has been described for the related kinase MAPKAP-K2. Active ERK1/2 or p38 MAPK is able to bind to the docking motif in the C-terminal region of MSK1, resulting in the phosphorylation of three sites, Ser360, Thr581 and Thr700 in MSK1 (red circles, B). Thr581 is in the activation loop of the C-terminal kinase domain and its phosphorylation may be required to stabilise the active conformation of the activation loop. Thr700 is proposed to lie in a hinge region between the C-terminal kinase domain and the inhibitory C-terminal sequence and phosphorylation of this residue would promote the dissociation of the inhibitory sequence from the kinase domain. MSK1 is additionally phosphorylated on a further 4 sites by an unidentified kinase (green circles), and the function of these phosphorylations is unclear. The activated C-terminal kinase domain then activates the N-terminal kinase domain via the phosphorylation of three sites Ser212, Ser376 and Ser381 (yellow circles, D). Once activated the N-terminal kinase domain is able to phosphorylate substrates, and additionally phosphorylates three sites, Ser750, Ser752 and Ser758 (blue circles, E) at the C-terminus of MSK1.










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MSK1      344 DVSNFAEEFTEMDFTYSPALFOSSE-KLFQGYSFVAPSILFKRNAAVI-DPLQFHMGEVDFPGVTVNVARSMMKDSFPFYQ
MSK2      311 DVGNFAEFEFTRLEPVYSPGSPFPGDPRIHQGYSFVAPSILFDHNNAVMTDGLEAPGAGDFPGFAAVARSAMM-----Q
RSK1      347 DTFYFDTEFTSRTPKDSPPIPPSAGAHQLFRGFVAVTGLMEDDGK-----PRAPQAPLHSSVQQLHGK-----N
MAPKAPK-2  1 ---MLSNSQGQSPVPFPPAPAPFPQPP-----TPALPHPPAQPPPP-----PPQFPQFHVRSGL-QIKKNAI-----
MNK1      1 ---MVSSQKLEKPIE-MESSEELPIA-----DGD-----RRRKKKRGKATDSLPGKF-----
MNK2a     1 -----MPASQPIDIP-----DAK-----KRGKKKRGKATDSFSRF-----

MSK1      422 HY--ELDKDKPLGGSSSICRKCVRHKSNQAFVAVKIISKR---MEANTQKEITALKLCEGHPNIVKLEHVVHHD----QL
MSK2      385 QY--ELDREPALGGSSSVCRRRCRQOSGQEFVAVKIISRR---LEANTQREVAALRLCQSHPNVNVNHEVHHD----QL
RSK1      412 LVFSDEGYVVKETIGVGSYSECKRCVHKAATNMEYAVKVIDKS---KRDPSSEEIILRLRYGQHPNIITLTKDQVYDD---GK
MAPKAPK-2  60 --IDYKWTISQVVLGGINGKVLQIFNRTQEKFAIKMIDQDCP---KA--RREVELHWRASQCFEIVRIVDVENLYAGRK
MNK1      45 --EDMYKITSELLGCGAAKAVQGAVSLQNGKEVAVKIIEKQAGHSERSRVFREVELTYCCQGNKNIIELEIEFFED----DT
MNK2a     33 --EDVYQIQEDVVLGGGAHARVQTCINLIISQEVAVKIIEKQPGHISRVFREVELTYCCQGNKNIIELEIEFFEE----ED

MSK1      493 HTFLVMEILLNGGELFERIKKK--KHFSETEASYIMRKLVSAMSHMHD-----
MSK2      456 HTYLVLELLRGGELLEHIRKK--RHFSSEASQILRSIVSASFMHHE-----
RSK1      484 HVYLVTELLMRGGELLDKILRQ--KFFSEREASFVLTITGKTWEYLHSQ-----
MAPKAPK-2  133 CLLVMECLDGGELFSRIQDQGFATEREASEIMKSIQEAQYLLHS-----
MNK1      119 RFYLVFEEKLOGGSTLAHTCKQ--KHFNEREASRVVRDVAALDFLHTKDKVSLCHLGSWAMAPSGLTAAPTSLGSSDPPT
MNK2a     107 RFYLVFEEKVRGGSLLSHIRKK--RHFNLEASVIVQDVASALDELHNK-----

MSK1      538 -----GVVHRDLKPENILTDENDNL-EIKIIDFGFARLKPDP-----NQF-LKTPCFTLHYAAPEILNQNG---
MSK2      502 -----AGVHRDLKPENILMADDTPGA-PVKIIDFGFARLRPQSP-----GVF-VQTPCFTLQYAAPEILAQQG---
RSK1      530 -----GVVHRDLKPENILVDESGNPECLIKCDFGFARQLRAE-----NGL-LMTPCYTANFVAPEVLKROG---
MAPKAPK-2  180 -----INLAHRDLKPENILMTRKRPNA-ILKIDFGFAKETTSHNS-----LTPCYTPYVVAPEVLGPEK---
MNK1      197 SASQVAGTTGLAHRDLKPENILCESPEKVS-PVKICDFDLESGMKLNNSCTPITTPBLTTPCGSAEYVAPEVVEVFTDQA
MNK2a     153 -----GLAHRDLKPENILCEHPNQVS-PVKICDFDLESGIKLNGDCSPITPBLTTPCGSAEYVAPEVVEAFSEEA

MSK1      599 --YDESCDLWSLGVILYIMLSGQVPPQSHDRS-----LTCTS-AVEIMKKIKKQDFSEEGEAKNVSQEAKDLSKGLL
MSK2      564 --YDESCDLWSLGVILYIMLSGQVPPQASGQ-----GGQSQ-AAEIMKRIREGRFSLDGEAMQGVSEEAKEKLRGLL
RSK1      591 --YDEGCDLWSLGVILYIMLGGYTPFANGPS-----DT-PEEILTRIGSGKFTLSGGNNTVSEAKDLMSKML
MAPKAPK-2  240 --YDKSCDMWSLGVILYIMLSGYPPFYSNHGGL-----AI-SPGKTRIRMGQHEFPNPEWSEVSEVVKMLIRNLL
MNK1      276 TFYDKRCDLWSLGVILYIMLSGYPPFVGHCCADCGWDRGEVCRVCQNKIFESTQEGKYEEFPDKDWAHNSSEAKDLISKLL
MNK2a     223 SIYDKRCDLWSLGVILYIMLSGYPPFVGRGSDCGWDRGEACPACQNMIFESTQEGKYEEFPDKDWAHNSCAAKDLISKLL

MSK1      669 TVDPNKRLKMSGIRYNEWIQDGSQLSNPEMTPDNLGSSGAAVHTC-VKATFHAFNK-----
MSK2      634 TVDPAKRLKLEGLRGSSWIQDGSARSSPPELTPDVLLESSGPAVRSGLNATFMFNR-----
RSK1      657 HVDPHQRLTAKQVLOHPWVTKDKLQSQLSHQDL-----QLVKGA-MAATYSALNS-----
MAPKAPK-2  307 KTEPTQRMTIITFMNHPWIMQSTKVPQTPLEHTSRVLIKEDKERWE---DVKGCLHE-----
MNK1      356 VRDAKQRLSAAQVLOHPWVQGA--PEKGLTTPQVLRNSSTMVLTFLFAEAIALNSQLSQHEENELAE-----PEAL
MNK2a     303 VRDAKQRLSAAQVLOHPWVQGA--PENTLETPVVLQRNSCAKDLTSFAEAIAIANNQLAQHDEDLAEAAAGQGPVLV

MSK1      725 --YKREGFLQNVKAPLAKRRKMKKTSTSTETRSSSESSHSSSHSHGKTTPTKTLQPSNPADSNNPETLQFSDSVA
MSK2      690 --GKREGFFLKSVENAPL-----
RSK1      708 --SKPTP-QLKPIESSILAQRRVRKLPSTTL-----
MAPKAPK-2  359 -----KNSQATWLTRL-----
MNK1      428 ADGLCSM-KLSPPCKSRLLARRRRLAQAARG-----EDRSPPTAL-----
MNK2a     381 RATSRCLELSPPSQSKLAQRQRASLSSA-----PVVLVGDHA-----
    
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