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A novel method to identify protein kinase substrates: eEF2 kinase is phosphorylated and inhibited by SAPK4/p38δ

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We have developed a method of general application for identifying putative substrates of protein kinases in cell extracts. Using this procedure, we identified the physiological substrates of several mitogen-activated protein kinase kinases and an authentic substrate of stress-activated protein kinase (SAPK) 2a/p38. A 120 kDa protein was detected in skeletal muscle extracts that was phosphorylated rapidly by SAPK4/p38δ, but poorly by SAPK2/p38, SAPK3/p38γ, SAPK1/JNK or extracellular signal-regulated kinase 2 (ERK2). It was purified and identified as eukaryotic elongation factor 2 kinase (eEF2K). SAPK4/p38δ phosphorylated eEF2K at Ser359 *in vitro*, causing its inactivation. eEF2K became phosphorylated at Ser359 and its substrate eEF2 became dephosphorylated (activated) when KB cells were exposed to anisomycin, an agonist that activates all SAPKs, including SAPK4/p38δ. The anisomycin-induced phosphorylation of Ser359 was unaffected by SB 203580, U0126 or rapamycin, and was prevented by overexpression of a catalytically inactive SAPK4/p38δ mutant, suggesting that SAPK4/p38δ may mediate the inhibition of eEF2K by this stress. The phosphorylation of eEF2K at Ser359 was also induced by insulin-like growth factor-1. However, this was blocked by rapamycin, indicating that Ser359 is targeted by at least two signalling pathways.

Keywords: IGF-1/p38/protein synthesis/SAPK/stress

Introduction

Nearly all aspects of cell life are controlled by the reversible phosphorylation of proteins. Approximately one-third of mammalian proteins contain covalently bound phosphate, and protein kinases represent the largest single family of enzymes in the human genome, accounting for 2% of gene products. Since 30 000 proteins are encoded by the human genome and many proteins are phosphorylated by more than one protein kinase, an 'average' protein kinase phosphorylates at least 20 substrates *in vivo*. The unambiguous identification of the physiological substrates of each protein kinase is therefore a major challenge of the post-genomic era and will require the development of improved methodology. Approaches that have been used with some success include the screening of expression libraries for protein kinase substrates (Fukunaga and Hunter, 1997), yeast 'two-hybrid' analysis (McLaughlin *et al*., 1996; Balendran *et al*., 1999), the screening of protein sequence databases with known consensus sequences for particular protein kinases (New *et al*., 1998) and the use of ATP derivatives that can only be used as substrates by mutated forms of protein kinases (Shah *et al*., 1997; Liu *et al*., 1998).

In principle, a simple approach to identify protein kinase substrates would be to incubate cell extracts with the protein kinase of interest and Mg[γ-^{32}P]ATP and to analyse the proteins that become phosphorylated. However, this approach has rarely been attempted for several reasons. First, cell extracts contain hundreds of protein kinases and their substrates, so that the substrates of interest may be obscured by the high background phosphorylation. Secondly, because many protein kinases phosphorylate proteins *in vitro* that are not phosphorylated *in vivo*, many of the substrates identified might not be relevant physiologically. Thirdly, until recently, it was a major challenge to purify and identify each of the putative protein substrates detected in cell extracts.

In this study, we have introduced some simple modifications that greatly reduce the problem of high background phosphorylation in cell extracts. We have also increased the probability of identifying potentially interesting substrates by comparing the specificities of protein kinases that are closely related in structure. These proteins can then be purified relatively easily using modern high resolution methods of protein purification and identified by tryptic mass fingerprinting. Using this method, which we have termed KESTREL (kinase substrate tracking and elucidation), we have been able to identify known physiological substrates of several protein kinases, sometimes even in crude cell extracts. The further application of this method has led to a significant advance in our understanding of how eukaryotic elongation factor 2 kinase (eEF2K) is regulated by phosphorylation.

Results

Identification of the substrates of mitogen-activated protein kinase kinases (MKKs)

In order to develop KESTREL, we initially selected the MKK family of protein kinases for study, because they are believed to have a restricted substrate specificity *in vivo* and to only phosphorylate particular mitogen-activated protein kinase (MAPK) family members. The cell extracts were prepared in the absence of protein phosphatase inhibitors, so that the MKKs and their substrates would be present in their inactive, dephosphorylated forms. In order to maximize the sensitivity of the method, we used [γ-^{32}P]ATP of high specific radioactivity and then incubated ATP-depleted HeLa cell extracts for just a few

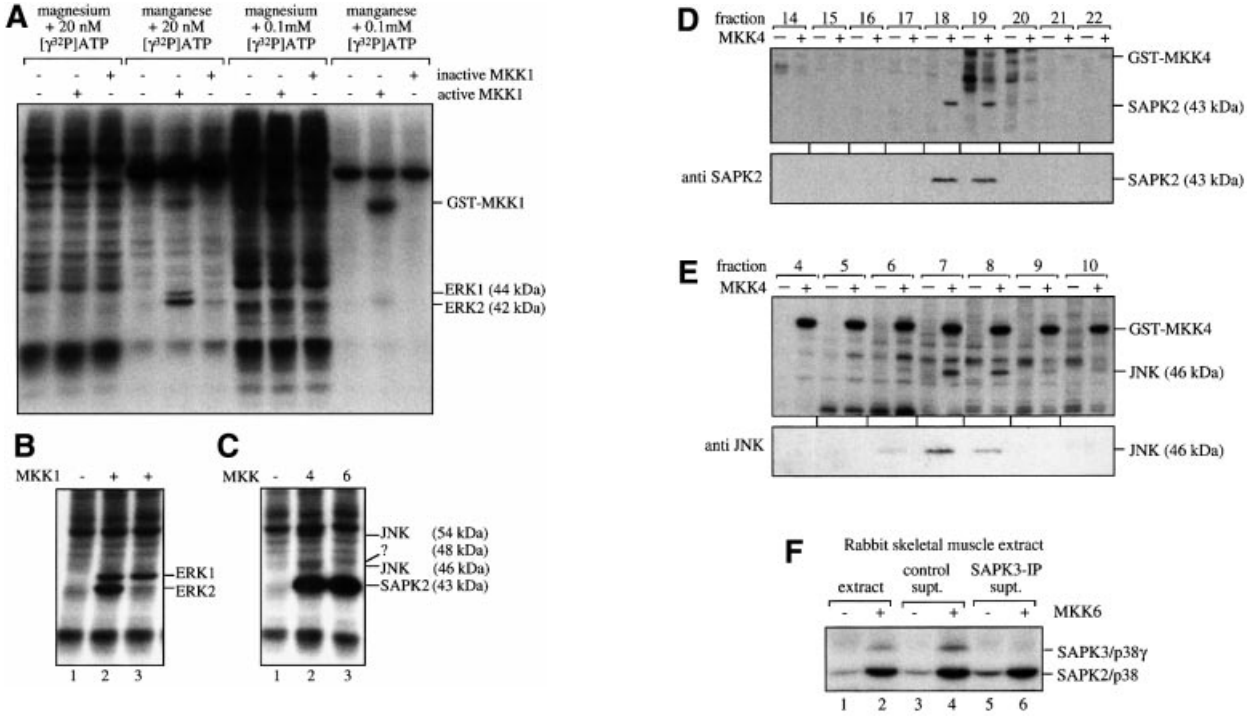

Fig. 1. Identification of substrates for MAPK kinases. (**A**) Desalted HeLa cell extracts (see Materials and methods) were supplemented with 0.5 μM constitutively active GST–MKK1 mutant (active MKK1) or 0.5 μM catalytically inactive GST–MKK1 (inactive MKK1), 10 mM magnesium acetate or 2 mM $MnCl_2$, and 20 nM [γ-^{32}P]ATP (2.5 × 10^6 c.p.m.) or 0.1 mM [γ-^{32}P]ATP (10^6 c.p.m./nmol) as indicated. The assay volumes were 0.025 ml. After 5 min at 30°C, the reactions were stopped with SDS/EDTA, subjected to SDS–PAGE, transferred to a PVDF membrane and autoradiographed. (**B**) An ATP-depleted HeLa cell extract was phosphorylated with or without active MKK1, in the presence of 2 mM $MnCl_2$ and [γ-^{32}P]ATP (20 nM), and analysed as in (A). In lane 3, ERK2 was first depleted from the extract with an immunoprecipitating antibody bound to protein G–Sepharose before phosphorylation. Lanes 1 and 2 show control experiments using protein G–Sepharose without antibody attached. (**C**) The same as (A) using manganese ions (2 mM) and [γ-^{32}P]ATP (20 nM), except that the active mutants of MKK4 and MKK6 (also at 0.5 μM) were used instead of MKK1. (**D**) An ATP-depleted HeLa cell extract (2 mg of protein) was applied to a Mono Q HR5/5 column equilibrated in 30 mM Tris pH 7.5, 5% (v/v) glycerol, 0.03% (w/v) Brij 35, 0.1% (v/v) 2-mercaptoethanol, and the column was eluted with a 20 ml salt gradient to 1 M NaCl. Fractions of 0.7 ml were collected and aliquots of the fractions indicated were diluted 8-fold into 30 mM Tris–HCl pH 7.5, 0.1 mM EGTA, 0.1% (v/v) 2-mercaptoethanol, then phosphorylated for 5 min at 30°C in a 0.03 ml assay with 10 mU of active MKK4 in the presence of 2 mM $MnCl_2$ and 20 nM [γ-^{32}P]ATP. The reactions were then analysed as in (A). A further aliquot of the same fractions was electrophoresed on a separate gel and immmunoblotted with a SAPK2a/p38-specific antibody (lower panel). The 43 kDa substrate of MKK4 co-eluted with SAPK2a/p38 in fractions 18 and 19, but was absent from all the other column fractions. (**E**) The same experiment as (D), except that the fractions were immunoblotted with an SAPK1/JNK-specific antibody. The 46 kDa substrate of MKK4 co-eluted with the 46 kDa form of SAPK1/JNK in fractions 7 and 8, but was absent from all other fractions. (**F**) An ATP-depleted rabbit muscle extract (extract) was phosphorylated with or without active MKK6, in the presence of 2 mM $MnCl_2$ and [γ-^{32}P]ATP (20 nM) as in (B) (lanes 1 and 2). In lanes 5 and 6, SAPK3/p38γ was first depleted from the extract with an immunoprecipitating SAPK3/p38γ-specific antibody bound to protein G–Sepharose before phosphorylation. Lanes 3 and 4 show a control experiment using protein G–Sepharose without antibody attached.

minutes with high concentrations of a constitutively active mutant of MKK1. Using Mg[γ-^{32}P]ATP, we were unable to detect the known substrates of MKK1, namely extracellular signal-regulated protein kinases 1 and 2 (ERK1 and ERK2). However, when the substrate was Mn[γ-^{32}P]ATP, which is used even more efficiently by MKK1, two protein substrates with the apparent molecular masses of ERK1 (44 kDa) and ERK2 (42 kDa) were clearly detectable in the cell extracts, because the background phosphorylation was reduced considerably (Figure 1A). The identity of the 42 kDa protein as ERK2 was confirmed by immunodepletion experiments (Figure 1B). The only other phosphoprotein detected upon addition of MKK1 was the added MKK1 itself (Figure 1A), which underwent autophosphorylation.

We next extended these studies to MKK4 and MKK6. When the ATP-depleted HeLa extracts were supplemented with a constitutively active form of MKK4, three new ^{32}P-labelled bands appeared upon incubation with Mn[γ-^{32}P]ATP (Figure 1C, lane 2). The most prominent migrated between ERK1 and ERK2 with an apparent molecular mass of 43 kDa, which also appeared when HeLa cell extracts were incubated with MKK6 in the presence of Mn[γ-^{32}P]ATP (Figure1C, lane 3). MKK4 and MKK6 are both known to phosphorylate stress-activated protein kinase 2a (SAPK2a, also called p38). The identity of the 43 kDa protein phosphorylated by MKK4 as SAPK2a/p38 was confirmed by its co-elution with immunoreactive SAPK2a/p38 after chromatography on Mono Q (Figure 1D). MKK4 is also known to phosphorylate the isoforms of SAPK1 (also called JNK), which migrate on SDS–polyacrylamide gels with apparent molecular masses of 46 and 54 kDa (Figure 1C, lane 2) (Hibi *et al*., 1993). The identity of the 46 kDa band as a SAPK1/JNK isoform was also established by its co-elution from Mono Q with immunoreactive SAPK1/JNK (Figure 1E).

Table I. Identification of SAPK3/p38γ as a substrate of MKK6

Masses submitted	Masses matched	Start	End	Peptide
761.3774	761.3946	19	24	TAWEVR
805.4189	805.4096	336	341	TLEEWK
904.5403	904.5256	169	176	ILDFGLAR
1072.6305	1072.6155	347	355	EVLSFKPPR
1136.6310	1136.6329	143	153	YIHAAGVIHR
1282.6966	1282.7483	130	139	IQFLVYQMLK
1282.6966	1282.7483	356	367	QLGARVPKETAL
1498.8136	1498.8058	58	69	LYRPFQSELFAK
1626.9003	1626.9008	57	69	KLYRPFQSELFAK
1862.9270	1862.9322	153	168	DLKPGNLAVNEDCELK
2518.1649	2518.2036	25	48	AVYQDLQPVGSGAYGAVCSAVDSR

A putative MKK6 substrate of 48 kDa was purified to near homogeneity as described in Materials and methods. The protein was subjected to SDS–PAGE, stained with Sypro-orange, excised and digested with trypsin. The tryptic peptides were subjected to mass spectrometry fingerprinting. Eleven of 21 submitted masses (53%) were matched with SAPK3, covering 30% of the protein.

An additional 48 kDa band was observed upon addition of MKK6 to the cell extracts. This appeared as a relatively minor component in HeLa cell extracts (Figure 1C, lane 3), but was much more prominent in skeletal muscle extracts, where the level of the 43 kDa band (SAPK2a/p38), as well as that of the 48 kDa band, was much higher (Figure 1F). This made it possible to detect these substrates after much shorter exposure of the X-ray film, thereby minimizing the background phosphorylation (Figure 1F, lanes 1 and 2). The 48 kDa MKK6 substrate cross-reacted with the same phospho-specific antibody that recognized SAPK2a/p38 (data not shown), suggesting that it might be a closely related protein or a splice variant. In order to identify the 48 kDa protein, we purified it to near homogeneity from rabbit skeletal muscle extracts (see Materials and methods) and subjected it to tryptic digestion and mass spectrometric analysis. These experiments revealed that it was SAPK3/p38γ (Table I), a known substrate of MKK6 (Cuenda *et al*., 1997). This identification was confirmed by immunodepletion experiments using a SAPK3/p38γ-specific antibody (Figure 1F, lanes 5 and 6).

Identification of specific substrates for SAPK2a/p38, SAPK3/p38γ and SAPK4/p38δ

The finding that the authentic substrates for MKKs can be detected in crude cell extracts encouraged us to apply this method to the SAPK family of enzymes. SAPK2a/p38, SAPK3/p38γ and SAPK4/p38δ are 60% identical in amino acid sequence to one another. Indeed, they have similar substrate specificities *in vitro* and are often referred to as isoforms. In order to compare their specificities, we examined their ability to phosphorylate proteins in rabbit skeletal muscle extracts. Like the MKKs, each SAPK was able to use MnATP with similar efficiency to MgATP. However, in contrast to the MKKs, we were unable to detect any proteins phosphorylated specifically by the SAPKs in cell extracts, presumably due to their low abundance.

In order to improve the sensitivity of the assay, we chromatographed the extracts on Mono S using a salt gradient, which separated the endogenous protein kinases from many of their substrates and therefore eliminated much of the background phosphorylation. Each fraction was incubated with Mn[γ-^{32}P]ATP in the absence or presence of activated, GST-tagged versions of SAPK2a/p38, SAPK3/p38γ or SAPK4/p38δ. These experiments revealed a number of proteins that were phosphorylated by these protein kinases. Much to our surprise, there was little overlap in specificity between the three SAPKs. For example, in two fractions that eluted between 0.21 and 0.23 M NaCl, several substrates were detected that were phosphorylated specifically by just one of the three SAPKs (Figure 2A). SAPK2a/p38 phosphorylated a 50 kDa

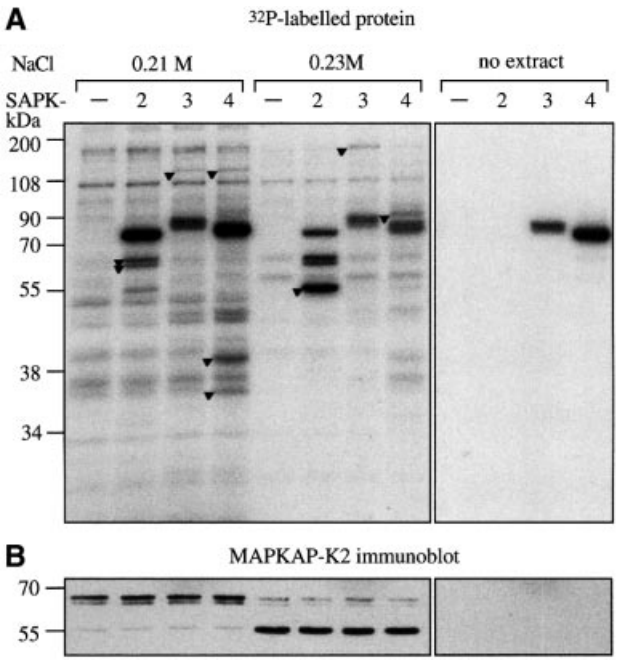

Fig. 2. Stress-activated protein kinases phosphorylate distinct proteins. A rabbit skeletal muscle extract (400 mg protein) was applied to a Mono S column (5 × 1 cm) equilibrated in 30 mM MOPS pH 7.0, 0.1 mM EGTA, 5% (v/v) glycerol, 0.03% (w/v) Brij 35, 0.1% (v/v) 2-mercaptoethanol and the column eluted with a 20 ml linear salt gradient to 1 M NaCl. Aliquots of fractions eluting at 0.21 and 0.23 M NaCl were diluted 5-fold in 30 mM Tris–HCl pH 7.5, 0.1 mM EGTA, 0.1% (v/v) 2-mercaptoethanol, then phosphorylated for 5 min at 30°C in a 0.03 ml assay with 10 mU of activated GST–SAPK2a/p38, GST–SAPK3/p38γ or GST–SAPK4/p38δ in the presence of 2 mM $MnCl_2$ and 20 nM [γ-^{32}P]ATP. (**A**) The reactions were then analysed as described in Figure 1A. (**B**) The membrane was immunostained with an antibody that recognizes the different forms of rabbit muscle MAPKAP-K2.

Table II. Identification of eEF2K as a substrate of SAPK4

Masses submitted	Masses matched	Start	End	Peptide
718.3267	718.3273	532	537	YHEGGR
855.4292	855.4616	596	602	GFDYLLK
893.4398	893.4303	132	139	MASQPFGR
893.4398	893.4780	356	362	CGSPRIR
917.5325	917.5242	610	617	QSMILVAR
999.5531	999.5651	508	516	LNALDLGRK
1043.5244	1043.4910	238	246	YNSNSGFVR
1066.5030	1066.4958	192	199	LWGEEYNR
1309.7668	1309.7956	363	374	TLSGSRPPLLLR
1309.7668	1309.7778	520	531	SVLGKVHLAMVR
1580.7688	1580.7862	252	264	LTPQAFSHFTFER
1621.7361	1621.7408	303	315	GMALFFYSHACNR
1786.7453	1786.7992	688	704	SGDLYTQAAEAAMEAMK
1877.9302	1878.0013	116	131	YNAVTGEWLKDEVLIK
3251.3886	3251.4286	375	405	LSENSGDENMSDVTFDSLPSSPSSATPHSQK

A 120 kDa putative substrate of SAPK4 was purified from rabbit muscle, subjected to SDS–PAGE, excised from the gel and digested with trypsin. The peptides were subjected to mass spectrometry. Forty-six peptides were detected, 13 of which could be matched with the sequence of rat eF2K.

protein and a 60 kDa doublet that were not substrates for SAPK3/p38γ or SAPK4/p38δ. This pattern of bands was identical to that observed for purified MAP kinase-activated protein kinase 2 (MAPKAP-K2) from rabbit skeletal muscle (Stokoe *et al*., 1992), a known physiological substrate of SAPK2a/p38 (Rouse *et al*., 1994; Cuenda *et al*., 1995). The identity of these proteins as forms of MAPKAP-K2 was confirmed by immunoblotting experiments with a MAPKAP-K2-specific antibody (Figure 2B). In contrast, SAPK3/p38γ phosphorylated a protein of ~200 kDa specifically, while SAPK4/p38δ phosphorylated several proteins of lower molecular mass that were not phosphorylated by SAPK2a/p38 or SAPK3/p38γ (Figure 2A).

The active forms of GST–SAPK3 and GST–SAPK4 became autophosphorylated when they were incubated with MgATP (Figure 2A, right hand panel), whereas SAPK2a/p38 did not. However, SAPK2a/p38 added to the fractions shown from Mono S became highly phosphorylated, presumably due to the presence of endogenous MKK6 activity in these fractions (Figure 2A, left hand panel).

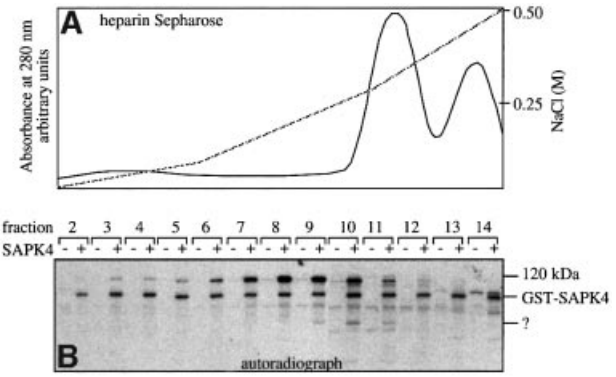

Fig. 3. Detection of a 120 kDa SAPK4/p38δ substrate. A rabbit muscle extract was chromatographed on SP-Sepharose, followed by fractionation from 8–16% PEG6000 and further chromatography on Mono S. (**A**) The Mono S eluate was chromatographed on heparin–Sepharose (see Materials and methods). The absorbance at 280 nM is shown by the full line and the NaCl gradient by the broken line. (**B**) Aliquots of each fraction were incubated for 5 min at 30°C in a 0.03 ml assay with 10 mU of activated GST–SAPK4/p38δ in the presence of 2 mM $MnCl_2$ and 20 nM [γ-^{32}P]ATP, denatured in SDS and analysed as in Figure 2. The positions of the autophosphorylated GST–SAPK4/p38δ and the 120 kDa substrate are indicated.

Identification of eEF2K as a protein that is phosphorylated relatively specifically by SAPK4/p38δ

A protein of apparent molecular mass 120 kDa that was phosphorylated by SAPK4, but much less efficiently by SAPK2/p38 or SAPK3/p38γ, was purified to near homogeneity from rabbit skeletal muscle extracts as described in Materials and methods. Figure 3 shows the elution profile from chromatography on heparin–Sepharose, which separated this substrate from two major contaminating proteins. Note that this protein is not the 120 kDa protein phosphorylated by both SAPK3/p38γ and SAPK4/p38δ that was detected after chromatography of cell extracts on Mono S (Figure 2). The 120 kDa protein phosphorylated specifically by SAPK4/p38δ was eluted from Mono S at a much lower concentration of NaCl (data not shown).

At the final step, chromatography on Mono Q, the 120 kDa protein phosphorylated specifically by SAPK4/p38δ was the major band staining with Sypro-orange (data not shown). It was identified by tryptic mass fingerprinting (Table II) as a protein that was very similar to human eEF2K. This identification was confirmed by Edman sequencing of two peptides (LDHLHWPVF and AFDTGGNLSPER) that correspond to residues 407–415 and 619–630 of human eEF2K. eEF2K is a protein kinase that is dependent on calcium ions and calmodulin for activity and which phosphorylates and inactivates eEF2 *in vivo*. It has therefore also been termed calcium/calmodulin-dependent protein kinase III (Nairn *et al*., 1985; Kigoshi *et al*., 1989; Ryazanov *et al*., 1997).

Phosphorylation and inactivation of eEF2K by SAPK4

GST–eEF2K was expressed in *Escherichia coli* and purified as described in Materials and methods. It was phosphorylated much more rapidly by SAPK4/p38δ than

Table III. Rate of phosphorylation of eEF2K by different MAPK family members

Protein kinase	Relative activity towards eEF2K (%)
SAPK1/JNK	0.6
SAPK2a/p38	1.4
SAPK3/p38γ	1.9
SAPK4/p38δ	100.0
ERK2	8.6

Active SAPK2a/p38, SAPK3/p38γ, SAPK4/p38δ, ERK2 and SAPK1/JNK (each at 3 nM) were used to phosphorylate bacterially expressed GST–eEF2K (2 μM) under initial rate conditions. The rate of phosphorylation of GST–eEF2K is given relative to SAPK4/p38δ (100%).

by any other MAPK family member tested (Table III) and phosphorylation reached 1.3 mol phosphate incorporated per mol protein after 1 h (Figure 4A). Phosphorylation (carried out in the absence of calcium ions and calmodulin) was accompanied by a large decrease in eEF2K activity, when this was measured by the phosphorylation of its substrate eEF2 in a subsequent assay performed in the presence of calcium ions and calmodulin. No phosphorylation or inactivation of eEF2K occurred if SAPK4 was omitted (Figure 4A).

^{32}P-labelled GST–eEF2K that had been phosphorylated by SAPK4/p38δ was digested with trypsin and chromatographed on a C_{18} column, which resolved one major and one minor tryptic phosphopeptide, termed T1 and T2 (Figure 5A). Peptide T1 was identified as residues 357–361 of eEF2K phosphorylated at Ser359 (Figure 5B). Peptide T2 corresponded to residues 364–406 plus one phosphate group. This peptide was subdigested with Asp-N proteinase, and the single phosphopeptide generated was identified as residues 391–406 phosphorylated at Ser396 (Figure 5C). Both residues are followed by a proline, consistent with the known specificity requirements of MAPK family members.

In order to establish that the SAPK4-catalysed inactivation of eEF2K resulted from the phosphorylation of the major site (Ser359), we mutated this residue to alanine. eEF2K[S359A] was phosphorylated (presumably at Ser396) by the high concentrations of SAPK4/p38δ used in this experiment. However, the inhibition of eEF2K under these conditions was reduced from 82% in the wild-type enzyme to 19% in eEF2K[S359A] (Figure 6). These experiments establish that the phosphorylation of Ser359 makes a major contribution to the SAPK4/p38δ-catalysed inhibition of eEF2K activity.

Anisomycin induces the phosphorylation of eEF2K in human epithelial KB cells

In order to examine whether Ser359 became phosphorylated in cells, we raised phospho-specific antibodies that recognize eEF2K only when it is phosphorylated at Ser359 (Figure 7). We deprived KB cells of serum and amino acids for 3 h, before exposing them to the protein synthesis inhibitor anisomycin, an agonist reported to activate transfected SAPK4/p38δ (Goedert *et al*., 1997). At various times, the cells were lysed, eEF2K immunoprecipitated from the extracts and the phosphorylation analysed by immunoblotting. These experiments showed that the

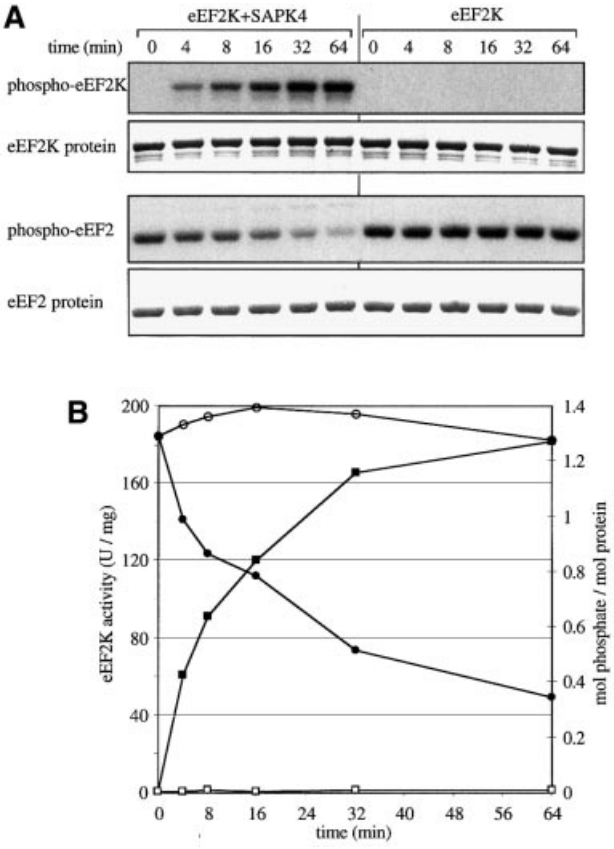

Fig. 4. SAPK4 inhibits eEF2K *in vitro*. (**A**) GST–eEF2K was phosphorylated with active GST–SAPK4/p38δ in the absence of calcium ions and calmodulin using 10 mM magnesium acetate and 0.1 mM [γ-^{32}P]ATP. Two aliquots were removed at various times. One was denatured in SDS and the other used to phosphorylate eEF2 in the presence of calcium ions and calmodulin, followed by denaturation in SDS. The two samples were subjected to SDS–PAGE and electrotransferred to a PVDF membrane. The membrane was autoradiographed and stained with Coomassie Blue. (**B**) The bands corresponding to eEF2K and eEF2 from (A) were excised from the membranes and analysed by Cerenkov counting to quantitate the incorporation of ^{32}P-radioactivity into eEF2K (squares) and the loss of eEF2K activity after phosphorylation (circles). Experiments were carried out in the presence (closed symbols) and absence (open symbols) of SAPK4/p38δ.

phosphorylation of Ser359 was low in the starved, unstimulated cells, but became phosphorylated in response to anisomycin (Figure 8A). Phosphorylation was detectable within a few minutes and maximal after ~30 min. Anisomycin also triggered the dephosphorylation (activation) of eEF2 at Thr56 (Figure 8A). eEF2K in starved cells migrated as a poorly resolved doublet, the lower band of the doublet disappearing upon stimulation with anisomycin.

If the cells were not starved of serum and amino acids prior to stimulation with anisomycin, Ser359 was already phosphorylated substantially. This suggested that mitogens/amino acids might also trigger the phosphorylation of Ser359. Indeed, this proved to be the case, Ser359 becoming phosphorylated when the starved cells were stimulated with insulin-like growth factor 1 (IGF-1; Figure 8B) or with epidermal growth factor (EGF) or serum (data not shown) in the absence of amino acids. This was also accompanied by the dephosphorylation of eEF2 (Figure 8B).

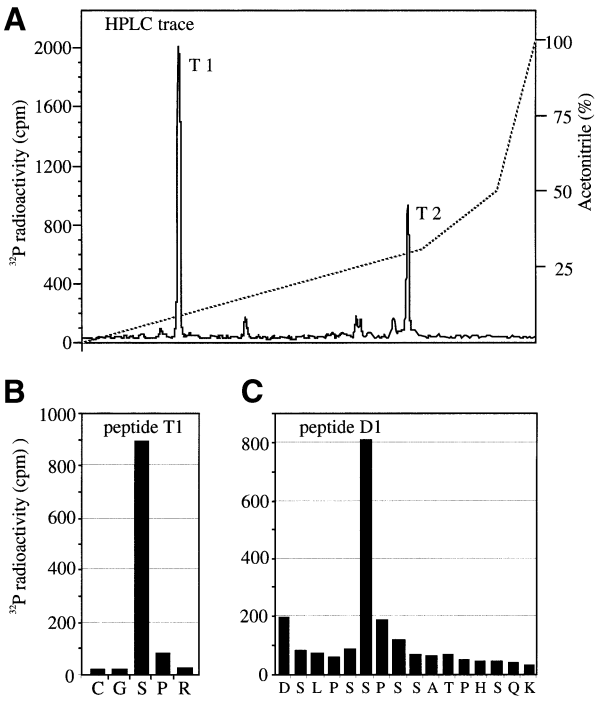

Fig. 5. Identification of the residues on eEF2K phosphorylated by SAPK4/p38δ. GST–eEF2K was phosphorylated with active GST–SAPK4/p38δ, denatured in SDS and subjected to SDS–PAGE. The band corresponding to ^{32}P-labelled eEF2K was excised and subjected to digestion with trypsin (Morrice and Powis, 1998). (**A**) The peptides were separated by reverse phase hydrophobic interaction chromatography on a Vydac C18 column equilibrated in 0.1% (v/v) trifluoroacetic acid. The column was then developed with an acetonitrile gradient in 0.1% trifluoroacetic acid (broken line). ^{32}P-Radioactivity is shown by the full line. (**B**) The mass of peptide T1 determined by mass spectrometry corresponded to residues 357–361 plus one phosphate group. The primary structure of the peptide was confirmed by Edman sequencing, and Ser359 was identified as the phosphorylation site in a separate solid phase sequencing experiment. The methodology is detailed elsewhere (Stokoe *et al*., 1992). (**C**) The mass of peptide T2 determined by mass spectrometry corresponded to residues 364–406 plus one phosphate group. This large peptide was subdigested with Asp-N proteinase, and rechromatography on the C_{18} column as in (A) revealed one ^{32}P-labelled peptide, termed D1, with a mass corresponding to residues 391–406 plus one phosphate group. This was confirmed by solid and gas phase sequencing of peptide D1, which identified Ser396 as the site of phosphorylation.

In order to obtain information about the signal transduction pathway that mediates the phosphorylation of Ser359, we examined the effects of several inhibitors of signal transduction cascades. These studies showed that SB 203580 (a specific inhibitor of SAPK2a/p38), U0126 (which prevents activation of the classical MAP kinase cascade) or rapamycin (a specific inhibitor of the protein kinase mTOR) did not affect the anisomycin-induced phosphorylation of Ser359 (Figure 9A), consistent with the involvement of SAPK4/p38δ. In contrast, the IGF-1-induced phosphorylation of Ser359 was prevented by rapamycin (Figure 9A) but not by U0126 (Figure 9B) or SB 203580 (data not shown). Control experiments showed that the anisomycin-induced phosphorylation of heat shock protein 27, a downstream component of the SAPK2a/p38 pathway, was prevented by SB 203580 (Figure 9) and that the IGF-1-induced activation of ERK1 and ERK2 was prevented by U0126, as expected (data not shown).

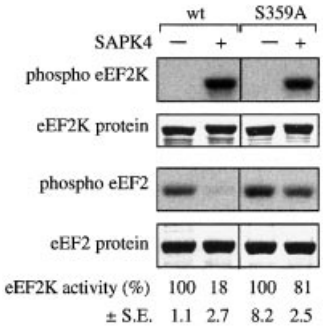

Fig. 6. Mutation of Ser359 to alanine attenuates the inactivation of eEF2K by SAPK4/p38δ. Wild-type GST–eEF2K or GST–eEF2K-S359A (3 μg) were phosphorylated for 1 h at 30°C in a 0.05 ml assay with or without active GST–SAPK4/p38δ (20 ng) as described in the legend to Figure 4. After 1 h, incorporation of ^{32}P-radioactivity into eEF2K was examined by SDS–PAGE and autoradiography and the activity of the eEF2K preparations in the presence of calcium ions and calmodulin was determined by the phosphorylation of eEF2 as in Figure 4.

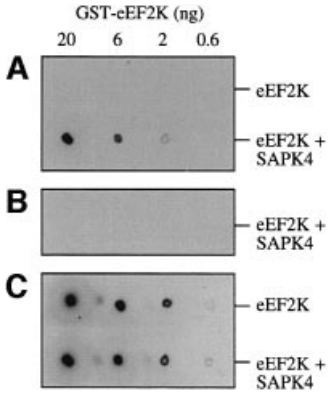

Fig. 7. Generation of an antibody that recognizes eEF2K only when phosphorylated at Ser359. The indicated amounts of unphosphorylated or SAPK4/p38δ-phosphorylated eEF2K were spotted onto an Immobilon P membrane, which was then immunostained with antibodies raised against the peptide TEEKCGpSPRVRTL, corresponding to residues 353–365 of eEF2K phosphorylated at Ser359 (where pS is phosphoserine). (**A**) Immunostaining in the presence of the unphosphorylated form of the peptide (5 μg/ml). (**B**) Immunostaining in the presence of the same concentration of the phosphopeptide antigen. (**C**) Immunostaining with an antibody raised against the whole eEF2K protein, which recognizes unphosphorylated and phosphorylated eEF2K equally well.

In order to obtain further evidence that SAPK4/p38δ is involved in the stress-induced phosphorylation of eEF2K at Ser359, we transfected a catalytically inactive mutant of SAPK4/p38δ (Goedert *et al*., 1997) into 293 cells, which prevented the anisomycin-induced phosphorylation of eEF2K at Ser359. In contrast, the overexpression of wild-type SAPK4/p38δ did not affect the phosphorylation of Ser359 in unstimulated cells, because it is inactive under these conditions, and only increased anisomycin-induced phosphorylation slightly, presumably because the endogenous Ser359 kinase activity is sufficient to induce near maximal phosphorylation (Figure 9C).

Discussion

Here we have demonstrated the feasibility of identifying physiologically relevant substrates of protein kinases, even

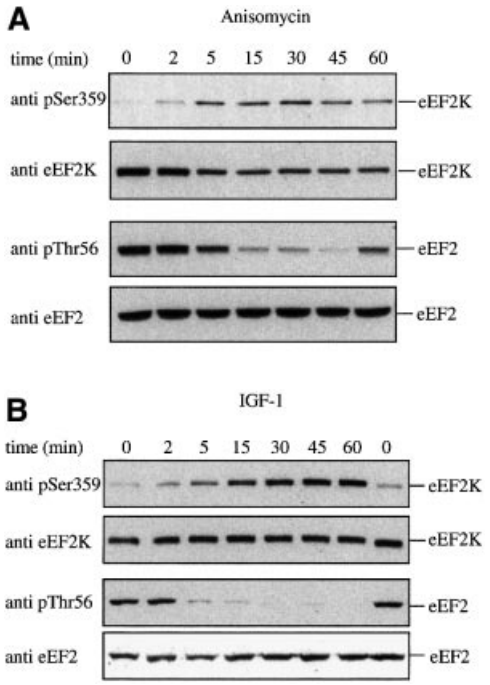

Fig. 8. Anisomycin and IGF-1 induce the phosphorylation of eEF2K in starved KB cells. The cells were starved of serum and amino acids for 3 h in Earl's balanced salt solution and then treated with either 40 μM anisomycin (**A**) or 50 ng/ml IGF-1 (**B**) for the times indicated. Endogenous eEF2K was immunoprecipitated from 2 mg of cell lysate protein, denatured in SDS and subjected to SDS–PAGE. After transfer to Immobilon P, the membranes were immunostained with the phospho-specific antibody that recognizes eEF2K only when phosphorylated at Ser359 (Figure 7). After removing these antibodies by washing extensively with Tris-buffered saline containing 1.5 M NaCl and 2.5% (v/v) Tween-20, the membranes were reprobed with the antibody used for immunoprecipitation, which recognizes phosphorylated and unphosphorylated eEF2K with similar efficiency (see Figure 7). Further aliquots of the cell lysates (20 μg protein) were subjected to SDS–PAGE and immunoblotting using an antibody that recognizes eEF2 phosphorylated at Thr56, followed by immunostaining with an antibody that recognizes phosphorylated and unphosphorylated eEF2 equally well.

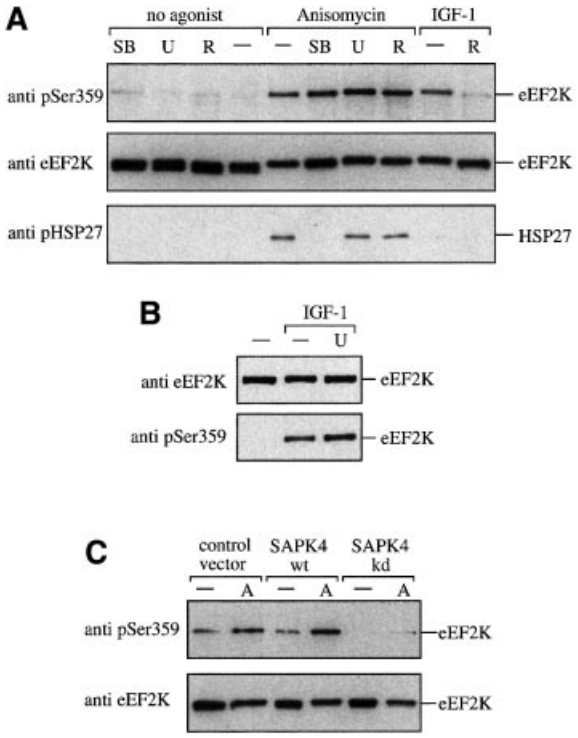

Fig. 9. Effects of protein kinase inhibitors and catalytically inactive SAPK4/p38δ on anisomycin- and IGF-1-induced phosphorylation of eEF2K at Ser359. (**A**) KB cells were starved for 3 h in Earl's balanced salt solution and then treated or not for 45 min with 10 μM SB 203580, 10 μM U0126 or 100 nM rapamycin. The cells were then stimulated for 30 min with 40 μM anisomycin or 50 ng/ml IGF-1, lysed and eEF2K immunoprecipitated and analysed for Ser359 phosphorylation and eEF2K protein as in Figure 8. Further aliquots of the lysate (20 μg protein) were subjected to SDS–PAGE and immunoblotting using an antibody that recognizes heat shock protein 27 phosphorylated at Ser15 (pHSP27), a downstream target of the SAPK2a/p38 pathway. (**B**) The KB cells were treated or not with 10 μM U0126, then stimulated or not with IGF-1 and analysed as in (A). (**C**) 293 cells were transfected with either a control vector pEGFP (Clontech, Basingstoke, UK) or with pcDNA3.1-SAPK4wt or pcDNA3.1-SAPK4[D168A] (kinase-dead, kd) (Goedert *et al*., 1997). After 36 h, the cells were either left untreated (–) or exposed for 20 min to 20 μM anisomycin (A). Phosphorylation of eEF2K at Ser359 was analysed with a phospho-specific antibody (anti-pSer359) as in Figure 8.

in crude cell extracts. The problem of high background phosphorylation in the extracts obscuring the identification of putative substrates could be improved by carrying out the phosphorylation reactions for just a few minutes, using high concentrations of the protein kinase of interest and [γ-^{32}P]ATP of very high specific radioactivity. A further significant improvement in signal to noise ratio was obtained by using MnATP as the substrate instead of MgATP. This made it possible to identify nearly all the major substrates of MKK1, MKK4 and MKK6 in crude cell extracts, even though these substrates (i.e. different MAPK family members) represented no more than 0.1% of the total protein content of the extracts. This trick is applicable to all protein kinases that use MnATP with similar efficiency to MgATP, such as MKKs and MAPKs.

The signal to noise ratio can be improved greatly by subjecting the cell extracts to a single step of ion-exchange chromatography before performing the analysis. Indeed, after such a chromatography, we have found that the signal to noise ratio is so much improved that substrates of protein kinases are now easily detectable using MgATP. In order to maximize the probability of identifying physiologically relevant substrates, we always carry out the analyses using two or three closely related protein kinases whose specificities towards synthetic peptide substrates are similar, trying to detect proteins that are phosphorylated specifically by just one of these kinases. Following the purification of each putative substrate to near homogeneity, it is important to check that they can be phosphorylated stoichiometrically and at similar rates to authentic physiological substrates, if such substrates are known and available. False positives tend to be very abundant proteins that are phosphorylated to low stoichiometries and these are not analysed further. In order to establish physiological relevance, it is then necessary to identify the site(s) of phosphorylation, to raise appropriate phospho-specific antibodies and to examine whether the protein becomes phosphorylated at these sites in response to signals that activate the protein kinase.

In the present study, we exploited KESTREL to identify eEF2K as a protein that is phosphorylated and inactivated by SAPK4/p38δ rapidly and stoichiometrically *in vitro*,

but four other MAPK family members tested were less effective in phosphorylating eEF2K *in vitro* (Table III). We identified Ser359 as the major site whose phosphorylation triggers inactivation, and showed that this site becomes phosphorylated in cells in response to anisomycin, an agonist that we (Goedert *et al*., 1997) and others previously have reported to activate transfected SAPK4/p38δ. Consistent with a role for SAPK4/p38δ, SB 203580, an inhibitor of SAPK2a/p38 that does not inhibit SAPK4/p38δ (Eyers *et al*., 1998), and U0126, a specific inhibitor of the classical MAP kinase cascade that also does not inhibit SAPK4/p38δ (Davies *et al*., 2000), did not affect the anisomycin-induced phosphorylation of eEF2K at Ser359. Moreover, the overexpression of a catalytically inactive mutant of SAPK4/p38δ prevented the anisomycin-induced phosphorylation of eEF2K at Ser359. However, a specific inhibitor of SAPK4/p38δ or a mouse 'knockout', which are not yet available, will be needed to establish whether this enzyme or another stress- and cytokine-activated protein kinase with similar specificity is responsible for the inhibition of eEF2 kinase via the phosphorylation of Ser359.

To our knowledge, this is the first report that eEF2K can be inactivated and eEF2 activated in response to a cellular stress. This may be one of the mechanisms required to stimulate the translation of mRNAs encoding proteins whose levels are enhanced by stress.

In the course of these studies, we noticed that Ser359 was significantly phosphorylated in normally growing cells and that serum and amino acid starvation was necessary to suppress the phosphorylation of this site. This was not surprising because an earlier study had reported that insulin was able to induce the inhibition of eEF2K and hence the dephosphorylation and activation of eEF2 in Chinese hamster ovary (CHO) cells overexpressing the insulin receptor by a rapamycin-sensitive pathway (Redpath *et al*., 1996). We confirmed that IGF-1 (Figure 8B) induces the dephosphorylation of eEF2 in KB cells and that this was also accompanied by the phosphorylation of Ser359 (Figure 8B). This was also true for EGF and serum (data not shown). Consistent with the earlier report (Redpath *et al*., 1996), the IGF-1-induced phosphorylation of Ser359 was prevented by rapamycin. However, this was not true for anisomycin (Figure 8A), indicating that Ser359 is targeted by at least two different signal transduction pathways.

Ser359 is in close proximity to Ser366, and Wang *et al*. (2001) demonstrated that the latter residue also becomes phosphorylated in response to growth factors. The growth factor-induced phosphorylation of Ser366 appears to be mediated by S6 kinase-1 (S6K1) via a phosphatidylinositol 3-kinase-dependent, rapamycin-sensitive pathway as well as by MAP kinase-activated protein kinase-1 (MAPKAP-K1, also called RSK), the most downstream component of the classical MAP kinase cascade (Wang *et al*., 2001). These findings and the present study indicate that the regulation of eEF2K is complex and involves the phosphorylation of at least two sites by several pathways.

In summary, the present report has demonstrated that KESTREL can be a powerful method for identifying putative physiological substrates of protein kinases and novel regulatory sites of phosphorylation. The method is quite fast, because it only takes one person a day or two to detect a number of promising ^{32}P-labelled substrates and only a few weeks to purify and identify nearly all of them. Phosphorylation sites are usually identified within a few days, and the slowest step is the generation and exploitation of appropriate phospho-specific antibodies. We have now applied KESTREL to 11 protein kinases in several different subclasses, some of which use MgATP, but not MnATP. These studies, which will be reported in future papers, have emphasized the general utility of the method, even for identifying and purifying low abundance proteins. Indeed, eEF2K, which was identified in the present report as a substrate of SAPK4/p38δ, comprises <0.01% of the protein in cell extracts.

Materials and methods

Materials

[γ-^{32}P]ATP and materials for protein purification were obtained from Amersham Pharmacia Biotech, UK. Unlabelled ATP and dithiothreitol (DTT) were from Roche Molecular Biochemicals (Lewes, UK), cell culture media and human IGF-1 from Gibco (Paisley, UK), SB 203580, rapamycin and U0126 from Calbiochem (Nottingham, UK), and Immobilon P membranes from Millipore (Bedford, UK). Other chemicals were of the highest purity available and were purchased from Merck (Poole, UK) or Sigma-Aldrich (Poole, UK). Calmodulin was purified from bovine brain.

Antibodies

Peptides were synthesized by Dr Graham Blumberg (University of Bristol, UK), coupled to bovine serum albumin (BSA) and keyhole limpet haemocyanin and injected into sheep at the Scottish Antibody Production Unit (Carluke, UK). The antisera were then affinity purified on phosphopeptide antigen–Sepharose columns followed by passage through an unphosphorylated peptide–Sepharose column, and the flowthrough fractions collected and used for experimentation. The antibodies were used at a concentration of 0.5 μg/ml in the presence of 5 μg/ml of the unphosphorylated peptide antigen. Antibodies that recognize SAPK3 phosphorylated at Thr183 and Tyr185 were raised against the peptide ADSEMpTGpYVVTRW (where pT and pY represent phosphothreonine and phosphotyrosine, respectively). This antibody also recognizes the phosphorylated forms of SAPK2a/p38 and SAPK4/p38δ. The phospho-specific SAPK3/p38γ antibody, a phospho-specific antibody that recognizes heat shock protein 27 phosphorylated at Ser15, immunoprecipitating antibodies raised against GST–eEF2K, GST–SAPK3/p38γ and His6-JNK1, and further antibodies that immunoprecipitate ERK2 (raised against the peptide EETARFQPGYRS) and MAPKAP-K2 (raised against the C-terminal peptide MTSALATMRVDYEQIK) were all raised in sheep and provided by Drs Jane Leitch and Chris Armstrong, Division of Signal Transduction Therapy, University of Dundee. The eEF2K antibody was affinity purified on glutathione–Sepharose to remove anti-GST antibodies and then affinity purified on GST–eEF2K–Sepharose. Rabbit antibodies that recognize eEF2 phosphorylated at Thr56 and the eEF2 protein were provided by Professor Chris Proud, University of Dundee. An antibody that recognizes SAPK2a/p38 specifically was purchased from New England Biolabs (Hitchin, UK). Rabbit anti-sheep IgG and goat anti-rabbit IgG antibodies, both conjugated to peroxidase, were obtained from Perbio Science Ltd (Tattenhall, UK).

Protein expression, mutagenesis and activation

All recombinant protein kinases were expressed in *E.coli* BL21 as GST-tagged proteins, except for MKK6, which was expressed as a maltose-binding protein (MBP) fusion protein, and SAPK1/JNK1, which was expressed with an N-terminal His6 tag. Each protein was purified to 60–90% homogeneity by affinity chromatography on glutathione–Sepharose, maltose–Sepharose or nickel nitrilo-triacetate (Ni-NTA)–agarose as appropriate, dialysed into 50 mM Tris–HCl pH 7.5, 10 mM DTT, 50% (v/v) glycerol, and stored at –20°C. MKK1 (rabbit), MKK4 (mouse) and MKK6 (human) were converted to constitutively active forms, namely MKK1[S218D,S222D], MKK4[S257D,T261D] and MKK6[S207D,T211D], by mutating the residues involved in activation by phosphorylation to aspartic acid using 'Quickchange' (Stratagene). The catalytically inactive MKK1[D208A] mutant was also generated. Ser359 in human eEF2K, the residue phosphorylated by SAPK4/p38δ,

was mutated to alanine to generate eEF2K[S359A]. Human SAPK2/p38, SAPK3/p38γ and SAPK4/p38δ were activated by incubation with MKK6-DD and subsequently repurified by chromatography on glutathione–Sepharose. SAPK1/JNK1 was activated with MKK4[S257D,T261D] and repurified on Ni-NTA–agarose. ERK2 was activated with MKK1[S218D, S222D] and repurified on Mono Q.

Preparation of ATP-depleted cell extracts

Ten confluent 15 cm diameter dishes of HeLa tk– cells [cultured in Dulbecco's modified Eagle's medium (DMEM), 10% fetal calf serum (FCS) containing penicillin and streptomycin] were washed twice with 10 ml of 50 mM Tris–HCl pH 7.0, 140 mM NaCl, 10 mM EDTA, 10 mM 2-mercaptoethanol and left for 10 min in 5 ml of the same buffer to achieve detachment from the dishes. The cells were collected, sedimented for 4 min at 500 *g* and resuspended in 2 ml of lysis buffer [40 mM Tris–HCl pH 7.0, 1% (v/v) Triton X-100, 2.5 mM EDTA, 15 mM DTT, 1 mM phenylmethylsulfonyl fluoride (PMSF), 10 μg/ml aprotinin, 10 μg/ml leupeptin]. The nuclei and cellular debris were sedimented by centrifugation at 13 000 *g* for 15 min at 4°C. The supernatant was decanted and passed through Sephadex G-25 equilibrated in 40 mM Tris–HCl pH 7.0, 0.1% (v/v) 2-mercaptoethanol, 0.1 mM EGTA, 0.1% Triton X-100 in order to remove ATP, ADP and other small molecules. The desalted extracts were incubated for 20 min at 30°C to promote protein dephosphorylation.

Protein kinase assays

SAPKs and ERK2 were incubated for 10 min at 30°C with myelin basic protein (0.4 mg/ml) [except for SAPK1/JNK1 where myelin basic protein was replaced by 0.3 mg/ml GST–ATF2(19–96)] in 0.05 ml of 50 mM Tris–HCl pH 7.5, 1 mg/ml BSA, 0.1 mM EGTA, 0.1% (v/v) β-mercaptoethanol, 5 mM sodium β-glycerophosphate, 1 mM Na_3VO_4, 0.1 mM [γ-^{32}P]ATP and either 10 mM magnesium acetate or 2 mM $MnCl_2$. The reactions were terminated by spotting 0.04 ml aliquots onto 2 × 2 cm squares of Whatman P81 phosphocellulose paper, followed by immersion in 75 mM phosphoric acid. After washing four times with 75 mM phosphoric acid and then once with acetone, the papers were dried and analysed by Cerenkov counting. The incorporated radioactivity was subtracted from control incubations in which myelin basic protein had been omitted. Each protein kinase was diluted appropriately to ensure that initial rate conditions were met. One unit of SAPK2/p38, SAPK3/p38γ or SAPK4/p38δ was that amount of enzyme that phosphorylated 1 nmol of myelin basic protein in 1 min. One unit of SAPK1/JNK was the amount that phosphorylated 1 nmol of GST–ATF2(19–96).

Substrates of SAPKs in cell extracts or fractions from ion-exchange chromatography were identified as follows. Fractions were first diluted with 9 vols of 30 mM Tris–HCl pH 7.5, 2 mM $MnCl_2$, 10 mM DTT, 0.1 mM EGTA. Aliquots (25 μl) were then incubated for 5 min at 30°C with or without 10 mU of activated SAPK and 2.5 × 10^6 c.p.m. of 20 nM [γ-^{32}P]ATP in a total volume of 0.03 ml. The reactions were stopped by the addition of 0.25 vol. of 320 mM Tris–HCl pH 6.8, 8% (w/v) SDS, 20 mM EDTA, 32% (v/v) glycerol, 1.14 M 2-mercaptoethanol, 0.02% (w/v) bromophenol blue heated for 3 min at 100°C, subjected to SDS–PAGE, electroblotted onto Immobilon P membranes and autoradiographed to reveal substrate proteins.

In order to phosphorylate substrates stoichiometrically, the concentration of [γ-^{32}P]ATP was increased to 0.1 mM and its specific radioactivity decreased to 10^6 c.p.m./nmol. $MnCl_2$ at 2 mM was replaced by 10 mM magnesium acetate where indicated.

Phosphorylation of eEF2K and eEF2 by SAPK4/p38δ

GST–eEF2K (400 nM) was incubated at 30°C in 30 mM Tris–HCl pH 7.5, 0.1% (v/v) 2-mercaptoethanol, 3.3 nM active GST–SAPK4/p38δ, 5 μM EGTA, 10 mM magnesium acetate and 0.1 mM [γ-^{32}P]ATP (10^6 c.p.m./nmol) in a 0.05 ml assay. At particular times, two aliquots were removed. One (40 μl) was denatured in SDS, while a second 1 μl aliquot was removed and added to another 30°C assay (0.05 ml) containing 800 nM eEF2, 0.3 μM calmodulin, 5 μM EGTA, 10 μM $CaCl_2$, 10 mM magnesium acetate and 0.1 mM [γ-^{32}P]ATP (10^6 c.p.m./nmol). After incubation for 5 min, the reactions were stopped with SDS. All the samples were subjected SDS–PAGE, electroblotted onto Immobilon P membranes and autoradiographed. Control experiments were performed in which GST–SAPK4/p38δ was omitted.

Purification of a 48 kDa MKK6 substrate (SAPK3)

A rabbit skeletal muscle extract (10 ml, 150 mg of protein), prepared as described previously (Cuenda *et al*., 1996), was centrifuged for 5 min at 2000 *g*. The supernatant was removed, filtered through a 0.2 μm membrane, diluted with 3 vols of buffer A [30 mM Tris–HCl pH 7.5, 0.1 mM EGTA, 0.1% (v/v) 2-mercaptoethanol, 5% (v/v) glycerol, 0.03% (w/v) Brij 35, 0.1 mM PMSF, 1 mM benzamidine] and incubated for 1 h on a rotating shaker with 0.4 ml of Fast-Flow Q-Sepharose (Pharmacia) equilibrated in buffer A. The Q-Sepharose was sedimented by centrifugation, the supernatant discarded, the Q-Sepharose was washed five times with 10 ml of buffer A and the 48 kDa protein was eluted with 3 ml of buffer A plus 0.3 M NaCl. The eluate was diluted with 5 vols of buffer A without NaCl and chromatographed on Mono Q equilibrated in buffer A. The column was developed at 1 ml/min with a 20 ml linear NaCl gradient to 0.5 M. Fractions of 0.5 ml were collected and the 48 kDa protein (eluting at 0.16–0.17 M NaCl) was detected by incubating aliquots of each fraction with active MKK6, 2 mM $MnCl_2$ and 0.1 mM [γ-^{32}P]ATP, followed by SDS–PAGE and autoradiography. These fractions were pooled, and the 48 kDa protein maximally phosphorylated by incubation for 15 min at 30°C with 0.4 μM active MKK6, 2 mM $MnCl_2$ and 0.1 mM [γ-^{32}P]ATP (10^6 c.p.m./nmol). The preparation was then added to 0.1 mg of the phospho-specific antibody that recognizes SAPK2/p38, SAPK3/p38γ and SAPK4/p38δ and which had been coupled covalently to 0.25 ml of protein A/G–Sepharose. After mixing for 2 h, the protein A/G–Sepharose was collected by sedimentation and washed four times with 50 mM Tris–HCl pH 7.5, 0.1 mM EGTA, 140 mM NaCl, 0.1% (v/v) Triton X-100. Bound proteins were eluted with 1 ml of 50 mM Tris–HCl pH 7.5, 0.1 mM EGTA, 0.1% (v/v) 2-mercaptoethanol, 1.5 M LiBr, concentrated to 0.02 ml and desalted in a Centricon 10 membrane (Millipore, Bedford, UK). After denaturation in SDS, the sample was electrophoresed on a 10% polyacrylamide gel. The gel was stained with Coomassie Blue, destained and autoradiographed. The ^{32}P-labelled 48 kDa band, which co-migrated with a protein band that stained with Coomassie blue, was excised and subjected to tryptic digestion, mass spectrometry and database analysis in order to identify it (Morrice and Powis, 1998).

Purification of eEF2K and eEF2 from rabbit skeletal muscle extracts

Rabbit skeletal muscle (140 ml, 15 mg/ml) was diluted with an equal volume of buffer B [30 mM MOPS/NaOH pH 7.0, 5% glycerol, 0.1% (v/v) 2-mercaptoethanol, 0.03% Brij 35, 0.1 mM EGTA] and applied to a 200 ml bed of SP-Sepharose HP in a 500 ml Millipore Express 0.22 μM Vacuum Filter Unit, washed with three bed volumes of buffer B and eluted with two bed volumes of buffer B plus 1.0 M NaCl. The eluate was taken to 8% (w/v) in polyethylene glycol (PEG) 6000 and, after standing for 2 h, the suspension was centrifuged for 15 min at 5000 *g*. The supernatant was decanted and the precipitate redissolved in buffer B. The supernatant was then taken to 16% PEG6000 and allowed to stand for a further 2 h. After centrifugation, the pellet was redissolved in buffer B, clarified by passage through a 0.22 μm filter and chromatographed on Mono S (HR5/5) equilibrated in buffer B. The column was developed with a 20 ml linear salt gradient to 1 M NaCl. Each 1 ml fraction collected was incubated for 5 min with 50 ng of activated SAPK4/p38δ and Mn[γ-^{32}P]ATP, subjected to SDS–PAGE, electrotransferred to Immobilon P and the membranes autoradiographed. The fractions containing the 120 kDa substrate (eluting between 0.16 and 0.2 M NaCl) were pooled, diluted in 5 vols of buffer C [30 mM MES/NaOH pH 6.0, 5% (v/v) glycerol, 0.1% (v/v) 2-mercaptoethanol, 0.03 % (w/v) Brij 35, 0.1 mM EGTA] and applied to a 1 ml heparin–Sepharose column (HiTrap from Amersham Pharmacia Biotech) equilibrated in the same buffer. The column was developed with a 20 ml linear NaCl gradient in buffer C and the fractions (1 ml) containing the 120 kDa substrate (eluting between 0.18 and 0.22 M NaCl, see Results) were pooled, diluted 10-fold in buffer D (30 mM Tris–HCl pH 8.0, 5% glycerol, 0.1% 2-mercaptoethanol, 0.03% Brij 35, 0.1 mM EGTA) and chromatographed on Mini Q (3.2 × 0.3 cm) equilibrated in the same buffer. The column was developed with a 4 ml gradient to 1.0 M NaCl in buffer D and the fractions (0.05 ml) containing the 120 kDa substrate (eluting from 0.33 to 0.37 M NaCl) were collected.

Rabbit eEF2 co-purified with eEF2K through SP-Sepharose and fractionation with PEG6000, but was resolved in Mono S, from which it eluted at a much higher concentration of NaCl (0.3–0.4 M). The fractions containing eEF2 (detected by immunoblotting with an EF2-specific antibody) were pooled and subjected to chromatography on heparin–Sepharose as described above for eEF2K. The eEF2-containing fractions were then purified on Mono Q equilibrated in buffer C, from which it eluted in a homogeneous form between 0.11 and 0.13 M NaCl. About 3 mg of pure eEF2 was obtained from 2 g of rabbit muscle extract.

Mass spectrometry

Tryptic peptides were analysed on a Perseptive Biosystems (Framingham, MA) Elite STR matrix-assisted laser desorption time of flight (MALDI-TOF) mass spectrometer with saturated α-cyanocinnamic acid as the matrix. The mass spectrum was acquired in the reflector mode and was internally mass calibrated. The tryptic peptide ions obtained were scanned against the Swiss-Prot and Genpep databases using the MS-FIT program of Protein Prospector.

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