

Leptomycin B-sensitive nuclear export of MAPKAP kinase 2 is regulated by phosphorylation

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To study the intracellular localization of MAPKAP kinase 2 (MK2), which carries a putative bipartite nuclear localization signal (NLS), we constructed a green fluorescent protein–MAPKAP kinase 2 fusion protein (GFP–MK2). In transfected cells, this protein is located predominantly in the nucleus; unexpectedly, upon stress, it rapidly translocates to the cytoplasm. This translocation can be blocked by the p38 MAP kinase inhibitor SB203580, indicating its regulation by phosphorylation. Molecular mimicry of MK2 phosphorylation at T317 in GFP–MK2 led to a mutant which is located almost exclusively in the cytoplasm of the cell, whereas the mutant T317A shows no stress-induced redistribution. Since leptomycin B, which inhibits the interaction of exportin 1 with the Rev-type leucine-rich nuclear export signal (NES), blocks stress-dependent translocation of GFP–MK2, it is supposed that phosphorylation-induced export of the protein causes the translocation. We have identified the region responsible for nuclear export in MK2 which is partially overlapping with and C-terminal to the autoinhibitory motif. This region contains a cluster of hydrophobic amino acids in the characteristic spacing of a leucine-rich Rev-type NES which is necessary to direct GFP–MK2 to the cytoplasm. However, unlike the Rev-type NES, this region alone is not sufficient for nuclear export. The data obtained indicate that MK2 contains a constitutively active NLS and a stress-regulated signal for nuclear export.

Keywords: nuclear export/nuclear import/protein phosphorylation/signal transduction/stress response

Introduction

Different MAP kinase cascades mediate signal transduction from the cell surface to the nucleus (for a review see Karin and Hunter, 1995). In parallel to the classical MAP kinase cascade which leads to stimulation of p42/44 MAP/extracellular signal-regulated kinases (ERKs), there are at least two stress-activated cascades which activate the stress-activated protein kinases (SAPKs)/c-Jun N-terminal kinases (JNKs) and the p38

MAP/reactivating kinases (RKs). Activation of MAP kinases leads to their nuclear translocation (Chen *et al.*, 1992; Lenormand *et al.*, 1993) and phosphorylation of certain DNA-binding proteins which mainly contributes to transcriptional regulation (reviewed in Karin and Hunter, 1995). So far, it is not clear by which mechanism nuclear translocation of the MAP kinases proceeds, since no nuclear localization signal (NLS) could be identified in these enzymes. However, the importance of the translocation of, for example, the JNKs to the nucleus for stress-induced signalling has been demonstrated recently by inhibition of signalling through cytoplasmic retention of the enzyme by the specific JNK-interacting protein JIP (Dickens *et al.*, 1997).

In contrast to the MAPKs, there is evidence that the upstream kinase MAPKK/MEK is localized constitutively in the cytoplasm (Lenormand *et al.*, 1993) as a consequence of a nuclear export signal (NES) in its N-terminus (Fukuda *et al.*, 1996). MAPKK may also function as a cytoplasmic anchor protein for non-activated MAPK (Fukuda *et al.*, 1997a) or as a shuttle for activated MAPK to the nucleus (Jaaro *et al.*, 1997). Interestingly, deletion of the NES in MAPKK leads to malignant cell transformation which is probably caused by increased nuclear MAPK activity and which can be reverted by overexpression of the MAPK-specific phosphatase CL100 (Fukuda *et al.*, 1997c).

Apart from phosphorylation of transcription factors, activation of several protein kinases is downstream in MAPK signalling. p42/44 MAPKs activate p90 RSK, also designated as MAPK-activated protein kinase 1. As a result of activation, p90 RSK is also translocated from the cytoplasm to the nucleus (Chen *et al.*, 1992). The stress-stimulated p38 MAPK is the activator for MAPKAP kinase 2 (MK2) (Freshney *et al.*, 1994; Rouse *et al.*, 1994) and MAPKAP kinase 3 (McLaughlin *et al.*, 1996) which have been shown to phosphorylate the cytoplasmic small heat shock protein Hsp25/27 (Stokoe *et al.*, 1992a; Clifton *et al.*, 1996; Ludwig *et al.*, 1996) and, as demonstrated for MK2, the nuclear transcription factor CREB (Tan *et al.*, 1996). Although p38 MAPK is detected both in the nucleus and in the cytoplasm (Raingeaud *et al.*, 1995; Read *et al.*, 1997), it is not clear whether this kinase changes its subcellular localization upon stress. For its targets, MK2 and 3, a conserved putative NLS has been identified (Engel *et al.*, 1993; Stokoe *et al.*, 1993; McLaughlin *et al.*, 1996; Sithanandam *et al.*, 1996).

Here we examined the signal-dependent intracellular distribution of MK2 using mainly green fluorescence protein (GFP)–kinase hybrids. We provide evidence for a functional NLS and, in addition, for a functional NES in the C-terminal part of MK2. Both signals together influence the subcellular distribution of MK2 in a phosphorylation-dependent manner.

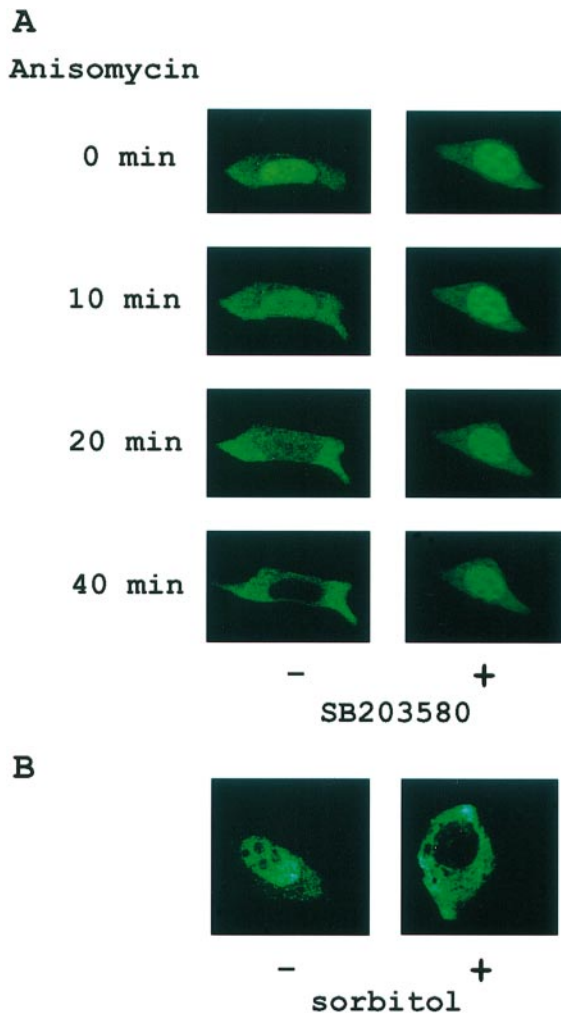


Fig. 1. Stress-induced phosphorylation-dependent export of GFP-MK2 and Myc-MK2. **(A)** Swiss 3T3 cells transiently transfected with GFP-MK2 were treated with anisomycin (Cano *et al.*, 1996) to stimulate the p38 MAP kinase cascade in the presence (+) and absence (-) of the p38 MAP kinase inhibitor SB203580 (Lee *et al.*, 1994). The subcellular localization of MK2 was analysed in single cells at different times after stimulation by confocal fluorescence microscopy. **(B)** Swiss 3T3 cells transiently transfected with Myc-tagged MK2 were subjected to a hyperosmotic stress treatment with 0.4 M sorbitol (+sorbitol) and, as a control, were left untreated (-sorbitol). After 20 min, cells were fixed and the subcellular localization of Myc-MK2 was analysed using the anti-Myc antibody 9E10.

Results

Stress-induced phosphorylation-dependent changes in subcellular localization of GFP-MK2 and Myc-MK2 in Swiss 3T3 cells

To analyse the subcellular distribution of mouse MK2, we constructed a GFP-kinase hybrid (GFP-MK2) which was transfected into Swiss 3T3 cells. We found that in contrast to GFP alone, which is distributed diffusely both in the cytoplasm and the nucleus, the GFP-MK2 is located mainly in the nucleus of the cells (Figure 1A), indicating that the putative NLS of the kinase (amino acids 356–372) is functional. Remarkably, stress-inducing conditions such as treatment with anisomycin (10 µg/ml; Figure 1A), UV (30 J/m²), H₂O₂ (4 mM) or arsenite (200 µM) (data not shown), which activate the p38 MAP kinase cascade, lead to rapid translocation of GFP-MK2 to the cytoplasm

which is almost complete after 20 min at 25°C. The inhibitor SB203580 (Lee *et al.*, 1994), which is highly specific for the p38 MAP kinases and blocks stress-dependent MK2 phosphorylation and activation (Cuenda *et al.*, 1995), completely prevents this translocation of the kinase (Figure 1A). These data suggest a stress-induced translocation of GFP-MK2 which is regulated by phosphorylation.

In a second experiment, we used Myc-tagged MK2 (Myc-MK2) to make sure that the stress-induced translocation is not an artificial result of the GFP domain fused to the kinase. After transfection with the Myc-MK2 expression vector, Swiss 3T3 cells were subjected to hyperosmotic stress (0.4 M sorbitol) which is also known to stimulate the p38 MAPK effectively in mammalian cells (Brunet and Pouyssegur, 1996). The Myc-MK2 is localized predominantly in the nucleus of control cells whereas in hyperosmotic stimulated cells the tagged enzyme is seen mainly in the cytoplasm (Figure 1B). This finding suggests that the stress-dependent translocation of MK2 is independent of the N-terminal fused peptide or protein.

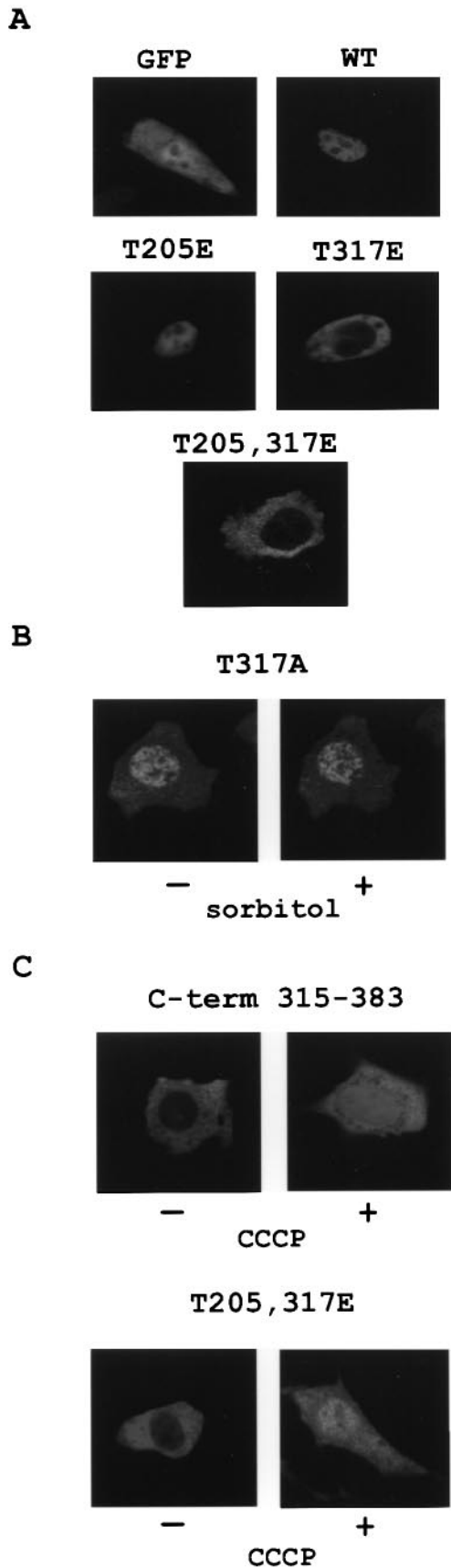
Subcellular distribution of GFP-MK2 mutants mimicking regulatory phosphorylation of MK2

In MK2 there are at least two regulatory phosphorylation sites: threonine 205 (T205; Ben-Levy *et al.*, 1995; Engel *et al.*, 1995), which is located at the activation loop of the kinase between subdomains VII and VIII, and threonine 317 (T317; Stokoe *et al.*, 1992b), which is outside of the catalytic domain adjacent to the autoinhibitory A-helix (Engel *et al.*, 1995; Figure 4A). To understand further the molecular mechanism of the nuclear-cytoplasmic translocation, constitutively active mutants (Engel *et al.*, 1995) were used in which the regulative phosphorylation sites are replaced by glutamic acid. Mutant GFP-MK2-T205E was found in the nucleus but the mutants GFP-MK2-T317E and -T205,317E localized exclusively in the cytoplasm (Figure 2A). This indicated that phosphorylation of T317 but not of T205 is essential for initiating translocation. To prove this idea, we constructed the mutant GFP-MK2-T317A which should mimic a kinase which is non-phosphorylatable at this site. Although GFP-MK2-T317A showed an increased cytoplasmic localization compared with the wild-type protein, it still accumulated predominantly in the nucleus of the transfected cells (Figure 2B). More importantly, stress treatment, for example, addition of 0.4 M sorbitol, did not change the intracellular localization of this mutant (Figure 2B). This strengthens the notion that phosphorylation of T317 is necessary for the translocation.

The C-terminus of MK2 is translocated constitutively to the cytoplasm in an ATP-dependent manner

Phosphorylation of T317 has been supposed to weaken the interaction between the autoinhibitory motif and the catalytic core and thereby to regulate the accessibility of the C-terminus of the kinase (Engel *et al.*, 1995). This process might also be involved in the phosphorylation-induced translocation of MK2. To check this idea, we deleted the entire catalytic core and the N-terminal part of MK2 from the construct. The resulting fusion protein GFP-C-term containing amino acids 315–383 carries the

phosphorylation site T317, the autoinhibitory motif (with its functionally relevant tryptophan residue W332) and the bipartite NLS (356–372) (Figure 4A). In transfected



Swiss 3T3 cells, the GFP-C-term protein is localized completely in the cytoplasm of the cell independently of T317 phosphorylation (Figure 2C, C-term 315–383, –CCCPC), supporting the hypothesis that a release of the autoinhibitory motif from the catalytic domain might be sufficient to lead to cytoplasmic localization of the kinase.

Recent advances in understanding intracellular protein transport between the cytoplasm and the nucleus have demonstrated that signal-dependent processes are responsible for both nuclear import and export of cellular and viral proteins (Nigg, 1997; Ullman *et al.*, 1997). Hence, the release of the autoinhibitory motif from the catalytic domain may deactivate the NLS or activate an NES or a cytoplasmic anchor sequence in the C-terminus of the kinase.

To characterize further the translocation, the energy dependence was analysed by depletion of intracellular ATP using different reagents (e.g. CCCP, azide). These reagents depleted the intracellular ATP level to ~10% after 30 min and caused a significant reversible redistribution of GFP-C-term as well as of GFP-MK2-T205,317E; both mutants were then also detected in the nucleus (Figure 2C), suggesting an energy-dependent export or cytoplasmic anchoring of the protein.

Leptomycin B inhibits stress-dependent export of GFP-MK2 and reverses the translocation of cytoplasmic GFP-MK2 mutants

The rapid translocation of GFP-MK2 in response to stress (Figure 1A) and the data shown above indicate that the subcellular distribution of this protein is not controlled entirely by phosphorylation-dependent masking of the NLS and a resulting passive diffusion which would be significantly slower (Laskey and Dingwall, 1993). To decide between a phosphorylation-dependent export and a phosphorylation-dependent cytoplasmic retention of the protein, we used leptomycin B (LMB), a specific inhibitor of nuclear export (Wolff *et al.*, 1997), which interferes with the binding of the leucine-rich Rev-type NES to the export receptor exportin 1 (Fornerod *et al.*, 1997; Fukuda *et al.*, 1997b; Ossareh-Nazari *et al.*, 1997). When we were confident that LMB did not interfere with stress-dependent stimulation of MK2 activity (data not shown), we analysed the influence of LMB on the sorbitol-induced nuclear export of GFP-MK2 in Swiss 3T3 cells stably transfected with the expression construct. In the control (Figure 3A, –LMB), there is an almost complete nuclear export of

Fig. 2. Analysis of the subcellular localization of GFP-MK2 mutants and its ATP dependence. (A) The subcellular localization of GFP, GFP-wild-type MK2 (WT) and constitutively active MK2 mutants where the regulatory phosphorylation sites were mutated to glutamate (T205E, T317E, T205,317E; Engel *et al.*, 1995) was analysed in Swiss 3T3 cells by confocal fluorescence microscopy. (B) The mutant GFP-MK2-T317A was analysed in untreated 3T3 cells (–sorbitol) and in cells stimulated for 40 min with 0.4 M sorbitol. (C) Constitutive cytoplasmic translocation of the C-terminus of MK2 and energy dependence of the translocation. The C-terminus of MK2 (amino acids 315–383) was fused to GFP and expressed in Swiss 3T3 cells. Similarly to the mutant T205,317E, this mutant also shows a cytoplasmic localization. In ATP-depleted cells (+CCCPC), which contain ~10% of the physiological ATP level after a 30 min treatment with 20 μM CCCPC, these mutants are distributed equally between cytoplasm and nucleus, indicating an energy-dependent nuclear export or cytoplasmic retention.

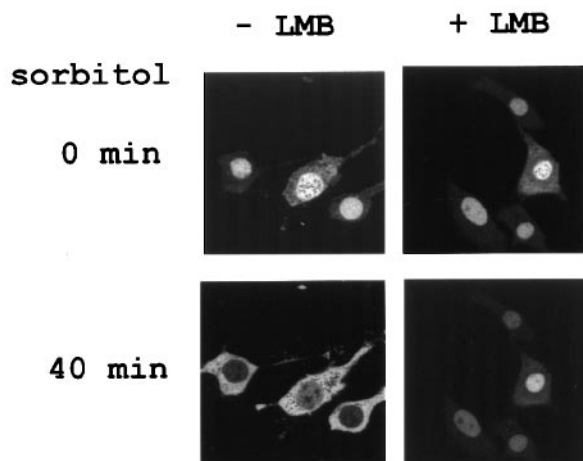
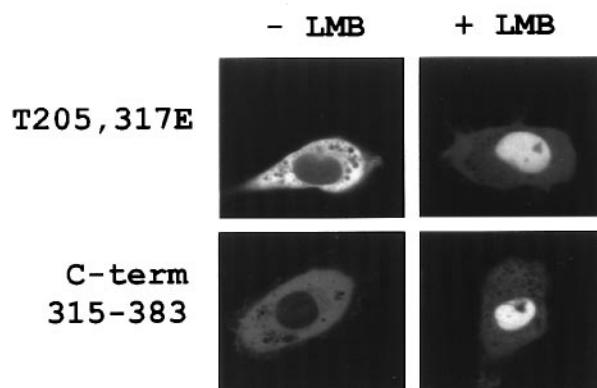
A**B**

Fig. 3. Influence of LMB on stress-dependent translocation of GFP-MK2 and subcellular distribution of constitutively cytoplasmic GFP-MK2 mutants. **(A)** Swiss 3T3 cells stably transfected with GFP-MK2 were stimulated by treatment with 0.4 M sorbitol for 40 min in the presence (+LMB) and absence (-LMB) of 200 nM LMB. **(B)** Swiss 3T3 cells transiently expressing the constitutively cytoplasmic mutants T205,317E and C-term (amino acids 315–383) were analysed by confocal microscopy before (-LMB) and after a 20 min treatment with 200 nM leptomycin B (+LMB).

GFP-MK2 after 40 min of treatment of the cells with 0.4 M sorbitol. In contrast, addition of 200 nM LMB (Figure 3A, +LMB) before sorbitol treatment leads to a block of nuclear export of the kinase which is still found predominantly in the nucleus after 40 min. In a second approach, we examined the influence of LMB on the intracellular distribution of constitutively cytoplasmic mutants of GFP-MK2. After 20 min of treatment with 200 nM LMB, the cytoplasmic localization of GFP-MK2-T205,317E and GFP-C-term was reversed completely to a nuclear one (Figure 3B). These data indicate an LMB-dependent nuclear export of the protein and a NES or a binding region for an NES-containing adaptor molecule in the C-terminal part of MK2. In addition, the rapid re-entrance of the mutants into the nucleus under conditions where the export is inhibited strengthens the notion that

the NLS in GFP-MK2 is functionally active and that there is no phosphorylation-induced block of import or cytoplasmic anchoring for GFP-MK2.

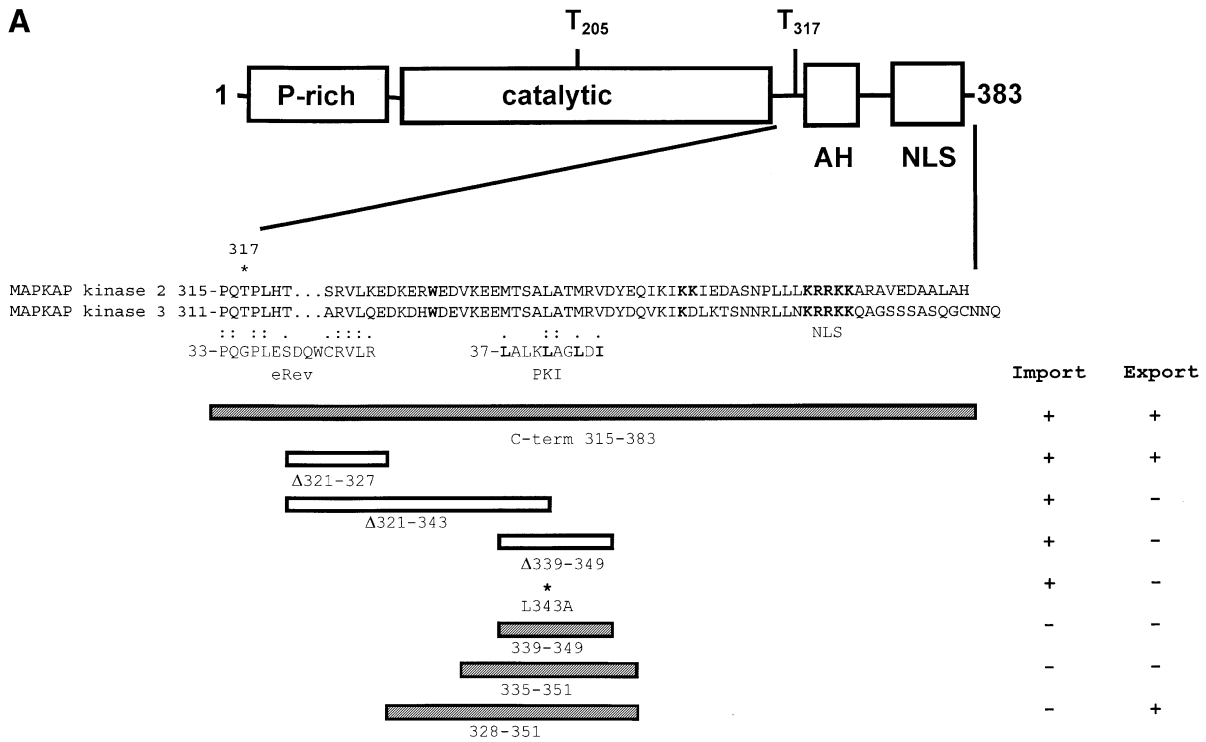
Characterization of the NES in MK2

The finding that LMB inhibits the export of MK2 suggests that a leucine-rich NES is involved (Fornerod *et al.*, 1997). However, the leucine-rich NES has not been completely defined so far since rapid nuclear export of proteins which carry a NES with only one leucine residue (Bogerd *et al.*, 1996) and of certain viral proteins (FIV, Mancuso *et al.*, 1994; EIAV, Fridell *et al.*, 1993) which have atypical NES sequences with no homology to the leucine-rich sequence has been observed. In the C-terminus of MK2 we have identified a region which shows weak sequence similarity to the atypical NES of the equine infectious anaemia virus Rev (eRev) (Fridell *et al.*, 1993) protein (amino acids 315–326) and a second region with four hydrophobic residues present at a characteristic spacing of typical leucine-rich NESs (amino acids 339–348), e.g. similar to the inhibitor of protein kinase A, PKI (Wen *et al.*, 1995) (Figure 4A). Deletion of the region corresponding to the eRev similarity (amino acids 321–327; Figure 4A) in the constitutively exported mutant T205,317E does not block nuclear export of the resulting mutant (Figure 4B). In contrast, deletion of a larger C-terminal region (amino acids 321–343) overlapping the autoinhibitory A-helix region (amino acids 318–338; Engel *et al.*, 1995) and two of the four characteristically spaced hydrophobic residues or deletion of the region of the four hydrophobic residues (amino acids 339–349) in T205,317E renders the resulting mutant constitutively nuclear (Figure 4B). Replacement of one of the characteristically spaced hydrophobic residues, Leu343, by alanine also blocks nuclear export (Figure 4B). However, the fusion of the hydrophobic motif of MK2 (amino acids 339–349 or 335–351) to GFP does not lead to export of GFP. Only a larger peptide including a part of the autoinhibitory motif (amino acids 328–351) is sufficient for LMB-sensitive nuclear export of GFP (Figure 4B). These data suggest that MK2 carries an NES overlapping with and C-terminal to the autoinhibitory region of the enzyme.

Mammalian MK2 is activated and exported in parallel also in yeast in a HOG1-dependent manner

In *Saccharomyces cerevisiae*, no MK2 was identified by homology analysis (Hunter and Plowman, 1997) and in cell lysates of budding yeast no kinase activity phosphorylating the MK2 substrate Hsp25 (Stokoe *et al.*, 1992a) was detected (data not shown). We expressed mammalian GFP-MK2 in yeast as a C-terminal fusion to the DNA-binding domain of GAL4. The transformed cells showed a basal MK2 activity measured by phosphorylation of Hsp25 which can be stimulated by treatment with sorbitol, an activator of the yeast p38 MAPK homologue HOG1 (Brewster *et al.*, 1993) (Figure 5A, WT). As a control, we transfected the expression construct into the *hog1*⁻ yeast strain (Brewster *et al.*, 1993). In this strain, no basal activity and stress inducibility of the enzyme could be detected (Figure 5A, *hog1*⁻). The hybrid protein was located mainly in the nucleus of the wild-type and *hog1*⁻ strain due to the MK2 NLS and/or the SV40 NLS in front of the GAL4 domain. However, stimulation with sorbitol

A



B

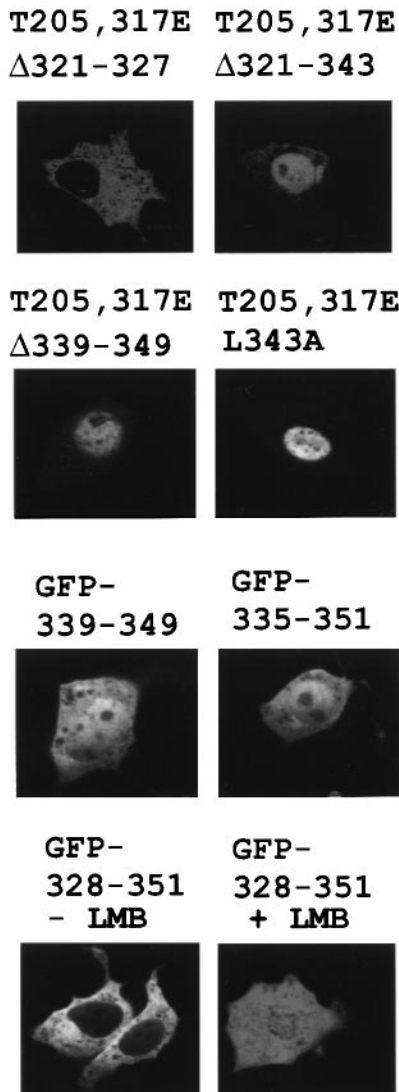
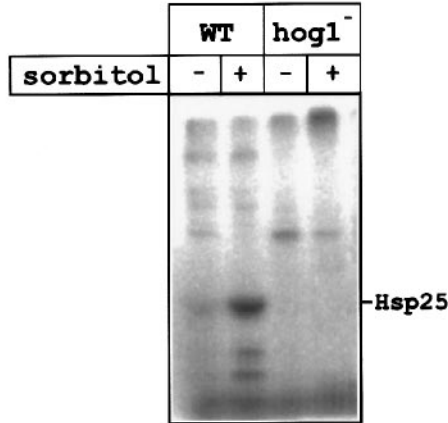
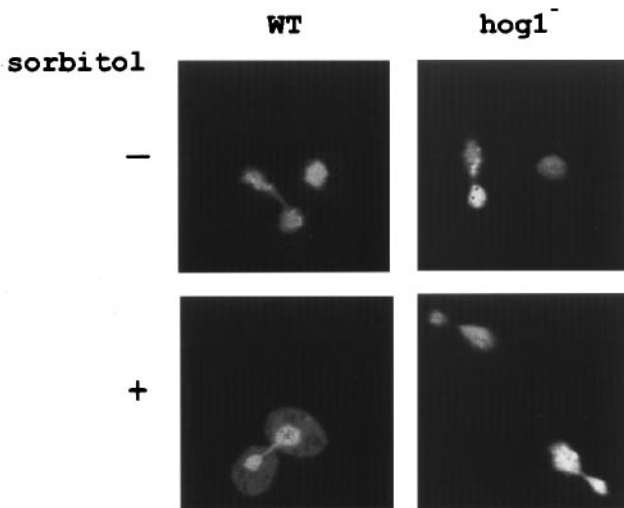


Fig. 4. (A) Schematic representation of the MK2 structure, the mutants and the localization of the NES (P-rich, proline-rich N-terminal part; Catalytic, catalytic core; AH, autoinhibitory A-helix; NLS, bipartite nuclear localization signal; NES, nuclear export signal). The phosphorylation site T317 is marked by an asterisk. The sequence of the C-terminus of MAPKAP kinase 3 (McLaughlin *et al.*, 1996) is aligned to MK2. The sequence similarity to the effector region of eRev (amino acids 33–48) and to the NES of PKI (amino acids 37–46) is indicated. The tryptophan residue of the autoinhibitory motif and the bipartite NLS are in bold. The different regions fused to GFP (grey bars) or deleted from GFP-MK2-T205,317E (open bars) are shown. (B) Identification of the NES in MK2. The subcellular localization of the MK2 mutants T205,317E Δ321–327, Δ321–343 and L343A, and the fusion proteins GFP-amino acids 339–349, GFP-amino acids 335–351 and GFP-amino acids 328–351 was analysed in transfected Swiss 3T3 cells. The C-terminal region between amino acids 328 and 351 is sufficient to trigger nuclear export of GFP which can be inhibited by LMB (GFP-328–351, +LMB).

A



B



C

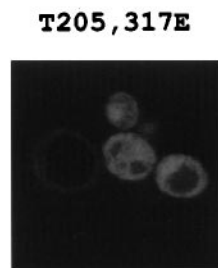


Fig. 5. Stress-induced, HOG1-dependent activation and nuclear export of mammalian MK2 in the yeast *S.cerevisiae*. (A) GFP-MK2 activity in wild-type (WT) or *hog1*⁻ yeast transfected with GFP-MK2 before (-) and after 10 min (+) of treatment with 1 M sorbitol was analysed by immunoprecipitation of the kinase and subsequent kinase assay using recombinant Hsp25 as substrate. The relative MK2 activity was quantified by phosphoimaging. (B) Subcellular distribution of GFP-MK2 in wild-type (WT) or *hog1*⁻ yeast before (-) and after a 20 min osmotic stimulation by 1 M sorbitol treatment (+sorbitol). Cells dividing by mitosis in which the nucleus can be clearly distinguished from the vacuole by the mitotic bridge were chosen for the fluorescence microscopic pictures. (C) Subcellular distribution of the constitutively exported MK2 mutant T205,317E in wild-type yeast.

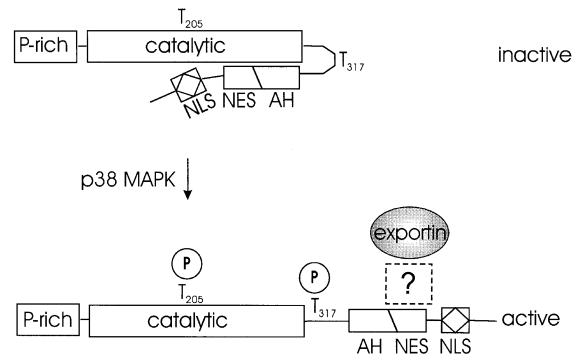


Fig. 6. A model for MK2 activation and translocation as a result of phosphorylation. MK2 carries an NLS which is accessible in both the active and inactive enzyme. Phosphorylation of T205 contributes to activation of the kinase by changing the conformation of the activation loop within the catalytic domain. Phosphorylation of T317 contributes to activation probably by neutralizing the interaction between the autoinhibitory A-helix (AH) and the catalytic domain. In addition to that, release of the autoinhibitory motif from the catalytic domain leads to unmasking of the NES overlapping with the autoinhibitory A-helix. The NES is now recognized by an exportin or an exportin-binding adaptor. In the active state, both the NES and the NLS are accessible. The active enzyme shuttles between the nucleus and cytoplasm but is localized predominantly in the cytoplasm because its export is more efficient than its import (see Discussion).

induced increased cytoplasmic localization of the hybrid protein only in the wild-type yeast (Figure 5B). Interestingly, the constitutively exported mutant T205,317E could also be detected to a substantial degree in the cytoplasm of wild-type yeast when expressed as a NLS-GAL4-GFP-kinase hybrid protein (Figure 5C). These findings indicate that the phosphorylation-dependent nuclear export of MK2 functions in principle also in yeast, and support the idea that the export machinery involved is evolutionarily conserved.

Discussion

Using a GFP fusion protein, we provided evidence for the existence of both a functional NLS and an NES in the C-terminal part of MK2. The NLS is accessible in the inactive and active form of the kinase whereas the NES is only functional in the phosphorylated and activated form of the enzyme. The model shown in Figure 6 is proposed to explain the coupling of activation and translocation of the kinase at the molecular level. The accessibility of the NES, which overlaps with the autoinhibitory region of the kinase, might be reduced by intramolecular interaction of the autoinhibitory motif with the catalytic domain. Phosphorylation at T317 could weaken the interaction between these parts and/or release the autoinhibitory motif. This contributes to activation of the enzyme and, in parallel, might unmask the NES (Figure 6). As a result, the activated kinase is exported rapidly from the nucleus. Since the intramolecular interaction of the autoinhibitory motif has not been analysed experimentally so far, this model needs further support. However, the coupling of activation and nuclear export is clearly shown here and represents a new mechanism by which intracellular signal transduction can occur. In contrast to MAPK kinase which is constitutively exported from the nucleus (Fukuda *et al.*, 1996, 1997a) and to the MAP kinases which translocate to the nucleus upon

activation (Chen *et al.*, 1992; Lenormand *et al.*, 1993), the data presented here suggest that MK2 in its inactive form is mainly nuclear and its stress-induced activation is paralleled by a rapid export to the cytoplasm. This phenomenon is explained by the fact that both enzymatic activity and nuclear export of MK2 are regulated by phosphorylation at T317.

It is interesting to note that, when fused to GFP, the constitutively active MK2 (T205,317E) and the C-terminal part (amino acids 315–383), carrying both the NES and the bipartite NLS, lead to almost exclusive cytoplasmic localization of the fusion proteins in Swiss 3T3 cells although the rapid re-entry of the proteins into the nucleus after LMB treatment clearly indicates a functional NLS. This suggests that nuclear export of these constructs is more effective than its import, leading to a steady-state where most of the continuously shuttling protein is cytoplasmic. A similar observation has been made very recently in the yeast *S.cerevisiae* for a GFP reporter protein carrying the SV40 large T NLS and the leucine-rich NES of PKI (Stade *et al.*, 1997). This reporter protein is also localized mainly in the cytoplasm because its rate of export is higher than its rate of import. It is also interesting to note that conditions of ATP depletion to ~10% lead to a redistribution of the constitutively exported mutants from a mainly cytoplasmic localization to a nearly equal distribution between nucleus and cytoplasm. Probably as a consequence of ATP depletion, the RanGTP gradient across the nuclear membrane collapses. It has been shown recently that export pathways are more sensitive to RanGTP depletion than is NLS-dependent import (Izaurralde *et al.*, 1997). Hence, the shift of the MK2 distribution to the nucleus could be the result of a higher sensitivity of the export of the enzyme to ATP depletion.

We demonstrate that the region from amino acid 339 to 349 is necessary for nuclear export of MK2. This region shows weak homology to the Rev-type NES (Figure 4A), the interaction of which with exportin 1 has been shown to be inhibited by LMB (Fornerod *et al.*, 1997; Fukuda *et al.*, 1997b; Ossareh-Nazari *et al.*, 1997). One may speculate that the four hydrophobic residues, M339, L343, M346 and V348, present at a characteristic spacing for typical leucine-rich Rev-type NESs, form an exportin-interacting NES of MK2. This idea is supported by the finding that the single leucine to alanine replacement L343A blocks nuclear export, which is similar to results obtained, for example, for the NES of PKI (Wen *et al.*, 1995) or Gle1 (Murphy and Wentz, 1996). However, in contrast to the leucine-rich NES of PKI, which is sufficient for export of GST (Wen *et al.*, 1995) or GFP (Stade *et al.*, 1997), this region is not sufficient for export when fused to GFP (amino acids 339–349 or 335–351). Only the region between amino acids 328 and 351 which also contains part of the autoinhibitory region directs GFP to the cytoplasm in an LMB-sensitive manner. Hence, so far it cannot be ruled out that this region of MK2 binds in an LMB-sensitive manner to an exportin 1-related molecule which recognizes an NES different from the leucine-rich Rev-type NES or, alternatively, that an adaptor molecule carrying a Rev-type NES is involved in binding of the kinase to exportin 1. The existence of an adaptor molecule restricted to bind MK2 only is unlikely, since the export of the mammalian enzyme also functions in yeast where

no homologue for this kinase exists. Nevertheless, a common adaptor involved in the evolutionarily conserved nuclear export co-used by MK2 cannot be excluded. The incomplete nuclear export observed in yeast may reflect the inefficient binding of an evolutionarily distant adaptor molecule or may simply arise from the additional SV40 NLS in the GAL4 fusion construct. Further experiments analysing the direct interaction between recombinant exportins and MK2 could contribute to answering these questions.

The data for the intracellular localization of MK2 presented here are based predominantly on a GFP–MK2 fusion protein expressed in eukaryotic cells. No immunocytochemical data about the stress-dependent subcellular distribution of endogenous MK2 are available so far, since all the different polyclonal antibodies raised against the enzyme are not suitable for immunocytochemistry, probably due to the low level of expression of endogenous MK2 and/or cross-reactivities of the antibodies with several other proteins. In addition, different methods of cell fractionation combined with Western blot detection of endogenous MK2 using the polyclonal antibodies available failed to give clear results, whereas we can exclude detectable kinase activity in the nucleus (data not shown). Hence, it is still an open question as to whether the endogenous enzyme shows the same subcellular distribution as the fusion proteins investigated. The two artificial N-terminal tags, c-Myc and GFP, make it unlikely that these sequences themselves are responsible for the observed translocation of MK2 constructs. However, it cannot be excluded that N-terminal tagging interferes with the function of the proline-rich domain of MK2. C-terminal tagged variants of MK2 were not useful in our study because we had to retain the native structure of the C-terminal region important for export and import. Another point for discussion is the transfection approach used in the study. Overexpression could lead to a loss of function of an unknown protein involved in MK2 translocation by titration. This seems not to be the case, since in stably transfected cells which show much lower levels of GFP–MK2 we could not detect a qualitative difference in the stress-dependent redistribution of this molecule (data not shown).

Since there are potential nuclear (CREB; Tan *et al.*, 1996) and cytoplasmic substrates for MK2 (sHsp, Stokoe *et al.*, 1992a; tyrosine hydroxylase, Sutherland *et al.*, 1993), the coupling of activation and nuclear export of MK2 could have different functions. Export could be a means of rapid down-regulation of MK2 activity in the nucleus after a short pulse of nuclear phosphorylation. Alternatively, a different localization of the kinase and its cytoplasmic targets under physiological conditions could enhance the specificity of stress signalling further as is also presumed to be the case for signalling systems utilizing kinase targeting subunits (Hubbard and Cohen, 1993; Faux and Scott, 1996).

Materials and methods

Expression of GFP–MK2, its mutants and Myc-tagged MK2 in Swiss 3T3 cells

The cDNA of murine MK2 was cloned into *XhoI*–*Bam*HI-cut pEGFP-C1 (Clontech) as a PCR product amplified with the primers 5'-TGT

TCT CGA GGC TCT CCG GGC CAG ACT CC-3' (sense) and 5'-CGC GGA TCC AGT GGG TGG GAG ACA GAG CG-3' (antisense) using Expand™ high fidelity *Taq* polymerase (Boehringer Mannheim). Different phosphorylation mutants of MK2 were constructed by subcloning of *SacI*-*Bam*HI-cut PCR fragments amplified with the primers 5'-TGT GGA TCC CCC AGT TCC ACG TC-3' and the antisense primer using the appropriate templates (Engel *et al.*, 1995). The deletion mutants Δ321–327, Δ321–343 and Δ339–349 of GFP–MK2–T205,317E were generated by PCR with the QuickChange™ Site-Directed Mutagenesis Kit (Stratagene) using the primers 5'-GTG CAG TGG CTC CTG AGG GAC C-3' and 5'-GAC AAG GAA CGA TGG GAG GAT GTC-3' for Δ321–327 and appropriate primers for the other deletions. The fusion of amino acids 315–383 of the kinase to GFP was carried out by inserting an *Eco*RI-cut PCR fragment generated with *Pfu* polymerase (Stratagene) and primers 5'-CGG AAT TCC CCT CAG ACT CCA CTG C-3' and the antisense primer into *Eco*RI-*Sma*I-cut pEGFP-C1 (Clontech). The fusion of the smaller peptides to GFP was achieved by insertion of a cassette of two annealed oligonucleotides into *Xho*I-*Bam*HI-cut pEGFP-C1. In all constructs, mutations were verified by sequencing. For expression of epitope-tagged MK2, the vector pcDNA3–Myc–MK2 (Engel *et al.*, 1995) was used.

Transient transfections were carried out using the calcium phosphate precipitation technique. Half-confluent Swiss 3T3 cells in a chambered cover glass (Nunc) were transiently transfected with 0.5–1.0 μg of DNA. After 36–48 h, the subcellular distribution of GFP hybrid proteins was examined using confocal fluorescence microscopy (Leica TCS 4D). Stably transfected cell lines (used where mentioned) were derived by a 3 week selection using G418 (400 μg/ml). Myc–MK2 was detected after fixation of the cells with 4% formaldehyde using the monoclonal antibody 9E10 (European Collection of Animal Cell Culture, cell line 85102202) and a secondary anti-mouse immunoglobulin antibody conjugated to fluorescein isothiocyanate (FITC). Cells were stimulated by treatment with anisomycin and sorbitol (Sigma) at a final concentration of 10 μg/ml and 0.4 M, respectively.

ATP depletion, SB203580 and leptomycin B treatment

Swiss 3T3 cells were incubated in medium containing 10% fetal calf serum in phosphate-buffered saline (PBS) and 10 mM sodium azide (Sigma) or 20 μM CCCP (Sigma) for 30 min. ATP levels were measured in whole cell extracts using the ATP Bioluminescence CLS II kit (Boehringer Mannheim). SB203580 (a kind gift of Dr J.C.Lee, Smith-Kline Beecham) and LMB (kindly provided by Dr B.Wolff-Winiski, Novartis) were added to the medium of transfected cells to a final concentration of 20 μM and 200 nM, respectively.

Expression of GFP–MK2 in yeast

*Nco*I-*Bam*HI fragments from pEGFP-C1–MK2 vectors were cloned into the vector pAS1 (Clontech). Yeast YRG-2 (Stratagene) and W303 *hog1::LEU2* (kindly provided by Dr M.C.Gustin) were transformed using the lithium acetate technique and plated onto agar plates with synthetic medium including glucose and lacking tryptophan, or tryptophan and leucine, respectively. Cells were stimulated by 1 M sorbitol.

Measurement of kinase activity in GFP–MK2-transformed yeast

Yeast cells were lysed by vortexing them together with glass pearls in lysis buffer [20 mM Tris-acetate pH 7.0, 0.1 mM EDTA, 1 mM EGTA, 1 mM Na₃VO₄, 10 mM β-glycerophosphate, 50 mM NaF, 5 mM pyrophosphate, 1% Triton X-100, 1 mM benzamidine, 0.1% β-mercaptoethanol, 0.27 M sucrose, 0.2 mM phenylmethylsulfonyl fluoride (PMSF)]. Equal amounts of lysate protein were diluted in 500 μl of IP buffer (50 mM Tris–HCl pH 7.4, 150 mM NaCl, 50 mM NaF, 1% Triton X-100, 1 mM Na₃VO₄), and 4 μl of anti-MK2 antiserum (Schultz *et al.*, 1997) was added. After 2 h rotating end-over-end at 4°C, immunocomplexes were precipitated for 1 h at 4°C using protein A–Sepharose (Pharmacia). The kinase activity in the immunocomplex was measured by incubation with reaction mixture (50 mM β-glycerophosphate pH 7.4, 0.1 mM EDTA, 10 μg of Hsp25, 100 μM [γ-³²P]ATP, 10 mM magnesium acetate) for 15 min at 30°C. Labelling of Hsp25 was analysed and quantified by BioImaging (Fuji BAS 2000).

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References

- Ben-Levy,R., Leighton,I.A., Doza,Y.N., Attwood,P., Morrice,N., Marshall,C.J. and Cohen,P. (1995) Identification of novel phosphorylation sites required for activation of MAPKAP kinase-2. *EMBO J.*, **14**, 5920–5930.
- Bogerd,H.P., Fridell,R.A., Benson,R.E., Hua,J. and Cullen,B.R. (1996) Protein sequence requirements for function of the human T-cell leukemia virus type 1 Rex nuclear export signal delineated by a novel *in vivo* randomization-selection assay. *Mol. Cell. Biol.*, **16**, 4207–4214.
- Brewster,J.L., de Valoir,T., Dwyer,N.D., Winter,E. and Gustin,M.C. (1993) An osmosensing signal transduction pathway in yeast. *Science*, **259**, 1760–1763.
- Brunet,A. and Pouyssegur,J. (1996) Identification of MAP kinase domains by redirecting stress signals into growth factor responses. *Science*, **272**, 1652–1655.
- Cano,E., Doza,Y.N., Ben-Levy,R., Cohen P. and Mahadevan,L.C. (1996) Identification of anisomycin-activated kinases p45 and p55 in murine cells as MAPKAP kinase-2. *Oncogene*, **12**, 805–812.
- Chen,R.H., Sarnecki,C. and Blenis,J. (1992) Nuclear localization and regulation of erk- and rsk-encoded protein kinases. *Mol. Cell. Biol.*, **12**, 915–927.
- Clifton,A.D., Young,P.R. and Cohen,P. (1996) A comparison of the substrate specificity of MAPKAP kinase-2 and MAPKAP kinase-3 and their activation by cytokines and cellular stress. *FEBS Lett.*, **392**, 209–214.
- Cuenda,A., Rouse,J., Doza,Y.N., Meier,R., Cohen,P., Gallagher,T.F., Young,P.R. and Lee,J.C. (1995) SB 203580 is a specific inhibitor of a MAP kinase homologue which is stimulated by cellular stresses and interleukin-1. *FEBS Lett.*, **364**, 229–233.
- Dickens,K., Rogers,J.S., Cavanagh,J., Raitano,A., Xia,Z., Halpern,J.R., Greenberg,M.E., Sawyers,C.L. and Davis,R.J. (1997) A cytoplasmic inhibitor of the JNK signal transduction pathway. *Science*, **277**, 693–696.
- Engel,K., Plath,K. and Gaestel,M. (1993) The MAP kinase-activated protein kinase 2 contains a proline-rich SH3-binding domain. *FEBS Lett.*, **336**, 143–147.
- Engel,K., Schultz,H., Martin,F., Kotlyarov,A., Plath,K. Hahn,M., Heinemann,U. and Gaestel,M. (1995) Constitutive activation of mitogen-activated protein kinase-activated protein kinase 2 by mutation of phosphorylation sites and an A-helix motif. *J. Biol. Chem.*, **270**, 7213–7221.
- Faux,M.C. and Scott,J.D. (1996) Molecular glue: kinase anchoring and scaffold proteins. *Cell*, **85**, 9–12.
- Fornerod,M., Ohno,M., Yoshida,M. and Mattaj,I.W. (1997) CRM1 is an export receptor for leucine-rich nuclear export signals. *Cell*, **90**, 1051–1060.
- Freshney,N.W., Rawlinson,L., Guesdon,F., Jones,E., Cowley,S., Hsuan,J. and Saklatvala,J. (1994) Interleukin-1 activates a novel protein kinase cascade that results in the phosphorylation of Hsp27. *Cell*, **78**, 1039–1049.
- Fridell,R.A., Partin,K.M., Carpenter,S. and Cullen,B.R. (1993) Identification of the activation domain of equine infectious anemia virus rev. *J. Virol.*, **67**, 7317–7323.
- Fukuda,M., Gotoh,I., Gotoh,Y. and Nishida,E. (1996) 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.*, **271**, 20024–20028.
- Fukuda,M., Gotoh,Y. and Nishida,E. (1997a) Interaction of MAP kinase with MAP kinase kinase: its possible role in the control of nucleocytoplasmic transport of MAP kinase. *EMBO J.*, **16**, 1901–1908.
- Fukuda,M., Asano,S., Nakamura,T., Adachi,M., Yoshida,M., Yanagida,M. and Nishida,E. (1997b) CRM1 is responsible for intracellular transport mediated by the nuclear export signal. *Nature*, **390**, 308–311.
- Fukuda,M., Gotoh,I., Adachi,M., Gotoh,Y. and Nishida,E. (1997c) A novel regulatory mechanism in the mitogen-activated protein kinase cascade. Role of the nuclear export signal of MAP kinase kinase. *J. Biol. Chem.*, **272**, 32642–32648.
- Hubbard,M.J. and Cohen,P. (1993) On target with a new mechanism for the regulation of protein phosphorylation. *Trends Biochem. Sci.*, **18**, 172–177.

- Hunter,T. and Plowman,G.D. (1997) The protein kinases of budding yeast: six score and more. *Trends Biochem. Sci.*, **22**, 18–22.
- Izaurrealde,E., Kutay,U., von Kobbe,C., Mattaj,I. and Görlich,D. (1997) The asymmetric distribution of the constituents of the Ran system is essential for transport into and out of the nucleus. *EMBO J.*, **16**, 6535–6547.
- Jaaro,H., Rubinfeld,H., Hanoch,T. and Seger,R. (1997) Nuclear translocation of mitogen-activated protein kinase kinase (MEK1) in response to mitogenic stimulation. *Proc. Natl Acad. Sci. USA*, **94**, 3742–3747.
- Karin,M. and Hunter,T. (1995) Transcriptional control by protein phosphorylation: signal transmission from the cell surface to the nucleus. *Curr. Biol.*, **5**, 747–757.
- Laskey,R.A. and Dingwall,C. (1993) Nuclear shuttling: the default pathway for nuclear proteins? *Cell*, **74**, 385–386.
- Lee,J.C. *et al.* (1994) A protein kinase involved in the regulation of inflammatory cytokine biosynthesis. *Nature*, **372**, 739–746.
- Lenormand,P., Sardet,C., Pages,G., L'Allemain,G., Brunet,A. and Pouyssegur,J. (1993) Growth factors induce nuclear translocation of MAP kinases (p42mapk and p44mapk) but not of their activator MAP kinase kinase (p45mapkk) in fibroblasts. *J. Cell Biol.*, **122**, 1079–1088.
- Ludwig,S., Engel,K., Hoffmeyer,A., Sithanandam,G., Neufeld,B., Palm,D., Gaestel,M. and Rapp,U.R. (1996) 3pK, a novel mitogen-activated protein (MAP) kinase-activated protein kinase, is targeted by three MAP kinase pathways. *Mol. Cell Biol.*, **16**, 6687–6697.
- Mancuso,V.A., Hope,T.J., Zhu,L., Derse,D., Phillips,T. and Parslow,T.G. (1994) Posttranscriptional effector domains in the Rev proteins of feline immunodeficiency virus and equine infectious anemia virus. *J. Virol.*, **68**, 1998–2001.
- McLaughlin,M.M., Kumar,S., McDonnell,P.C., Van-Horn,S., Lee,J.C., Livi,G.P. and Young,P.R. (1996) Identification of mitogen-activated protein (MAP) kinase-activated protein kinase-3, a novel substrate of CSBP p38 MAP kinase. *J. Biol. Chem.*, **271**, 8488–8492.
- Murphy,R. and Wente,S.R. (1996) An RNA-export mediator with an essential nuclear export signal. *Nature*, **383**, 357–360.
- Nigg,E.A. (1997) Nucleocytoplasmic transport: signals, mechanisms and regulation. *Nature*, **386**, 779–787.
- Ossareh-Nazari,B., Bachelier,F. and Dargemont,C. (1997) Evidence for a role of CRM1 in signal-mediated nuclear protein export. *Science*, **278**, 141–144.
- Raingeaud,J., Gupta,S., Rogers,J.S., Dickens,M., Han,J., Ulevitch,R.J. and Davis,R.J. (1995) Pro-inflammatory cytokines and environmental stress cause p38 mitogen-activated protein kinase activation by dual phosphorylation on tyrosine and threonine. *J. Biol. Chem.*, **270**, 7420–7426.
- Read,M.A., Whitley,M.Z., Gupta,S., Pierce,J.W., Best,J., Davis,R.J. and Collins,T. (1997) Tumor necrosis factor alpha-induced E-selectin expression is activated by the nuclear factor-kappaB and c-JUN N-terminal kinase/p38 mitogen-activated protein kinase pathways. *J. Biol. Chem.*, **272**, 2753–2761.
- Rouse,J., Cohen,P., Trigon,S., Morange,M., Alonso-Llamazares,A., Zamanillo,D., Hunt,T. and Nebreda,A.R. (1994) A novel kinase cascade triggered by stress and heat shock that stimulates MAPKAP kinase-2 and phosphorylation of the small heat shock proteins. *Cell*, **78**, 1027–1037.
- Schultz,H., Engel,K. and Gaestel,M. (1997) PMA-induced activation of the p42/44ERK- and p38RK-MAP kinase cascades in HL-60 cells is PKC dependent but not essential for differentiation to the macrophage-like phenotype. *J. Cell. Physiol.*, **173**, 310–318.
- Sithanandam,G. *et al.* (1996) 3pK, a new mitogen-activated protein kinase-activated protein kinase located in the small cell lung cancer tumor suppressor gene region. *Mol. Cell Biol.*, **16**, 868–876.
- Stade,K., Ford,C.S., Guthrie,C. and Weis,K. (1997) Exportin 1 (Crm1p) is an essential nuclear export factor. *Cell*, **90**, 1041–1050.
- Stokoe,D., Engel,K., Campbell,D.G., Cohen,P. and Gaestel,M. (1992a) Identification of MAPKAP kinase 2 as a major enzyme responsible for the phosphorylation of the small mammalian heat shock proteins. *FEBS Lett.*, **313**, 307–313.
- Stokoe,D., Campbell,D.G., Nakielnny,S., Hidaka,H., Leever,S.J., Marshall,C. and Cohen,P. (1992b) MAPKAP kinase-2; a novel protein kinase activated by mitogen-activated protein kinase. *EMBO J.*, **11**, 3985–3994.
- Stokoe,D., Caudwell,B., Cohen,P.T. and Cohen,P. (1993) The substrate specificity and structure of mitogen-activated protein (MAP) kinase-activated protein kinase-2. *Biochem. J.*, **296**, 843–849.
- Sutherland,C., Alterio,J., Campbell,D.G., Le Bourdelles,B., Mallet,J., Haavik,J. and Cohen,P. (1993) Phosphorylation and activation of human tyrosine hydroxylase *in vitro* by mitogen-activated protein (MAP) kinase and MAP-kinase-activated kinases 1 and 2. *Eur. J. Biochem.*, **217**, 715–722.
- Tan,Y., Rouse,J., Zhang,A., Cariati,S., Cohen,P. and Comb,M.J. (1996) FGF and stress regulate CREB and ATF-1 via a pathway involving p38 MAP kinase and MAPKAP kinase-2. *EMBO J.*, **15**, 4629–4642.
- Ullman,K.S., Powers,M.A. and Forbes,D.J. (1997) Nuclear export receptors: from importin to exportin. *Cell*, **90**, 967–970.
- Wen,W., Meinkoth,J.L., Tsien,R.Y. and Taylor,S.S. (1995) Identification of a signal for rapid export of proteins from the nucleus. *Cell*, **82**, 463–473.
- Wolff,B., Sanglier,J.J. and Wang,Y. (1997) Leptomycin B is an inhibitor of nuclear export: inhibition of nucleocytoplasmic translocation of the human immunodeficiency virus type 1 (HIV-1) Rev protein and Rev-dependent mRNA. *Chem. Biol.*, **4**, 139–147.

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