Nuclear export of the stress-activated protein kinase p38 mediated by its substrate MAPKAP kinase-2

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Background: Mitogen-activated protein (MAP) kinases (or extracellular signal regulated kinases; Erks) and stress-activated protein (SAP) kinases mediate cellular responses to a wide variety of signals. In the Erk MAP kinase pathway, activation of MAP kinases takes place in the cytoplasm and the activated enzyme moves to the nucleus. This translocation to the nucleus is essential to MAP kinase signalling because it enables the kinase to phosphorylate transcription factors. Whether components of the pathway mediated by the SAP kinase p38 change their cellular location on activation is not clear; we have therefore studied the cellular localisation of components of this pathway before and after stimulation.

Results: The p38 SAP kinase substrate MAP-kinase-activated protein kinase-2 (MAPKAP kinase-2) contains a putative nuclear localisation signal which we show is functional and required for activation by a variety of stimuli. Following phosphorylation of MAPKAP kinase-2, nuclear p38 was exported to the cytoplasm in a complex with MAPKAP kinase-2. Export of MAPKAP kinase-2 required phosphorylation by p38 but did not appear to require the kinase activity of MAPKAP kinase-2. The p38 activators MKK3 and MKK6 were present in both the nucleus and the cytoplasm, consistent with a role in activating p38 in the nucleus.

Conclusions: In the p38 SAP kinase pathway, MAPKAP kinase-2 serves both as an effector of p38 by phosphorylating substrates and as a determinant of cellular localisation of p38. Nuclear export of p38 and MAPKAP kinase-2 may permit them to phosphorylate substrates in the cytoplasm.

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Background

The p38 stress-activated protein (SAP) kinases consist of p38α (also known as SAPK2a, RK, CSBPs, Mxi2 or Mpk2), p38β (SAPK2b), p38γ (SAPK3) and p38δ (SAPK4) and are activated by cell stresses such as DNA damage, heat shock, osmotic shock, anisomycin and sodium arsenite, as well as pro-inflammatory stimuli such as bacterial lipopolysaccharide and interleukin-1 (reviewed in [1]). Two splice forms of p38α exist and were originally termed CSBP1 and CSBP2 [2]. The p38 family mediate cellular responses such as inflammatory cytokine production and HSP27 phosphorylation [2,3]. Within the p38 family, p38α and p38β isoforms, but not p38γ and p38δ, are inhibited by the pyridinyl imidazole SB203580, which has proved extremely useful in delineating signalling pathways activated by cellular stresses and cytokines [1].

Like the mitogen-activated protein (MAP) kinases of the extracellular signal regulated kinase (Erk) family, which are mainly stimulated by growth factors, p38 SAP kinases are activated by phosphorylation on a threonine and a tyrosine residue in the T-loop; this phosphorylation results from a protein kinase cascade [4]. Although it is known that the phosphorylation is mediated by the dual-specificity kinases MKK3 and MKK6 [5–7], the regulation of MKK3 and MKK6 is poorly understood. One class of activators of MKK3 and MKK6 may be the MLK3 family of protein kinases [8] but there are likely to be others [9]. Activation of the kinase cascade leading to stimulation of p38 has been shown to require the Rho-subfamily GTPases Cdc42 and Rac [10] potentially working through p21-activated kinase (PAK) [11], but the details of this process remain poorly defined.

Substrates of p38 include MAP-kinase-activated protein kinase-2 (MAPKAP kinase-2) [12,13], MAPKAP kinase-3 [14] and MAP-kinase-interacting kinase (Mnk) [15,16] and the transcription factors CHOP [17], Elk-1 and Sap-1a [18–21]. In the Erk MAP kinase cascade it is clear that inactive Erk is mainly cytosolic, and that it moves to the nucleus upon activation [22,23] where it can phosphorylate nuclear Elk-1 [24]. Presumably active p38 needs to be nuclear to phosphorylate Elk-1 and other transcription factors. MAPKAP kinase-2 exists in two alternatively

spliced variants, one of which contains a potential nuclear localisation signal (NLS) close to its carboxyl terminus [25–27]. Nuclear MAPKAP kinase-2 may be required for the phosphorylation of the transcription factor CREB [28]. If MAPKAP kinase-2 is nuclear then it may need nuclear p38 to phosphorylate it. We have studied the cellular localisation of MAPKAP kinase-2 and p38 and investigated whether the potential NLS in the splice variant of MAPKAP kinase-2 is required for activation. We show that not only is the NLS-containing splice variant of MAPKAP kinase-2 nuclear and the NLS required for activation but that following phosphorylation this kinase exports activated p38 to the cytoplasm.

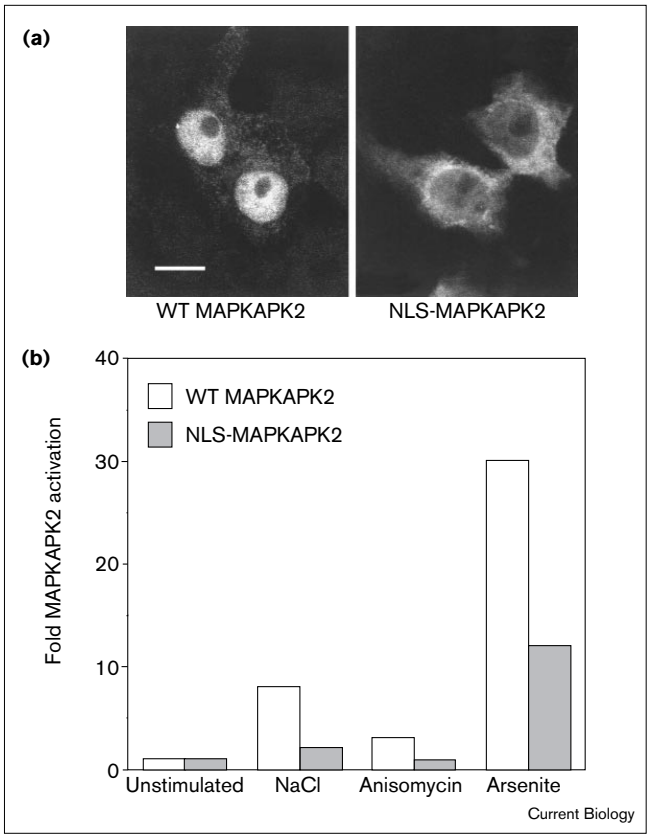
Results

MAPKAP kinase-2 has a nuclear localisation signal that is required for its activation by p38

The potential NLS in human MAPKAP kinase-2 is at residues 373–389 (**KK**IEDASNPLLL**KRRKK**; in single-letter amino acid code with conserved residues of the NLS sequence in bold) [26,27] and is conserved in other species such as the long-tailed hamster (GenBank accession number X82220). To determine the cellular localisation of this splice variant we expressed a 9E10 epitope-tagged construct in 293T cells and examined the cells by immunofluorescence and confocal microscopy. Figure 1a shows that this protein is nuclear; similar results were found in PC12 cells (data not shown). To define whether the potential NLS is functional, we generated a mutant in which the putative NLS was disrupted by replacing four basic amino acids (385–388; KRRK) with asparagine residues (NLS-MAPKAP kinase-2 mutant). As shown in Figure 1a, the NLS-MAPKAP kinase-2 mutant protein was present mainly in the cytoplasmic compartment of the cell. We conclude, therefore, that the putative NLS is required for nuclear localisation of this kinase.

To determine whether the nuclear localisation of MAPKAP kinase-2 was required for activation by p38, we compared the activation of the NLS-MAPKAP kinase-2 mutant with its wild-type counterpart. As shown in Figure 1b, the activation of the NLS mutant was severely compromised in response to all stimuli examined, namely sodium arsenite, anisomycin and sodium chloride. A glutathione-S-transferase (GST) fusion protein of NLS-MAPKAP kinase-2 was activated to similar levels as the wild-type kinase by recombinant p38α *in vitro* (data not shown), arguing that the inability to activate NLS-MAPKAP kinase-2 *in vivo* is not because the mutant is a poor substrate of p38. Pretreatment with the p38 inhibitor SB203580 completely blocked activation of endogenous or overexpressed MAPKAP kinase-2 by sodium arsenite, anisomycin and sodium chloride, demonstrating that activation of this kinase was mediated principally by p38α or p38β (data not shown).

Figure 1

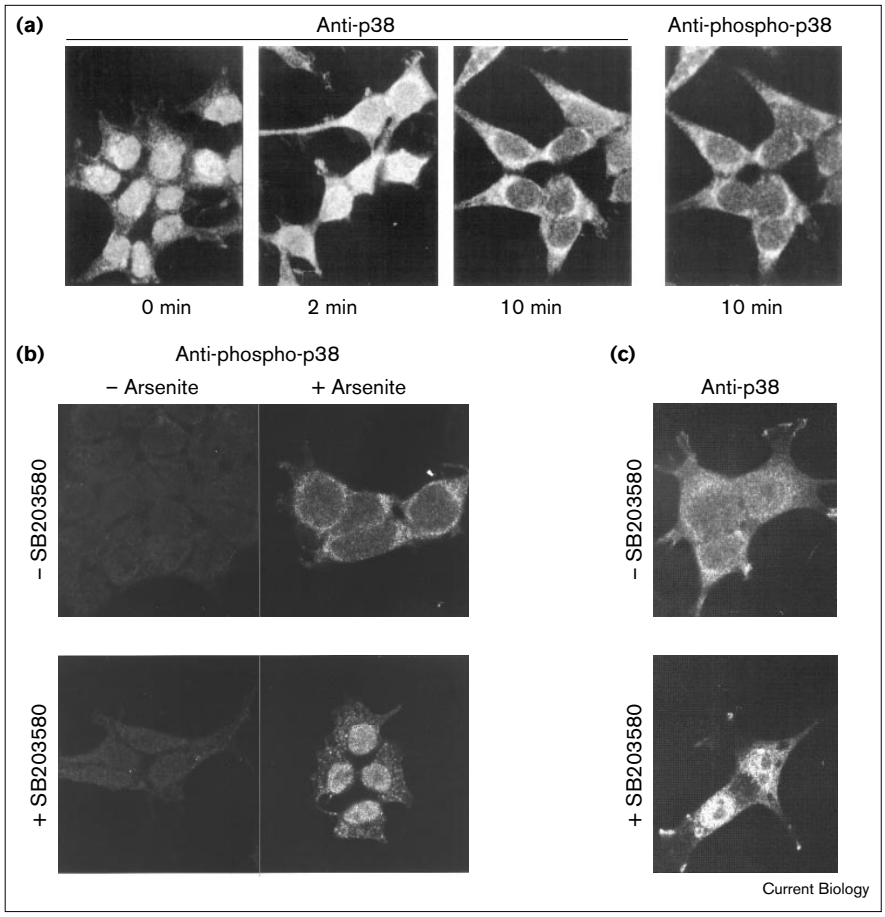


MAPKAP kinase-2 has a functional nuclear localisation signal that is required for activation by p38. **(a)** Localisation of MAPKAP kinase-2 (MAPKAPK2) and the NLS mutant. Myc-epitope-tagged wild-type (WT) MAPKAP kinase-2 or NLS-MAPKAP kinase-2 mutant (NLS-MAPKAPK2) were transfected into 293T cells, the cells fixed and the Myc epitope tag visualised by indirect immunofluorescence with mouse monoclonal antibody 9E10. The scale bar in (a) represents 10 μm. **(b)** Nuclear localisation of MAPKAP kinase-2 is required for activation. Wild-type MAPKAP kinase-2 or NLS-MAPKAP kinase-2 were transfected into 293T cells; after 40 h, cells were treated with anisomycin, NaCl or sodium arsenite, lysed, MAPKAP kinase-2 was immunoprecipitated with mouse monoclonal antibody 9E10 and the kinase activity measured.

Cellular localisation of p38

As nuclear localisation of MAPKAP kinase-2 was required for its activation we examined whether its activator, p38, was in the nucleus. 293T cells were stained by indirect immunofluorescence with a monoclonal antibody against p38. Figure 2a shows that endogenous p38 in 293T cells growing in 10% serum was mainly localised to the nucleus. Staining with an antibody raised against a phospho-peptide containing the phosphothreonine and phosphotyrosine in the T-loop — an antibody which recognises active p38 — showed little reactivity (Figure 2b). Strikingly, stimulation with sodium arsenite to activate the p38 protein kinase cascade led to accumulation of p38 in the cytoplasm. Ten

Figure 2



Endogenous p38 is nuclear and moves to the cytoplasm upon activation. **(a)** Unstimulated 293T cells or cells treated with 0.5 mM sodium arsenite for the indicated times were fixed and stained with a mouse monoclonal antibody against p38 (anti-p38) or a rabbit polyclonal serum raised against the phosphorylated peptide of the activation loop of p38 (anti-phospho-p38). **(b)** The activity of p38 is required for its exit from the nucleus. 293T cells were preincubated with 10 μM SB203580 for 30 min before they were treated with sodium arsenite for 10 min, fixed, and stained for phospho-p38 as before. **(c)** Inhibition of p38 activity results in its accumulation in the nucleus. In these cultures of cells, stained with anti-p38 to detect total p38, p38 was mainly cytosolic even before sodium arsenite treatment; SB203580 treatment resulted in p38 becoming nuclear, however.

minutes after stimulation the majority of p38 was found in the cytoplasm, and staining with the 'phospho-specific' antibody showed that this cytoplasmic p38 was active (Figure 2a). Similar results were found when p38 was activated by anisomycin (data not shown). In order to determine whether this relocalisation of p38 following activation required kinase activity, 293T cells were preincubated with the p38 inhibitor SB203580, treated with sodium arsenite and then examined by immunofluorescence. Figure 2b shows that SB203580 pretreatment resulted in activated p38 remaining in the nucleus rather than accumulating in the cytoplasm, as revealed by the phospho-specific antibody. Similar results were found for total p38 protein when SB203580-treated cells were stained with the monoclonal antibody against p38 (data not shown). These data suggest that p38 is activated in the nucleus but then exits to the cytoplasm. This export to the cytoplasm requires the kinase activity of p38 because export was inhibited by SB203580.

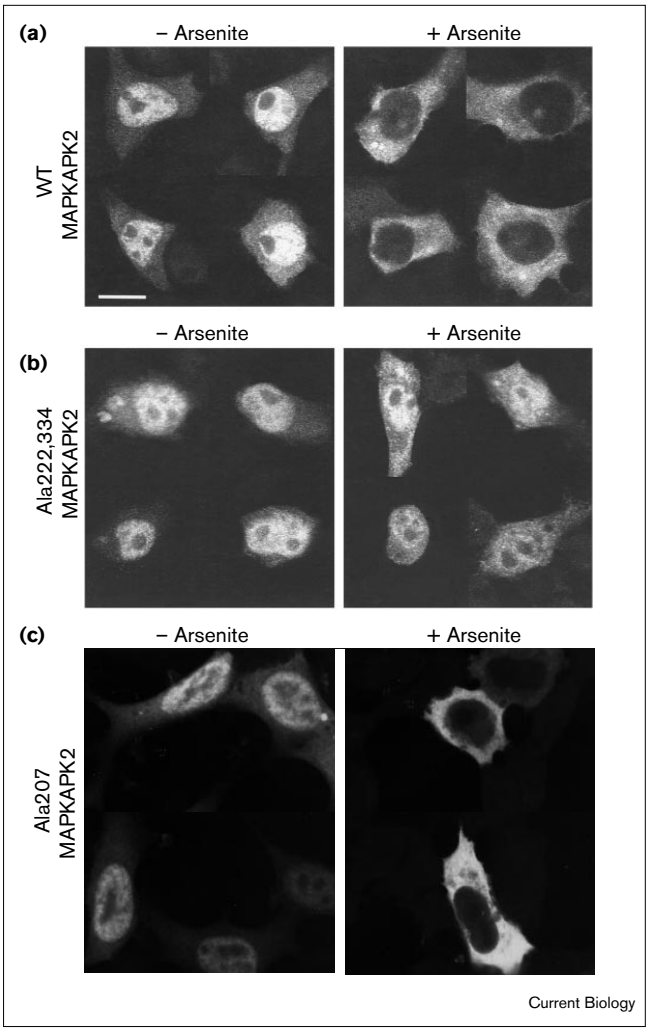
In some experiments with 293T cells, particularly when cells had been in culture for extended periods, we found that most of the cells had a high level of p38 in the cytoplasm before sodium arsenite stimulation (Figure 2c). When such cultures were treated with SB203580 before staining, however, p38 was found in the nucleus (Figure 2c). These observations suggest that culture conditions sometimes lead to activation of p38 and, as a consequence, it is exported to the cytoplasm.

MAPKAP kinase-2 moves to the cytoplasm upon activation induced by sodium arsenite

The observation that activated p38 does not move to the cytoplasm when cells are pretreated with the p38 inhibitor SB203580 suggests that phosphorylation of a substrate is required for nuclear export. Therefore, we examined the cellular localisation of MAPKAP kinase-2 before and after sodium arsenite stimulation. Figure 3a shows that MAPKAP kinase-2 moved to the cytoplasm on sodium arsenite treatment. Similar results were found in PC12 cells (data not shown).

To examine whether phosphorylation and activation of MAPKAP kinase-2 were required for nuclear export we

Figure 3



MAPKAP kinase-2 is exported to the cytoplasm following phosphorylation by p38. 293T cells were transfected with **(a)** Myc-epitope-tagged wild-type (WT), **(b)** Ala222,334 (non-phosphorylatable) or **(c)** Ala207 (kinase-dead) MAPKAP kinase-2 and treated with sodium arsenite for 15 min, followed by fixation and indirect immunofluorescence with the 9E10 monoclonal antibody to detect the Myc tag. The scale bar in (a) represents 10 μm.

expressed mutant versions of the protein, and examined their localisation following sodium arsenite treatment. The mutant Ala222,334 has two of the three phosphorylation sites in the protein that are required for its activation by p38 changed to alanine residues and cannot be activated by p38 *in vitro* or *in vivo* [29,30]. Figure 3b shows that the Ala222,334 mutant was nuclear in unstimulated cells and did not move to the cytoplasm following sodium arsenite stimulation of cells. This result was confirmed by showing that in cells treated with the p38 inhibitor SB203580 to block MAPKAP kinase-2 phosphorylation, MAPKAP kinase-2, like p38, was not exported to the cytoplasm (data not shown). These results demonstrate that phosphorylation of MAPKAP kinase-2 by p38 is required for nuclear export following activation of p38.

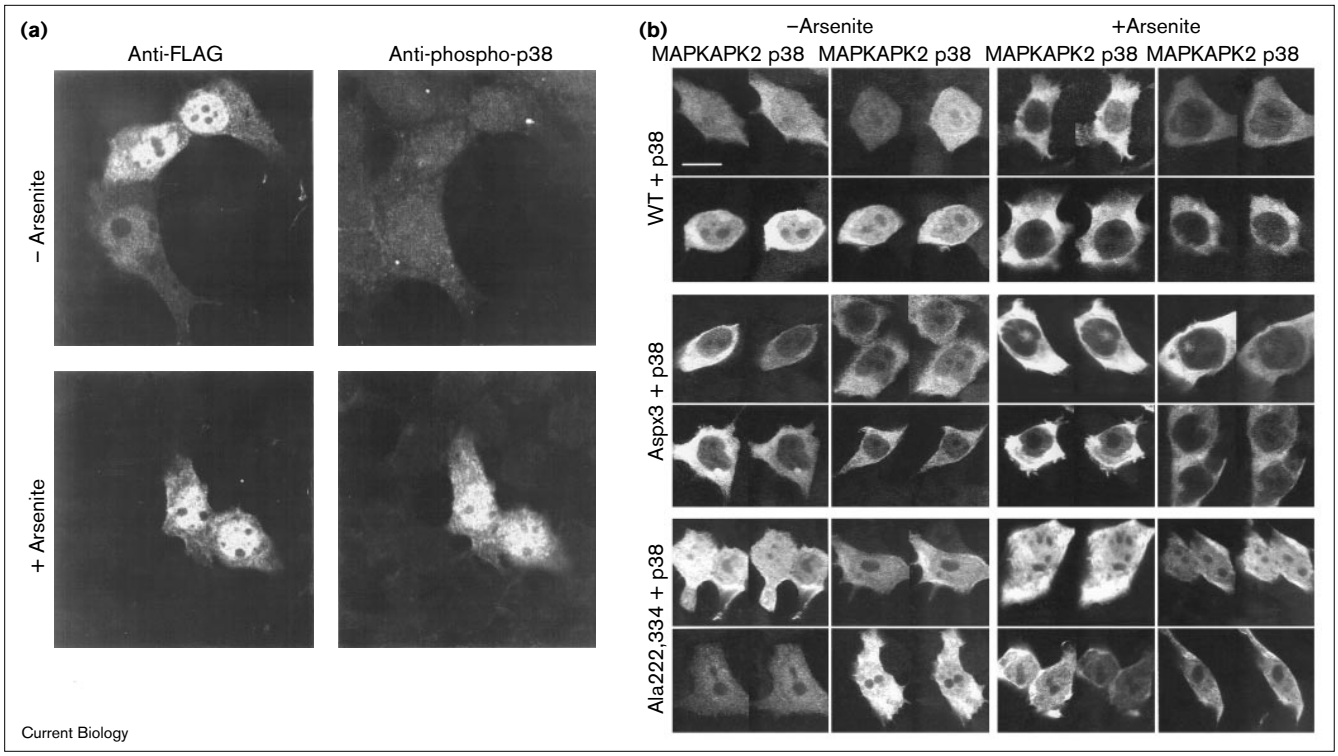
In order to determine whether the catalytic activity of MAPKAP kinase-2 was required for its export we expressed a mutant in which Asp207 of the conserved Asp–Phe–Gly motif was mutated to alanine. Asp207 is required to coordinate Mg^{2+} in the Mg^{2+}–ATP complex and substitution with alanine results in loss of catalytic activity [31]. Figure 3c shows that the kinase-dead Ala207 MAPKAP kinase-2 mutant was exported upon sodium arsenite stimulation, suggesting that the catalytic activity of MAPKAP kinase-2 is not required for export.

Nuclear export of p38 requires co-expression with MAPKAP kinase-2

To extend our findings on the export of endogenous p38 we examined the cellular localisation of overexpressed p38. Figure 4a shows that the overexpressed CSBP2 isoform of p38α, revealed by immunofluorescence for the FLAG epitope in the construct [14], was localised to the nucleus in growing cells but, unlike endogenous p38, was not exported to the cytoplasm on activation by sodium arsenite treatment. Similar results were found with the CSBP1 isoform (data not shown). The failure of the exogenous p38 to be exported could result from a failure of the overexpressed p38 to be activated; Figure 4a shows, however, that it did become activated because it was detected by the anti-phospho-p38 antibody following sodium arsenite treatment.

The results presented in Figure 4a show that when p38 is overexpressed it cannot be exported from the nucleus suggesting that an endogenous component may be limiting. Furthermore, the observation that treatment with the p38 inhibitor SB203580 blocks p38 export (Figure 2b,c) suggests that phosphorylation of this limiting component is required. As Figure 3a shows that the p38 substrate MAPKAP kinase-2 was exported from the nucleus following arsenite stimulation in a phosphorylation-dependent manner, we investigated whether MAPKAP kinase-2 was the limiting component. Figure 4b shows that when both p38 and MAPKAP kinase-2 were co-expressed, both the exogenous proteins were exported to the cytoplasm following activation of p38 by sodium arsenite treatment. Furthermore, if p38 was expressed together with Ala222,334 MAPKAP kinase-2, which fails to be exported, the exogenous p38 was not exported from the nucleus. We have previously described a constitutively activated mutant (Aspx3) of MAPKAP kinase-2 in which all three phosphorylation sites are replaced by aspartic acid residues to mimic phosphorylation [29]. This mutant was found to be cytosolic, consistent with the observations that nuclear export of MAPKAP kinase-2 requires phosphorylation. Figure 4b shows that when Aspx3 was co-expressed with p38, the p38 became cytosolic in the absence of sodium arsenite treatment.

Figure 4



MAPKAP kinase-2 and p38 are exported together from the nucleus. **(a)** Overexpressed p38 is nuclear and fails to be exported to the cytoplasm. 293T cells were transfected with FLAG-tagged p38, treated with sodium arsenite for 15 min, followed by fixation and indirect immunofluorescence with a mouse monoclonal antibody against the FLAG tag and rabbit antibody against phospho-p38. **(b)** Co-expression of wild-type MAPKAP kinase-2 with p38 results in nuclear export. 293T cells were transfected with p38 together with Myc-epitope-tagged wild-type (WT), Aspx3 or Ala222,334 MAPKAP kinase-2, treated with sodium arsenite, followed by fixation and indirect immunofluorescence with a goat polyclonal antibody against p38 or with the 9E10 antibody for the Myc tag on the MAPKAP kinase-2 proteins. The scale bar represents 10 μm.

These results argue that phosphorylation of MAPKAP kinase-2 by p38 not only activates the kinase activity of MAPKAP kinase-2, so that it can phosphorylate its substrates, but also results in nuclear export of both proteins.

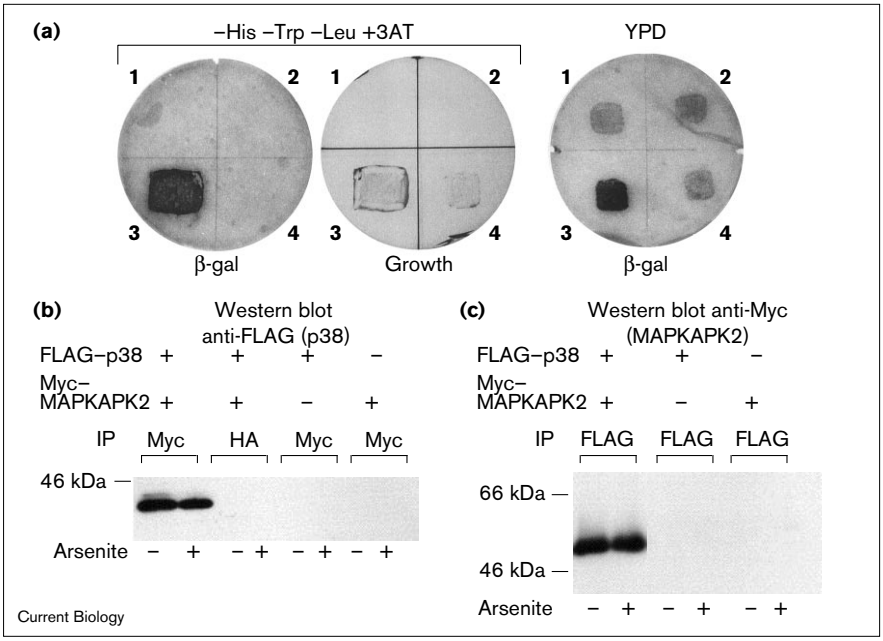
p38 and MAPKAP kinase-2 form a complex *in vivo*

The observation that export of overexpressed p38 requires co-expression of MAPKAP kinase-2 suggests that the two proteins form a complex. During screens of yeast two-hybrid libraries with MAPKAP kinase-2 as bait, we repeatedly isolated p38 (Figure 5a), suggesting that these proteins directly interact. To determine whether such a complex existed in mammalian cells, we expressed Myc-epitope-tagged MAPKAP kinase-2 and FLAG-epitope-tagged p38 in 293T cells and carried out reciprocal immunoprecipitations and western blots on the immunoprecipitates. Figure 5b,c shows that MAPKAP kinase-2 and p38 co-immunoprecipitated and that the amount of the proteins in the complex was unaffected by activation of the kinases by treatment of the cells with sodium arsenite.

Cellular localisation of MKK3 and MKK6

From the observations that nuclear p38 and MAPKAP kinase-2 are exported to the cytoplasm, it was of interest to determine the cellular localisation of MKK3 and MKK6, the dual-specificity kinase activators of p38 [5–7]. Staining of 293T cells with a sheep antibody raised against an amino-terminal peptide from MKK3 [32] revealed both cytosolic and nuclear staining (Figure 6). To confirm this localisation, MKK3 was overexpressed by transfection of a FLAG-epitope-tagged MKK3 expression vector [5] and the cells stained with the sheep anti-MKK3 antibody and a mouse monoclonal antibody against the FLAG epitope. Figure 6 shows that both antibodies gave an identical staining pattern consistent with the presence of MKK3 in both the cytoplasm and the nucleus. Staining for both endogenous MKK6 with a sheep anti-peptide antibody [32] and for FLAG-epitope-tagged overexpressed MKK6 [18] in transfected cells revealed a similar distribution to that of MKK3 (Figure 6). These results show that there is some MKK3 and MKK6 in the nucleus, consistent with activation of p38 in the nucleus.

Figure 5

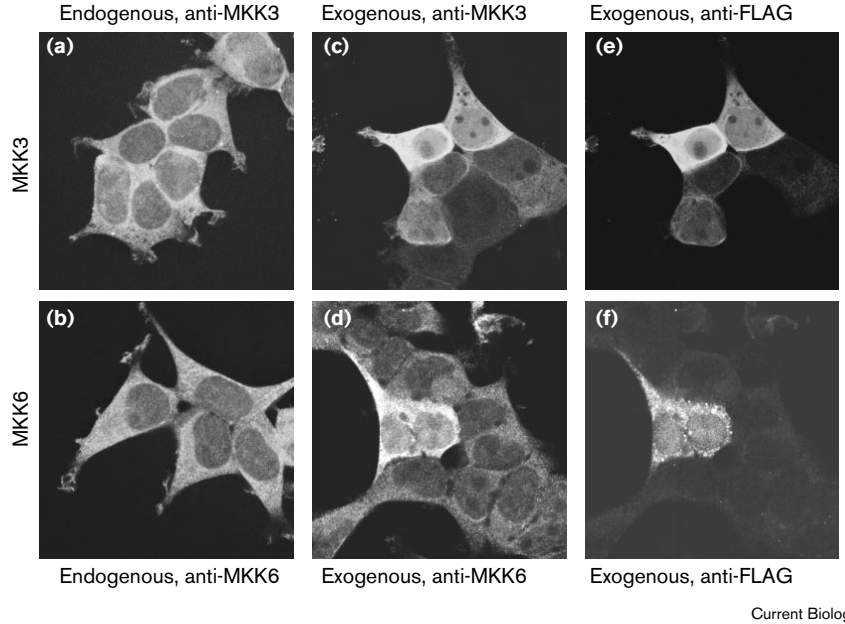


The p38 kinase and MAPKAP kinase-2 form a complex. **(a)** Two-hybrid analysis in yeast. On the left are β-galactosidase (β-gal) assays and growth on medium of the Y190 strain transformed with **1**, pYTH9 MAPKAPK2; **2**, pGADGH p38; **3**, pYTH9 MAPKAPK2 and pGADGH p38; **4**, pYTH9 and pGADGH p38 on medium lacking histidine, tryptophan and leucine (–His –Trp –Leu) and containing 3-aminotriazole (+3AT), which is selective for two-hybrid interaction. On the right is a β-galactosidase assay of the same transformants grown on non-selective YPD medium. **(b,c)** Complexes of MAPKAP kinase-2 and p38 in unstimulated mammalian cells and in cells stimulated with sodium arsenite. Myc-epitope-tagged MAPKAP kinase-2 and FLAG-epitope-tagged p38 were expressed in 293T cells, and immunoprecipitates (IP) with antibody against one epitope tag were immunoblotted with antibody against the other tag. In lanes labelled HA, immunoprecipitations were carried out with monoclonal antibody against the hemagglutinin (HA) epitope tag as an irrelevant antibody control.

Discussion

We have shown that MAPKAP kinase-2 has a functional nuclear localisation signal and that its activation by a variety of stimuli that activate p38 requires nuclear localisation. Our data are consistent with activation of p38 within the nucleus followed by its movement to the cytoplasm in a complex with phosphorylated MAPKAP kinase-2. It is, however, difficult to rule out cytoplasmic activation of p38 followed by rapid transport to the nucleus and then export to the cytoplasm. Export from

Figure 6



Cellular localisation of MKK3 and MKK6. For detection of endogenous proteins, 293T cells were fixed and **(a)** MKK3 and **(b)** MKK6 were detected by indirect immunofluorescence with sheep anti-peptide antibodies (anti-MKK3 or anti-MKK6). To detect exogenous overexpressed MKK3 or MKK6, 293T cells were transfected with expression vectors encoding FLAG-epitope-tagged proteins and, after fixation, proteins were visualised by indirect immunofluorescence with **(c)** sheep antibodies against MKK3 or **(d)** MKK6, and **(e,f)** a mouse monoclonal antibody against the FLAG tag. Note the similarity in staining patterns with the anti-FLAG and sheep antibodies in the overexpressing cells.

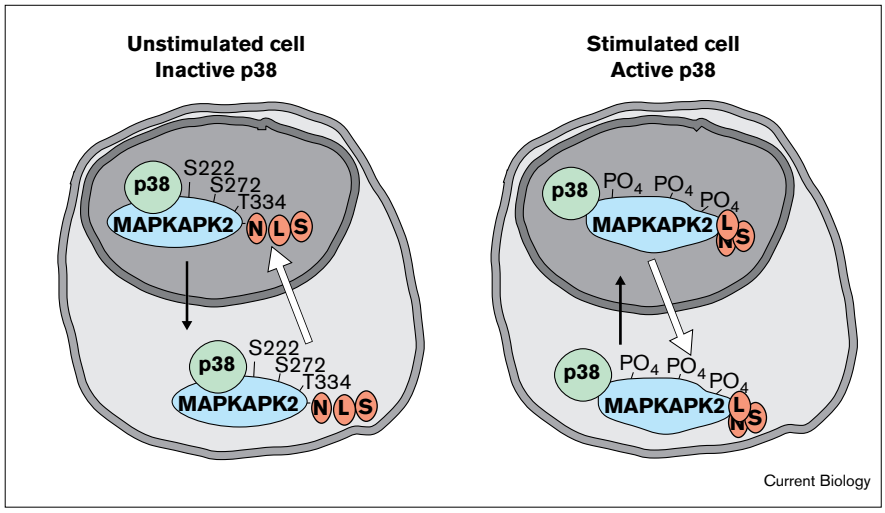
the nucleus may permit p38 and MAPKAP kinase-2 to be activated in the nucleus and then phosphorylate cytoplasmic substrates. The activation of p38 and MAPKAP kinase-2 in the nucleus contrasts with the Erk MAP kinase cascade where Erk and its protein kinase substrate $p90^{Rsk}$ are activated in the cytoplasm and move to the nucleus [22]. Thus, unlike the Erk MAP kinase cascade where the Erk activator — the dual-specificity protein kinase Mek — is actively excluded from the nucleus by its nuclear export signal [33], the p38 activators MKK3 and MKK6 must be able to activate p38 within the nucleus. Consistent with nuclear activation of p38, we find that MKK3 and MKK6 are localised in both the cytoplasm and nucleus, and thus could provide a link between cytoplasmic signalling events and nuclear activation of p38. Recent studies on the activation of p38 by DNA damage demonstrates that it requires c-Abl, which is activated by the nuclear kinase DNA-PK [34–36]. As c-Abl is nuclear, its activation could provide a mechanism for initiating the p38 cascade within the nucleus.

The localisation of p38 to the nucleus may depend on its association with nuclear MAPKAP kinase-2, but the observation that overexpressed p38 is nuclear, without the requirement for overexpression of MAPKAP kinase-2, suggests that nuclear localisation of p38 may not require MAPKAP kinase-2. Export of nuclear p38 to the cytoplasm does require MAPKAP kinase-2, because overexpressed p38 is not exported to the cytoplasm unless it is co-expressed with MAPKAP kinase-2, suggesting that the amount of MAPKAP kinase-2 is limiting. Comparison of the amounts of endogenous p38 with MAPKAP kinase-2 by immunoblotting with the monoclonal antibody against p38 and a polyclonal antibody against MAPKAP kinase-2 suggests that there is up to eight times more endogenous MAPKAP kinase-2 in the cells than p38 (data not shown). This suggests that not all the endogenous MAPKAP kinase-2 is complexed to p38. Perhaps the excess of MAPKAP kinase-2 is to ensure that all the p38 is complexed to it; alternatively, MAPKAP kinase-2 may have partners other than p38. In addition, p38 could form complexes with its substrates such as Mnk [16]. These issues will only be resolved when reagents become available to immunoprecipitate p38, MAPKAP kinase-2 and their complexes quantitatively.

The export of p38 and MAPKAP kinase-2 from the nucleus is dependent on the phosphorylation of MAPKAP kinase-2. As an overexpressed kinase-dead version of MAPKAP kinase-2 can be exported, this implies that export does not depend on catalytic activity unless the endogenous MAPKAP kinase-2 can provide this in *trans*. If export requires endogenous MAPKAP kinase-2 activity, however, then this is not sufficient on its own to permit export of overexpressed p38, as export of p38 is restored by overexpression of kinase-dead MAPKAP kinase-2. Phosphorylation might regulate the cellular location of MAPKAP kinase-2 by masking the function of the NLS and thereby altering the balance between nuclear export and import (see Figure 7 for a model). Phosphorylation of MAPKAP kinase-2 could also unmask a nuclear export signal, or permit formation of a complex with a protein containing an export signal. Export of nuclear MAPKAP kinase-2 appears to depend on a leucine-rich nuclear export signal as preliminary experiments (R.W., unpublished observations) show that export is blocked by leptomycin B [37]. While this work was in preparation Engel *et al.* [38] reported leptomycin-B-sensitive nuclear export of phosphorylated MAPKAP kinase-2 and suggested that there is a nuclear export signal within amino acids 328–351

Figure 7

A model for the regulation of the nuclear localisation of MAPKAP kinase-2 and p38 by phosphorylation. MAPKAP kinase-2 and p38 are complexed together. In unstimulated cells where p38 is inactive, MAPKAP kinase-2 is not phosphorylated and its nuclear localisation signal (NLS) is exposed permitting nuclear import. In stimulated cells, phosphorylation of MAPKAP kinase-2 by active p38 leads to a conformational change in MAPKAP kinase-2 resulting in masking of the NLS and perhaps unmasking of a nuclear export signal. The net effect is to lead to relocalisation of the p38–MAPKAP kinase-2 complex to the cytoplasm.



of the protein. They did not, however, describe the export of p38 in association with MAPKAP kinase-2.

These results show that the cellular localisation of p38 depends on the level of p38 activity and phosphorylation of MAPKAP kinase-2. From studies on other MAP kinase pathways it now appears that components of these cascades not only act as kinases but have other functions; for example, PBS2 in the p38/Hog1 pathway in *Saccharomyces cerevisiae* is not only an activator of p38/Hog1 MAP kinase but is also a 'scaffold' protein that brings the components of the cascade together [39]. Our studies with MAPKAP kinase-2 show that in the p38 SAP kinase pathway, MAPKAP kinase-2 serves a dual function, both as an effector of p38 by phosphorylating substrates such as Hsp27 and CREB and as a determinant of the localisation of p38 in the cell.

Materials and methods

Plasmid constructs

Human MAPKAP kinase-2 was subcloned into the *Nco*I–*Bam*HI sites of pEF vector [40] to create a 5′ Myc-tagged construct. The NLS-MAPKAP kinase-2 was generated by exchanging the 3′ end of the gene with a synthetic oligonucleotide in which the sequence encoding amino acids 386–389 (Lys–Arg–Arg–Lys) was replaced by four asparagine residues. The non-phosphorylatable (Ala222,334) and kinase-dead (Ala207) mutants were created by PCR-mediated mutagenesis using oligonucleotides to replace Ser222 and Thr334 or Asp207 with alanine residues. FLAG-tagged p38 (CSBP2) cloned in pCDN [14] was a gift from Peter Young, SmithKline Beecham.

Immunoprecipitation and kinase assays

The 293T cells were induced with agonists, and cell lysates prepared in lysis buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 10% glycerol, 1% NP-40, 1 mM EGTA, 1 mM sodium vanadate, 50 mM NaF, 3 mM PMSF, 10 μg/ml aprotinin, 10 μg/ml leupeptin and 10 μg/ml pepstatin). Soluble fractions were then immunoprecipitated with anti-MAPKAP kinase-2 antibodies bound to protein-G–Sepharose beads for 90 min at 4°C. Beads were washed three times in lysis buffer. Immunoprecipitates were resolved on 10% SDS–PAGE blotted and probed with anti-p38 antibodies (Santa Cruz) and anti-rabbit HRP followed by enhanced chemiluminescence. For kinase assays on immunoprecipitates from transfected cells, expression levels of the Myc-tagged MAPKAP kinase-2 were first estimated by immunoblotting with 9E10 monoclonal antibody and then equivalent amounts of Myc-tagged MAPKAP kinase-2 were immunoprecipitated from lysates. Kinase assays were performed with a peptide substrate as described [29].

Cell culture, transfection, microinjection and immunofluorescence staining

293T cells were transfected with $CaPO_4$ or microinjected using a Zeiss-Eppendorf microinjection workstation and analysed after 20 h. Cells were grown in DMEM plus 10% FCS and stimulated by addition of 0.5 mM sodium arsenite for 2–20 min, 50 mM anisomycin for 15 min, 300 mM NaCl for 15 min or 100 ng/ml EGF for 10 min. SB203580 was a gift from SmithKline Beecham and dissolved in dimethylsulphoxide. It was used at a final concentration of 10 μM and cells were pretreated for 0.5–1 h. After fixation [41], cells were stained with mouse monoclonal antibody against recombinant p38 (Zymed) and a rabbit polyclonal serum raised against amino acids 172–186 of p38, in which Thr180 and Tyr182 were phosphorylated (New England Biolabs), followed by TRITC-coupled anti-mouse IgG (Jackson Immunoresearch) and FITC-coupled anti-rabbit IgG. In some experiments, overexpressed p38 was visualised with a polyclonal goat serum against p38 (Santa Cruz). The Myc tag on Myc-tagged MAPKAP kinase-2 was visualised either with mouse monoclonal antibody 9E10 [42] or with a rabbit polyclonal serum (Santa Cruz). The FLAG tag on pCDN p38/CSBP2 was visualised either with a mouse monoclonal antibody (IBI-Kodak) or with a rabbit polyclonal serum (Santa Cruz). FLAG-tagged MKK3 (pRcRSVMKK3) [5] and FLAG-tagged MKK6 (pcDNA3 MKK6) [18] were visualised with a mouse monoclonal antibody against FLAG, or sheep anti-peptide antibodies against MKK3 or MKK6, respectively [32]. Stained cells were viewed on a confocal imaging system (BIORAD 1024) and fluorescence in each channel captured separately. Western blots showed that the rabbit anti-phospho-p38, sheep anti-MKK3, goat anti-p38 and monoclonal anti-p38 antibodies recognised one major band. Furthermore, when cells were transfected with expression constructs for the relevant target proteins, cells overexpressing the proteins showed more intense staining than untransfected surrounding cells.

Two-hybrid analysis in yeast

Human MAPKAP kinase-2 was subcloned into the vector pYTH9 and integrated into *S. cerevisiae* strain Y190. A transformant expressing the Gal4 DNA binding-domain fusion protein was then transformed with a HeLa two-hybrid library (Clontech). His^+ colonies were then tested for β-galactosidase activity. p38/CSBP2 was recovered many times from β-galactosidase-positive colonies as well as other sequences that will be described elsewhere.

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References

1. Cohen P: **The search for physiological substrates of MAP and SAP kinases in mammalian cells.** *Trends Cell Biol* 1997, **7:**353-361.
2. Lee JC, Laydon JT, McDonnell PC, Gallagher TF, Kumar S, Green D, *et al.*: **A protein kinase involved in the regulation of inflammatory cytokine biosynthesis.** *Nature* 1994, **372:**739-749.
3. Freshney NW, Rawlinson L, Guesdon F, Jones E, Cowley S, Hsuan J, *et al.*: **Interleukin-1 activates a novel protein kinase cascade that results in the phosphorylation of hsp27.** *Cell* 1994, **78:**1039-1049.
4. Galcheva-Gargova Z, Derijard B, Wu IH, Davis RJ: **An osmosensing signal transduction pathway in mammalian cells.** *Science* 1994, **265:**806-808.
5. Derijard B, Raingeaud J, Barrett T, Wu IH, Han J, Ulevitch RJ, *et al.*: **Independent human MAP-kinase signal transduction pathways defined by MEK and MKK isoforms**. *Science* 1995, **267:**682-685.
6. Han J, Lee JD, Jiang Y, Li Z, Feng L, Ulevitch RJ: **Characterization of the structure and function of a novel MAP kinase kinase (MKK6)**. *J Biol Chem* 1996, **271:**2886-2891.
7. Stein B, Brady H, Yang MX, Young DB, Barbosa MS: **Cloning and characterization of MEK6, a novel member of the mitogen-activated protein kinase kinase cascade**. *J Biol Chem* 1996, **271:**11427-11433.
8. Tibbles LA, Ing YL, Kiefer F, Chan J, Iscove N, Woodgett JR, *et al.*: **MLK-3 activates the SAPK/JNK and p38/RK pathways via SEK1 and MKK3/6**. *EMBO J* 1996, **15:**7026-7035.
9. Teramoto H, Coso OA, Miyata H, Igishi T, Miki T, Gutkind JS: **Signaling from the small GTP-binding proteins Rac1 and Cdc42 to the c-Jun N-terminal kinase/stress-activated protein kinase pathway: a role for mixed lineage kinase 3/protein-tyrosine kinase 1, a novel member of the mixed lineage kinase family.** *J Biol Chem* 1996, **271:**27225-27228.
10. Minden A, Lin A, Claret FX, Abo A, Karin M: **Selective activation of the JNK signaling cascade and c-Jun transcriptional activity by the small GTPases Rac and Cdc42Hs**. *Cell* 1995, **81:**1147-1157.
11. Zhang S, Han J, Sells M, Chernoff J, Knaus U, Ulevitch R, *et al.*: **Rho family GTPases regulate p38 mitogen-activated protein kinase through the downstream mediator PAK1**. *J Biol Chem* 1995, **270:**23934-23936.

12. Stokoe D, Campbell DG, Nakielny S, Hidaka H, Leevers SJ, Marshall CJ, *et al.*: **MAPKAP kinase-2; a novel protein kinase activated by mitogen-activated protein kinase**. *EMBO J* 1992, **11:**3985-3994.
13. Rouse J, Cohen P, Trigon S, Morange M, Alonso-Llamazares A, Zamanillo D, *et al.*: **A novel kinase cascade triggered by stress and heat shock that stimulates MAPKAP kinase-2 and phosphorylation of the small heat shock proteins.** *Cell* 1994, **78:**1027-1037.
14. McLaughlin MM, Kumar S, McDonnell PC, Van Horn S, Lee JC, Livi GP, *et al.*: **Identification of mitogen-activated protein (MAP) kinase-activated protein kinase-3, a novel substrate of CSBP p38 MAP kinase.** *J Biol Chem* 1996, **271:**8488-8492.
15. Waskiewicz AJ, Flynn A, Proud CG, Cooper JA: **Mitogen-activated protein kinases activate the serine/threonine kinases Mnk1 and Mnk2**. *EMBO J* 1997, **16:**1909-1920.
16. Fukunaga R, Hunter T: **MNK1, a new MAP kinase-activated protein kinase, isolated by a novel expression screening method for identifying protein kinase substrates**. *EMBO J* 1997, **16:**1921-1933.
17. Wang X, Ron D: **Stress-induced phosphorylation and activation of the transcription factor CHOP (GADD153) by p38 MAP kinase.** *Science* 1996, **272:**1347-1349.
18. Raingeaud J, Whitmarsh AJ, Barrett T, Derijard B, Davis RJ: **MKK3- and MKK6-regulated gene expression is mediated by the p38 mitogen-activated protein kinase signal transduction pathway.** *Mol Cell Biol* 1996, **16:**1247-1255.
19. Whitmarsh AJ, Yang SH, Su MS, Sharrocks AD, Davis RJ: **Role of p38 and JNK mitogen-activated protein kinases in the activation of ternary complex factors**. *Mol Cell Biol* 1997, **17:**2360-2371.
20. Price MA, Cruzalegui FH, Treisman R: **The p38 and ERK MAP kinase pathways cooperate to activate ternary complex factors and c-fos transcription in response to UV light**. *EMBO J* 1996, **15:**6552-6563.
21. Janknecht R, Hunter T: **Convergence of MAP kinase pathways on the ternary complex factor Sap-1a**. *EMBO J* 1997, **16:**1620-1627.
22. Chen R-H, Sarnecki C, Blenis J: **Nuclear localization and regulation of *erk*- and *rsk*-encoded protein kinases.** *Mol Cell Biol* 1992, **12:**915-927.
23. Traverse S, Gomez N, Paterson H, Marshall CJ, Cohen P: **Sustained activation of the mitogen-activated protein (MAP) kinase cascade may be required for differentiation of PC12 cells. Comparison of the effects of nerve growth factor and epidermal growth factor**. *Biochem J* 1992, **288:**351-355.
24. Hill CS, Marais R, John S, Wynne J, Dalton S, Treisman R: **Functional analysis of a growth factor-responsive transcription factor complex**. *Cell* 1993, **73:**395-406.
25. Zu YL, Wu F, Gilchrist A, Ai Y, Labadia ME, Huang CK: **The primary structure of a human MAP kinase activated protein kinase 2.** *Biochem Biophys Res Commun* 1994, **200:**1118-1124.
26. Stokoe D, Caudwell FB, Cohen PTW, Cohen P: **The substrate specificity and structure of mitogen-activated protein (MAP) kinase-activated protein kinase-2.** *Biochem J* 1993, **296:**843-849.
27. Dingwall C, Laskey RA: **Nuclear targeting sequences - A consensus?** *Trends Biochem Sci* 1991, **16:**478-481.
28. Tan Y, Rouse J, Zhang A, Cariati S, Cohen P, Comb MJ: **FGF and stress regulate CREB and ATF-1 via a pathway involving p38 MAP kinase and MAPKAP kinase-2**. *EMBO J* 1996, **15:**4629-4642.
29. Ben-Levy R, Leighton IA, Doza YN, Attwood P, Morrice N, Marshall CJ, *et al.*: **Identification of novel phosphorylation sites required for activation of MAPKAP kinase-2.** *EMBO J* 1995, **14:**5920-5930.
30. Engel K, Schultz H, Martin F, Kotlyarov A, Plath K, Hahn M, *et al.*: **Constitutive activation of mitogen-activated protein kinase-activated protein kinase 2 by mutation of phosphorylation sites and an A-helix motif.** *J Biol Chem* 1995, **270:**27213-27221.
31. Taylor SS, Radzio-Andzelm E: **Cyclic AMP-dependent protein kinase.** In *Protein Kinases*, Edited by Hames BD, Glover DM. Oxford: IRL Press; 1994:1-29.
32. Cuenda A, Alonso G, Morrice N, Jones M, Meier R, Cohen P, *et al.*: **Purification and cDNA cloning of SAPKK3, the major activator of RK/p38 in stress- and cytokine-stimulated monocytes and epithelial cells**. *EMBO J* 1996, **15:**4156-4164.
33. Fukuda M, Gotoh I, Gotoh Y, Nishida E: **Cytoplasmic localization of mitogen-activated protein kinase kinase directed by its NH2-terminal, leucine-rich short amino acid sequence, which acts as a nuclear export signal.** *J Biol Chem* 1996, **271:**20024-20028.
34. Pandey P, Raingeaud J, Kaneki M, Weichselbaum R, Davis RJ, Kufe D, *et al.*: **Activation of p38 mitogen-activated protein kinase by c-Abl-dependent and -independent mechanisms**. *J Biol Chem* 1996, **271:**23775-23779.
35. Kharbanda S, Ren R, Pandey P, Shafman TD, Feller SM, Weichselbaum RR, *et al.*: **Activation of the c-Abl tyrosine kinase in the stress response to DNA-damaging agents**. *Nature* 1995, **376:**785-788.
36. Kharbanda S, Pandey P, Jin S, Inoue S, Bharti A, Yuan ZM, *et al.*: **Functional interaction between DNA-PK and c-Abl in response to DNA damage**. *Nature* 1997, **386:**732-735.
37. Kudo N, Khochbin S, Nishi K, Kitano K, Yanagida M, Yoshida M, *et al.*: **Molecular cloning and cell cycle-dependent expression of mammalian CRM1, a protein involved in nuclear export of proteins**. *J Biol Chem* 1997, **272:**29742-29751.
38. Engel K, Kotlyarov A, Gaestel M: **Leptomycin B-sensitive nuclear export of MAPKAP kinase 2 is regulated by phosphorylation.** *EMBO J* 1998, **17:**3363-3371.
39. Posas F, Saito H: **Osmotic activation of the HOG MAPK pathway via Ste11p MAPKKK: scaffold role of Pbs2p MAPKK**. *Science* 1997, **276:**1702-1705.
40. Marais R, Light Y, Paterson HF, Marshall CJ: **Ras recruits Raf-1 to the plasma membrane for activation by tyrosine phosphorylation**. *EMBO J* 1995, **14:**3136-3145.
41. Traverse S, Cohen P, Paterson H, Marshall CJ, Rapp U, Grand RJA: **Specific association of activated MAP kinase kinase kinase (Raf) with the plasma membranes of *ras*-transformed retinal cells**. *Oncogene* 1993, **8:**3175-3181.
42. Evan GI, Lewis GK, Ramsay G, Bishop JM: **Isolation of monoclonal antibodies specific for human c-myc proto-oncogene product**. *Mol Cell Biol* 1985, **5:**3610-3616.

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