Stress-induced regulation of eukaryotic elongation factor 2 kinase by SB 203580-sensitive and -insensitive pathways

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Eukaryotic elongation factor 2 (eEF2) kinase, the enzyme that inactivates eEF2, is controlled by phosphorylation. Previous work showed that stress-activated protein kinase 4 (SAPK4, also called p38δ) inhibits eEF2 kinase *in vitro* by phosphorylating Ser-359, while ribosomal protein S6 kinases inhibit eEF2 kinase by phosphorylating Ser-366 [Knebel, Morrice and Cohen (2001) EMBO J. **20**, 4360–4369; Wang, Li, Williams, Terada, Alessi and Proud (2001) EMBO J. **20**, 4370–4379]. In the present study we have examined the effects of the protein synthesis inhibitor anisomycin and tumour necrosis factor-α (TNF-α) on the phosphorylation of eEF2 kinase. We demonstrate that Ser-359, Ser-366 and two novel sites (Ser-377 and Ser-396) are all phosphorylated in human epithelial KB cells, but only the phosphorylation of Ser-359 and Ser-377 increases in response to these agonists and correlates with the dephosphorylation (activation) of eEF2. Ser-377 is probably a substrate of MAPKAP-K2/K3 (mitogen-activated protein kinase-activated protein kinase 2/kinase 3) in cells, because eEF2 kinase is phosphorylated efficiently by these protein kinases *in vitro* and phosphorylation of this site, induced by TNF-α and low (but not high) concentrations of anisomycin, is prevented by SB 203580, which inhibits SAPK2a/p38, their 'upstream' activator. The phosphorylation of Ser-359 induced by high concentrations of anisomycin is probably catalysed by SAPK4/p38δ in cells, because no other stress-activated, proline-directed protein kinase tested phosphorylates this site *in vitro* and phosphorylation is insensitive to SB 203580. Interestingly, the phosphorylation of Ser-359 induced by TNF-α or low concentrations of anisomycin is suppressed by SB 203580, indicating that phosphorylation is also mediated by a novel pathway. Since the phosphorylation of Ser-377 does not inhibit eEF2 kinase *in vitro*, our results suggest that anisomycin or TNF-α inhibit eEF2 kinase via the phosphorylation of Ser-359.

Key words: elongation, mRNA translation, p38, SB 203580.

INTRODUCTION

The activity of eukaryotic elongation factor-2 (eEF2), which is critically important for the elongation of mRNA translation, is regulated by phosphorylation [1–3]. To be active, eEF2 must be dephosphorylated, and phosphorylation at Thr-56 [4] causes inactivation, resulting in the termination of mRNA translation. Thr-56 is phosphorylated by a specific, calcium and calmodulin (Ca/CaM)-dependent eEF2 kinase [5–8], which is itself regulated both negatively and positively by phosphorylation. First, eEF2 kinase is phosphorylated at Ser-359 and Ser-396 by stress activated protein kinase 4 (SAPK4; also called p38δ) *in vitro*, which reduces eEF2 kinase activity by 80 %. In cells deprived of serum and amino acids, Ser-359 is unphosphorylated but phosphorylation is induced by treatment with the protein synthesis inhibitor anisomycin. The anisomycin-induced phosphorylation of Ser-359 is resistant to SB 203580, an inhibitor of SAPK2a/p38 and SAPK2b/p38β2, and rapamycin, an inhibitor of the mammalian target of rapamycin ('mTOR') [9], an SB 203580-resistant enzyme [10,11]. The phosphorylation of Ser-359 is also induced when starved cells are treated with a mitogen, such as insulin-like growth factor ('IGF-1') or foetal calf serum (FCS) but, in this case, a rapamycin-sensitive pathway mediates the effect ([9] and A. Knebel, unpublished work). Growth factors and mitogens also increase the phosphorylation of Ser-366 and this appears to be mediated by ribosomal protein S6 kinase-1 ('S6K1') and ribosomal S6 kinase ('RSK'), which phosphorylate this site *in vitro* and partially inhibit eEF2 kinase activity [12]. In contrast, eEF2 kinase can also be phosphorylated at Ser-499 by cAMP-dependent kinase *in vitro* and in cells, which is reported to generate some activity in the absence of Ca/CaM [13,14,15].

These findings demonstrate that eEF2 kinase activity is not only dependent on Ca/CaM but is also controlled by the phosphorylation of four sites catalysed by at least five protein kinases. In the present study, we show that the phosphorylation of eEF2 kinase and its activity is also mediated by signalling pathways that are sensitive to SB 203580, and identify Ser-377 as a new site of phosphorylation in the human enzyme.

MATERIALS AND METHODS

Materials

[γ-^{32}P]ATP and materials for protein purification were obtained from Amersham Biosciences (Little Chalfont, Bucks., U.K.). Unlabelled ATP and dithiothreitol were from Roche Molecular Biochemicals (Lewes, East Sussex, U.K.), cell culture media from Gibco (Paisley, Renfrewshire, Scotland, U.K.), human recombinant tumour necrosis factor (TNF)-α from Alexis (Nottingham, U.K.), SB 203580 from Calbiochem–Novabiochem (Nottingham, U.K.) and Immobilon P membranes from Millipore (Bedford, U.K.). Other chemicals were of the highest purity available and purchased from VWR (Poole, Dorset, U.K.) or Sigma-

Abbreviations used: Ca/CaM, calcium and calmodulin; EBSS, Earl's balanced salt solution; eEF2, eukaryotic elongation factor 2; FCS, foetal calf serum; GST, glutathione S-transferase; HSP27, heat shock protein 27; JNK, c-Jun N-terminal kinase; MAPK, mitogen-activated protein kinase; MAPKAP-K, MAPK-activated protein kinase; MKK, MAPK kinase; S396A, a mutation of serine to alanine at position 396; SAPK, stress activated protein kinase; TNF, tumour necrosis factor.

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Aldrich (Poole, Dorset, U.K.). Calmodulin was purified from bovine brain.

Antibodies

Peptides were synthesized by Graham Bloomberg (Department of Biochemistry, University of Bristol, U.K.), coupled separately to bovine serum albumin and keyhole limpet haemocyanin, mixed and injected into sheep at Diagnostics Ltd. (Edinburgh, U.K.). The antisera were then affinity purified on phosphopeptide antigen-Sepharose columns followed by passage through an unphosphorylated peptide-Sepharose column, and the flow-through fractions were collected and used for experiments. The antibodies were used at a concentration of 1 μg/ml in the presence of 10 μg/ml of the unphosphorylated peptide antigen. The antibodies recognizing eEF2 kinase phosphorylated at Ser-377 or Ser-396 were raised against the peptides LLRPL-pSENSG or FDSLPSpSPSSATPH respectively (the one-letter symbols for amino acids are used and pS represents phosphoserine). Antibodies against eEF2 phosphorylated at Thr-56 were raised against the peptide AGETRFpTDTRKD, where pT is phosphothreonine. The eEF2 kinase (pSer-396) antibody was raised in rabbit. Procedures for the preparation of antibodies recognizing eEF2, eEF2 kinase, mitogen-activated protein kinase (MAPK)-activated protein kinase (MAPKAP-K) 2, heat shock protein 27 (HSP27) (phosphorylated at Ser-15), phosphorylated eEF2 kinase (pSer-359 or pSer-366) were described previously [9,12]. All these antibodies were provided by Dr Jane Leitch and Dr Chris Armstrong (Division of Signal Transduction Therapy, University of Dundee, U.K.). The rabbit antibodies that recognize the eEF2 protein were provided by Professor Chris Proud (Division of Molecular Sciences, University of Dundee, U.K.). Rabbit anti-(sheep IgG) antibodies and goat anti-(rabbit IgG), both conjugated to peroxidase, were obtained from Perbio Science UK Ltd (Tattenhall, Cheshire, U.K).

Protein expression, mutagenesis and activation

All recombinant protein kinases were expressed in *Escherichia coli* BL21 as glutathione S-transferase (GST)-tagged proteins and purified to 60–90 % homogeneity by affinity chromatography on GST-Sepharose, dialysed against 50 mM Tris/HCl (pH 7.5) containing 10 mM dithiothreitol and 50 % (v/v) glycerol, and stored at −20 °C. GST-eEF2 kinase was converted to GST-eEF2 kinase[S396A] (where S396A represents a mutation of serine to alanine at position 396) using the 'Quickchange' system (Stratagene, Cambridge, U.K.). Human SAPK2a/p38, SAPK2b/p38β2, SAPK3/p38γ and SAPK4/p38δ were activated by incubation with MAPK kinase (MKK)-6-DD, a constitutively active mutant in which Ser-207 and Thr-211 have been changed to aspartic acid, and subsequently repurified by chromatography on GST-Sepharose. His_6-tagged Jun N-terminal kinase (JNK), c-JNK1α1 and His_6-tagged JNK2α2 were activated using a combination of MKK4 and MKK7. GST-MAPKAP-K2, GST-MAPKAP-K3 and GST-MAPKAP-K5 [also called p38-regulated activated kinase (PRAK)] were activated by phosphorylation with SAPK2a/p38 and repurified by chromatography on S-Sepharose. eEF2 was purified from rabbit muscle as described previously [9].

Assay of eEF2 kinase activity

GST-eEF2 kinase (400 nM) was incubated in a 0.05 ml assay at 30 °C with 10 m-units of MAPKAP-K2, 100 nM GST-SAPK2a/p38, 100 nM GST-SAPK2b/p38β2 or 3.3 nM GST-SAPK4/p38δ in 30 mM Tris/HCl (pH 7.5) containing 0.1 % (v/v) 2-mercaptoethanol, 0.1 mM EGTA, 10 mM Mg-acetate and 0.1 mM [γ-^{32}P]ATP (10^6 c.p.m. per nmol). At various times, three aliquots were removed. One (40 μl) was denatured in SDS, and two (1 μl) aliquots were removed and added to another 30 °C assay (0.05 ml), containing 800 nM eEF2, 75 nM CaM, 1 μM EGTA, 2 μM $CaCl_2$, 10 mM Mg-acetate and 0.1 mM [γ-^{32}P]ATP (10^6 c.p.m. per nmol). After incubation for 5 min, the reactions were stopped with SDS. All the samples were subjected to SDS-PAGE, electroblotted on to Immobilon P membranes and autoradiographed. Control experiments were performed in the absence of any added protein kinase.

Cell culture and stimulation

KB cells (human oral epidermoid carcinoma cells) were cultivated in Dulbecco's modified Eagle's medium containing 10 % FCS and 100 units/ml penicillin and 0.1 mg/ml streptomycin. Cells were grown to 90 % confluency in 15 cm dishes, washed with 12 ml of Earl's balanced salt solution (EBSS; Invitrogen), cultivated for periods ranging from 5 min to 4 h in 20 ml of EBSS, then incubated with 10 μM SB 203580, followed by stimulation with anisomycin (0.1–10 μg/ml) or human TNF-α.

Cell lysis and immunoprecipitation of eEF2 kinase

The KB cells were washed once with 10 ml of PBS and collected in 0.8 ml of lysis buffer [50 mM Tris/HCl (pH 7.5), 1 % (v/v) Triton X-100, 20 mM Na-β-glycerophosphate, 10 mM NaF, 2 mM Na_3VO_4, 0.1 mM EDTA, 0.1 mM EGTA, 0.1 mM PMSF, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 1 μM Microcystin-LR]. The nuclei and cellular debris were sedimented by centrifugation at 13000 ***g*** for 15 min at 4 °C. Aliquots of the supernatant (400 μg of protein) were denatured in SDS for subsequent immunoblotting analysis. Aliquots (8 mg) of the protein extract were supplemented with 250 mM NaCl and used to immunoprecipitate eEF2 kinase with 50 μl of Protein G–Sepharose and 4 μg of anti-(eEF2 kinase) antibodies. The beads were washed three times with 50 mM Tris/HCl (pH 7.5) containing 0.1 % (v/v) Triton X-100, 250 mM NaCl and 20 mM Na-β-glycerophosphate, followed by one further wash with 30 mM Tris/HCl (pH 7.0) denatured in SDS, and centrifuged briefly to pellet the Protein G–Sepharose. Aliquots of the supernatant were subjected to SDS-PAGE and, after transfer to Immobilon P membranes, immunoblotted with various eEF2 kinase antibodies.

Mass spectrometry

Tryptic peptides were analysed on a Perseptive Biosystems Elite STR matrix-assisted laser desorption time of flight ('MALDI-TOF') mass spectrometer (Framingham, MA, U.S.A.) 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 SwissProt and Genpep databases using the MS-FIT programme of Protein Prospector [15a].

RESULTS

SB 230580 prevents the dephosphorylation of eEF2 induced by TNF-α and low, but not high, concentrations of anisomycin in human KB cells

We have reported previously that anisomycin (10 μg/ml) induces the activation of eEF2 by promoting its dephosphorylation at Thr-56 [9]. In the present study, we found that the dephosphorylation of Thr-56 was resistant to SB 203580 (Figure 1A), consistent with our earlier observation that dephosphorylation

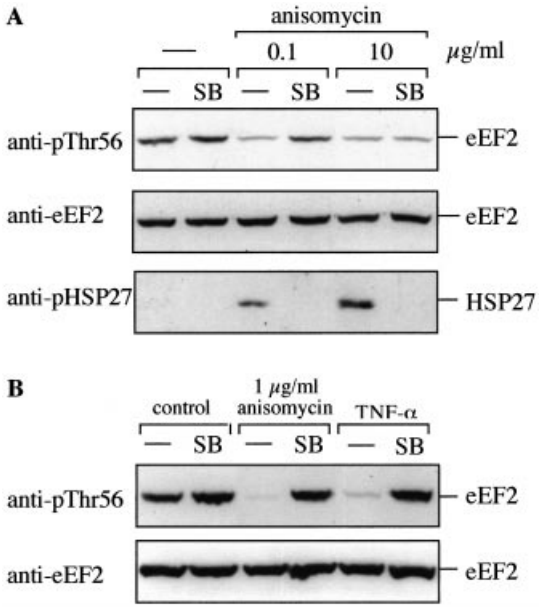

Figure 1 Effect of SB 203580 on anisomycin and TNF-α induced eEF2-dephosphorylation

(**A**) KB cells were grown to 90% confluency and kept in EBSS for 3 h. The cells were then incubated for 1 h with 10 μM SB 203580 (SB) or without this inhibitor (—) and then stimulated for 25 min with 0.1 μg/ml or 10 μg/ml anisomycin. The cells were then lysed and analysed by immunostaining for the phosphorylation of eEF2 at Thr-56 (anti-pThr56), for eEF2 protein (anti-eEF2) or phosphorylation of HSP27 at Ser-15 (anti-pHSP27). (**B**) The experiment was similar to that in (**A**), but the cells were treated with 1 μg/ml anisomycin or 50 ng/ml TNF-α. Similar results were obtained in four independent experiments.

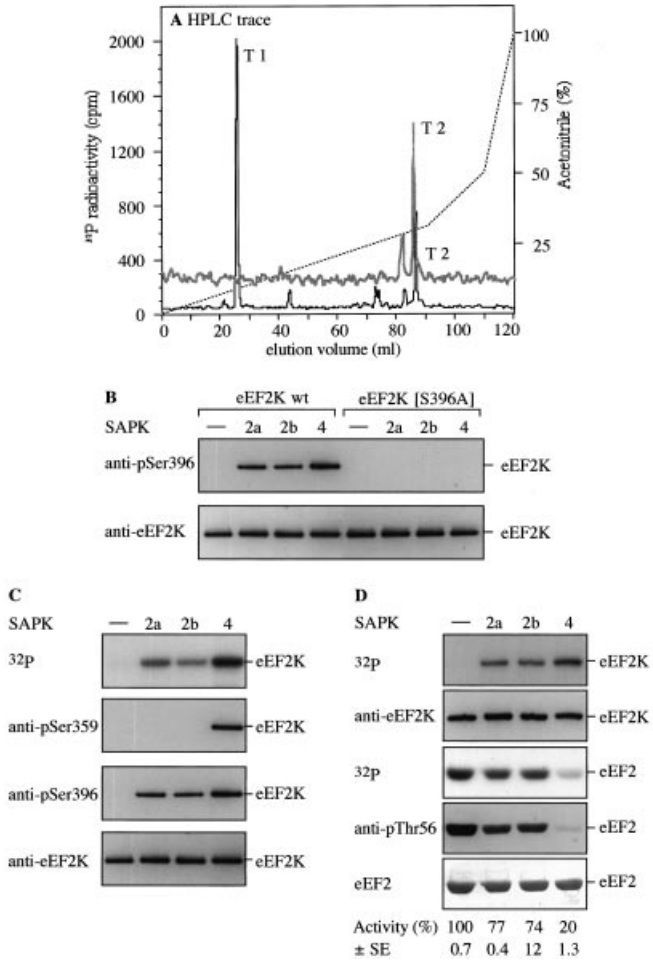

Figure 2 Phosphorylation of eEF2 kinase by SAPK2a/p38, SAPK2b/p38β2 and SAPK4/p38δ

(**A**) Human recombinant eEF2 kinase (3 μg) was phosphorylated with 10 m-units of active SAPK4/p38δ (black trace) or with 200 m-units of active SAPK2a/p38 (grey bold trace) and Mg-[γ-^{32}P]ATP (10^6 c.p.m. per nmole) in a 0.03 ml incubation. The protein was subjected to tryptic digestion and the resulting phosphopeptides separated by reverse-phase chromatography on a C_{18} column. The solid line shows the ^{32}P-radioactivity and the broken line shows the acetonitrile gradient. (**B**) Aliquots of 50 ng of wild-type eEF2 kinase (eEF2K wt) or the S396A mutant (eEF2K[S396A]) were phosphorylated in a 0.05 ml assay with SAPK2a/p38 (2a), SAPK2b/p38β2 (2b) or SAPK4/p38δ (4) and Mg-[γ-^{32}P]ATP (10^6 c.p.m. per nmole) as described in the Materials and methods section, or left unphosphorylated (—). The reaction mixtures were subjected to SDS/PAGE, followed by transfer to Immobilon membranes and immunoblotting with an eEF2 kinase-specific antibody (anti-eEF2K), or an antibody that recognizes eEF2 kinase when phosphorylated at Ser-396 (anti-pSer-396). (**C**) Conditions as in (**B**) but the reactions were also immunoblotted with a phospho-specific Ser-359 antibody (anti-pSer-359) or autoradiographed (^{32}P). (**D**) eEF2 kinase was phosphorylated as in (**B**) and aliquots were used to phosphorylate eEF2 in the presence of 2 μM Ca^{2+} and 0.12 μg/ml calmodulin in the presence of Mg-[γ-^{32}P]ATP (^{32}P). Phosphorylation of eEF2 kinase and eEF2 was detected by autoradiography (^{32}P) and then quantified by cutting out the radioactive bands and analysing by Cerenkov counting. 100% refers to the activity of unphosphorylated eEF2 kinase in this assay. The results are presented $\pm$S.E.M. for three determinations. The gels were also immunoblotted with an antibody that recognizes eEF2 phosphorylated at Thr-56 (anti-pThr56) and equal loading of eEF2 kinase or eEF2 was established by immunoblotting (anti-eEF2K) or by staining with Coomassie Blue (eEF2) respectively. Similar results were obtained in four independent experiments.

of eEF2 is mediated by inhibition of eEF2 kinase via an SB 203580-insensitive pathway catalysed by SAPK4/p38δ [9]. However, surprisingly we found that in serum and amino acid starved cells, the dephosphorylation of eEF2 at Thr-56 was prevented by SB 203580 if the anisomycin concentration was reduced to 0.1–1.0 μg/ml (Figures 1A and 1B). Similar results were seen if anisomycin was replaced by the proinflammatory cytokine TNF-α (Figure 1B). In contrast, SB 203580 prevented the phosphorylation of HSP27, a substrate of MAPKAP-K2 that is activated by SAPK2a/p38 [16–19], at high or low anisomycin concentrations (Figure 1A). The simplest interpretation of this result is that eEF2 phosphorylation is regulated by both an SB 203580-insensitive and an SB 203580-sensitive pathway at high anisomycin concentrations, the former being sufficient to induce the dephosphorylation of eEF2 at Thr-56, even when the SB 203580-sensitive pathway is blocked. On the other hand, only the SB 203580-sensitive pathway is activated at low concentrations of anisomycin.

The finding that SB 203580 effectively blocked the dephosphorylation of eEF2 induced by low anisomycin concentrations or TNF-α was surprising, because we have previously reported that SAPK2a/p38 phosphorylates eEF2 kinase 50–100 $\times$ slower than SAPK4/p38δ *in vitro* [9]. However, SAPK2a/p38 seems to be expressed at much higher levels in KB cells than SAPK4/p38δ, which might compensate for the much lower efficiency with which it phosphorylates eEF2 kinase. Alternatively, phosphorylation might have been catalysed by SAPK2b/p38β2, an SB 203580-sensitive protein kinase closely related to SAPK2a/p38, a hypothesis which was not investigated previously. Another possibility is that eEF2 kinase is inactivated by a protein kinase that lies 'downstream' of SAPK2a/p38, such as MAPKAP-K2 [18], MAPKAP-K3 [20,21] or MAPKAP-K5 [22,23]. We

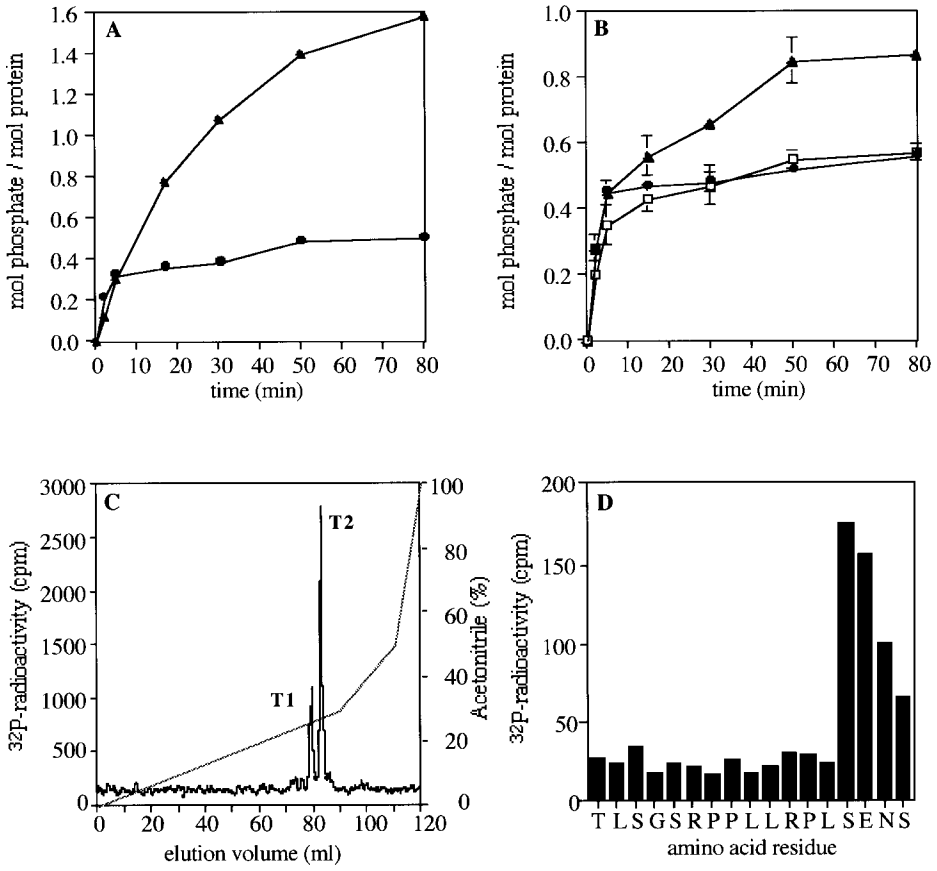

Figure 3 Phosphorylation of eEF2 kinase by MAPKAP kinases

(**A**) eEF2 kinase (circles) or HSP27 (triangles), both at 1 μM, were phosphorylated with 2 units/ml MAPKAP-K2, as described in the Materials and methods section. At the times indicated, aliquots were removed and precipitated with 25% (w/v) trichloroacetic acid and the precipitated protein was washed three times with 1 ml of 10% (w/v) trichloroacetic acid and then analysed by Cerenkov counting. (**B**), as in (**A**) except that 1 μM eEF2 kinase was phosphorylated with 2 units/ml MAPKAP-K2 (closed circles), MAPKAP-K3 (open squares) or MAPKAP-K5 (closed triangles). (**C**) eEF2 kinase was maximally phosphorylated with MAPKAP-K2 as in (**A**), digested with trypsin, and the resulting phosphopeptides separated by reverse-phase chromatography as in Figure 2(A). (**D**) The primary structure of peptide T2 from (**C**) was established by Edman sequencing and Ser-377 was identified as the site of phosphorylation in a separate solid-phase sequencing experiment. The methodology is detailed elsewhere [16]. Similar results were obtained in three independent experiments.

therefore examined the phosphorylation of eEF2 kinase by these enzymes in much greater detail.

Identification of Ser-396 as the site on eEF2 kinase phosphorylated *in vitro* by SAPK2a/p38 and SAPK2b/p38β2

We phosphorylated eEF2 kinase using very high concentrations of SAPK2a/p38 to achieve near stoichiometric phosphorylation and, after digestion with trypsin, separated the resulting phosphopeptides by chromatography on a C_{18} column. The profiles of the resulting ^{32}P-labelled peptides were then compared with those obtained after stoichiometric phosphorylation by much lower concentrations of SAPK4/p38δ (Figure 2A). As reported previously, SAPK4/p38δ phosphorylated two peptides (T1 and T2). T1 corresponds to residues 357–361, phosphorylated at Ser-359, while T2 corresponds to residues 364–406, phosphorylated at Ser-396 [9]. In contrast, SAPK2a/p38 only phosphorylated the peptide corresponding to T2, even after a long period of incubation (Figure 2A). We established that SAPK2a/p38 phosphorylates T2 at Ser-396, using the same methodology described previously [9].

In order to study the phosphorylation of Ser-396 in cells, we developed an antibody that only recognizes eEF2 kinase, when phosphorylated at Ser-396. This antibody did not recognize an eEF2 kinase mutant in which Ser-396 had been changed to Ala (eEF2K[S396A], Figure 2B). Experiments with this antibody confirmed that SAPK2a/p38, like SAPK4/p38δ, phosphorylates eEF2 kinase at Ser-396 *in vitro* (Figure 2B). Additionally, we established that SAPK2b/p38β2 also phosphorylates eEF2 kinase exclusively at Ser-396 *in vitro* (Figure 2B). Importantly, SAPK2b/p38β, like SAPK2a/p38, did not phosphorylate eEF2 kinase at Ser-359 (Figure 2C). We have also shown that JNK1α1, JNK2α2 and SAPK3/p38γ phosphorylate eEF2 kinase very poorly at Ser-396, but do not phosphorylate Ser-359 at all (results not shown).

Effect of phosphorylation of Ser-396 and Ser-377 on the activity of eEF2 kinase *in vitro*

eEF2 kinase was maximally phosphorylated with either SAPK2a/p38, SAPK2b/p38β2 or SAPK4/p38δ and then assayed for its ability to phosphorylate purified rabbit eEF2 in the presence of Ca/CaM. As reported previously [9], phosphorylation by SAPK4/p38δ inhibited eEF2 kinase by 80%, while phosphorylation by SAPK2a/p38 or SAPK2b/p38β only decreased eEF2 kinase activity slightly (Figure 2D). The extent of inhibition was quantified by excising the ^{32}P-labelled eEF2 band from the gel and analysing by Cerenkov counting. These

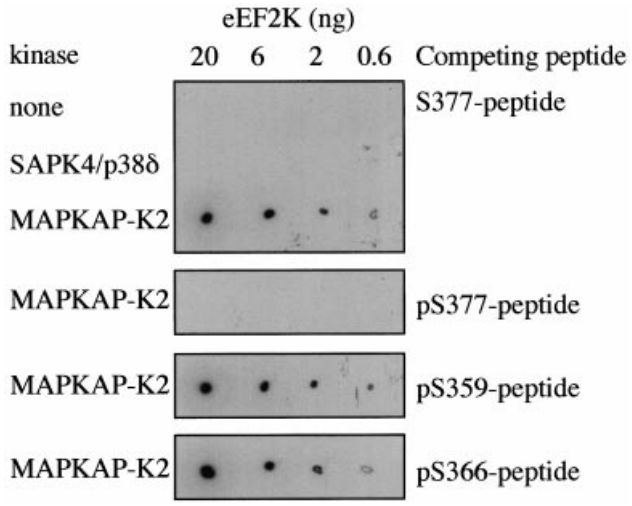

Figure 4 Generation of an antibody that recognizes eEF2 kinase only when phosphorylated at Ser-377

eEF2 kinase was left unphosphorylated or phosphorylated with SAPK4/p38δ or MAPKAP-K2 and the amounts indicated were spotted on to an Immobilon P membrane, which was then immunostained with antibodies raised against the peptide LLRPLpSENSG, corresponding to residues 372–381 of eEF2 kinase phosphorylated at Ser-377 (where pS is phosphoserine). Top panel, immunostaining in the presence of the unphosphorylated form of the peptide antigen (5 μg/ml). Second panel, immunostaining in the presence of the same concentration of the phosphopeptide antigen. Third panel, immunostaining in the presence of the peptide TEEKCGpSPRVRTL, corresponding to residues 353–365, phosphorylated at position Ser-359. Bottom panel, immunostaining in the presence of the peptide SPRVRTLpSGSRPPLLP, corresponding to residues 359–374, phosphorylated at Ser-366.

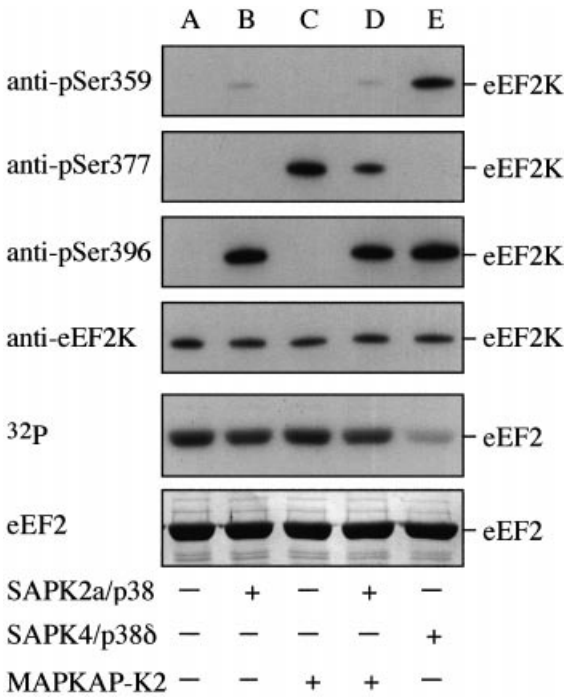

Figure 5 Effect of phosphorylation of eEF2 kinase on its Ca/CaM-dependent activity towards eEF2 *in vitro*

eEF2 kinase (3 μg/ml) was incubated for 40 min at 30 °C in the absence (A) or presence of SAPK2a/p38 (B), MAPKAP-K2 (C), SAPK2a/p38 plus MAPKAP-K2 (D) or SAPK4/p38δ (E) in 0.05 ml reactions, as described in the Materials and methods section. Aliquots (2 μl) were then used to phosphorylate 0.06 mg/ml eEF2 for 5 min in the presence of 0.12 μg/ml calmodulin and 2 μM $CaCl_2$ in 0.05 ml reactions. Another 48 μl aliquot was denatured in SDS, subjected to SDS-PAGE, electrotransferred to Immobilon P membranes, and analysed for the phosphorylation of eEF2 kinase at Ser-359, Ser-377 or Ser-396 by immunoblotting with appropriate phospho-specific antibodies. eEF2 phosphorylation was analysed by autoradiography (^{32}P). Equal loading was established by immunoblotting with anti-eEF2 kinase or by staining the Immobilon P membrane with Coomassie Blue to reveal the eEF2 protein.

experiments established that inhibition of eEF2 kinase resulting from phosphorylation of Ser-396 by SAPK2a/p38 was approx. 25%.

Identification of Ser-377 as the site on eEF2 kinase phosphorylated *in vitro* by MAPKAP-K2, MAPKAP-K3 and MAPKAP-K5; lack of effect on activity *in vitro*

MAPKAP-K2 phosphorylated eEF2 kinase at an initial rate similar to HSP27, a *bona fide* substrate of this enzyme. The phosphorylation reached a plateau at approx. 0.5 mol phosphate per mol protein, three times lower than HSP27, which is phosphorylated at three sites by MAPKAP-K2 [17,21] (Figure 3A). MAPKAP-K3 and MAPKAP-K5 phosphorylated eEF2 kinase to a similar extent (Figure 3B).

eEF2 kinase that had been phosphorylated by MAPKAP-K2 was digested with trypsin, and the resulting phosphopeptides separated by chromatography on a C_{18} column (Figure 3C). Two ^{32}P-labelled peptides were obtained, termed T1 and T2. These peptides had molecular masses of 4623.6 and 4607.6 respectively, corresponding to the predicted masses of oxidized and non-oxidized forms of the peptide, comprising residues 364–406 plus one phosphate group (with Met-385 converted to the sulphoxide in the oxidized derivative). This was confirmed by Edman and solid-phase sequencing, which identified Ser-377 as the site of phosphorylation (Figure 3D). The analysis was repeated using eEF2 kinase phosphorylated by MAPKAP-K3 or MAPKAP-K5 with identical results (results not shown). Thus MAPKAP-K2, MAPKAP-K3 and MAPKAP-K5 phosphorylate the same site on eEF2 kinase, namely Ser-377.

In order to confirm these results, we developed an antibody that recognizes only eEF2 kinase which is phosphorylated at Ser-377. This antibody recognized eEF2 kinase after phosphorylation by MAPKAP-K2, but did not recognize unphosphorylated eEF2 kinase or eEF2 kinase phosphorylated by SAPK4/p38δ (Figure 4, top panel). Recognition was prevented by incubation of the antibody with the phosphopeptide antigen but not by the unphosphorylated form of the peptide, or by the phosphopeptide used to generate the Ser-359 or the Ser-366 phospho-specific antibodies (Figure 4). Experiments with this antibody confirmed that SAPK2a/p38, and SAPK4/p38δ did not phosphorylate Ser-377 and that MAPKAP-K2 did not phosphorylate Ser-359 or Ser-396 *in vitro* (Figure 5).

Maximal phosphorylation of eEF2 kinase by MAPKAP-K2 (Figure 5) or MAPKAP-K5 (results not shown) had no effect on its activity. Moreover, the combined phosphorylation of eEF2 kinase by SAPK2a/p38 and MAPKAP-K2 did not decrease activity any more than SAPK2a/p38 alone (Figure 5). Phosphorylation by either SAPK2a/p38 or MAPKAP-K2 did not generate any eEF2 kinase activity measured in the absence of Ca/CaM (results not shown).

Ser-377 and Ser-396 are phosphorylated in KB cells

eEF2 kinase is present at very low concentrations in KB cells and phosphorylation could only be measured after immunoprecipitating the protein kinase from 2 mg of cell extract. In order to

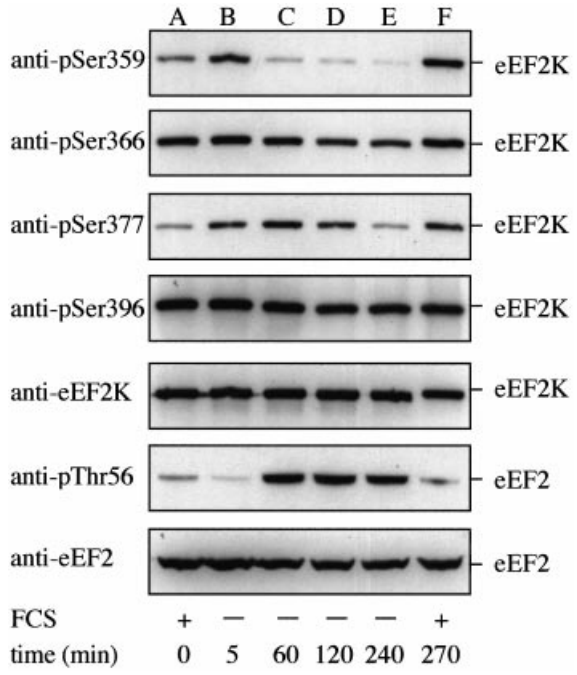

Figure 6 Effect of serum and amino acids on the phosphorylation of eEF2 kinase and eEF2

KB cells were grown to 90% confluency and then lysed (lane A) or incubated further in EBSS for the indicated periods of time (lanes B–F). A dish of cells that had been incubated for 240 min in EBSS was then stimulated for a further 30 min with 10% FCS in DMEM (lane F). eEF2 kinase was immunoprecipiated from the cell lysates and phosphorylation of the residues indicated were analysed by immunoblotting. A further aliquot of the extract was used to analyse the phosphorylation of eEF2 at Thr-56.

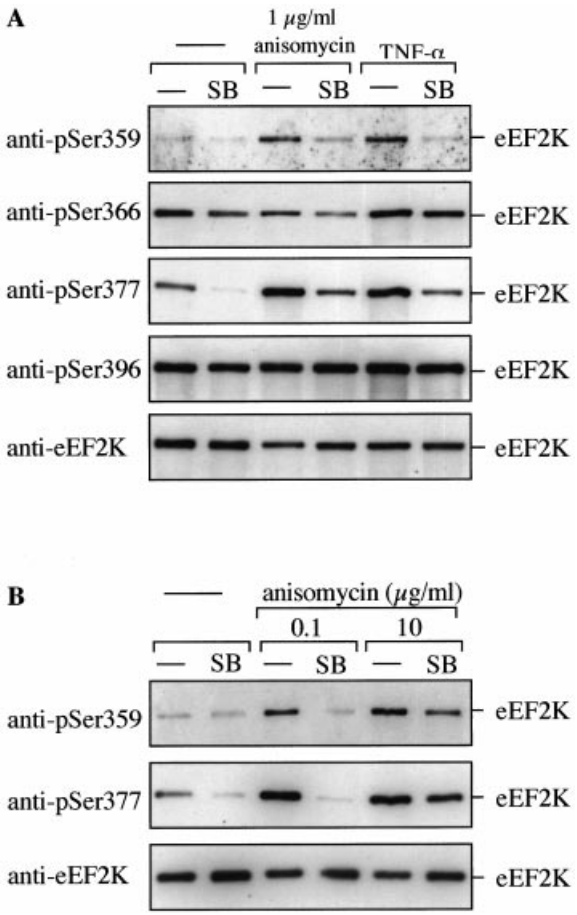

Figure 7 Effect of SB 203580 on anisomycin and TNF-α-induced site-specific phosphorylation of eEF2 kinase

(**A**) The extracts as in Figure 1(B) were used to analyse the phosphorylation of eEF2 kinase at Ser-359, Ser-366, Ser-377 and Ser-396. (**B**) The extracts as in Figure 1(A) were used to analyse the phosphorylation of eEF2 kinase at Ser-359 and Ser-377.

determine whether Ser-377 and Ser-396 are phosphorylated in cells, we immunoprecipitated eEF2 kinase from KB cells that were either growing in serum and amino acids, or were deprived of serum and amino acids for different periods of time. All the residues we looked at, namely Ser-359, Ser-366, Ser-377 and Ser-396, were found to be phosphorylated, but were regulated differentially. In cells growing in serum and amino acids, Ser-359 was partially phosphorylated (Figure 6, lane A). When the culture medium was exchanged to remove serum and amino acids, Ser-359 became transiently phosphorylated within 5 min (Figure 6, lane B) and then dephosphorylated after 60 min (Figure 6, lane C) or longer (Figure 6, lanes D and E). Ser-366 was also found to be phosphorylated in cells growing in serum and amino acids but, in contrast to the phosphorylation of Ser-359, the phosphorylation of Ser-366 did not increase 5 min after the withdrawal of serum and amino acids, and declined only slightly thereafter (Figure 6). In contrast, Ser-377 was only weakly phosphorylated in growing cells but became phosphorylated following the withdrawal of serum and amino acids, reaching a maximum phosphorylation after 1 h. Phosphorylation then gradually declined to basal levels after 4 h. Ser-396 was maximally phosphorylated under all of the conditions tested, and was unaffected by the presence or absence of serum and amino acids.

There was a good inverse correlation between the phosphorylation of eEF2 kinase at Ser-359 and the eEF2 phosphorylation at Thr-56 (Figure 6). The phosphorylation of the other residues on eEF2 kinase did not correlate well with eEF2 dephosphorylation.

SB 203580 inhibits the phosphorylation of Ser-359 and Ser-377 by TNF-α and low concentrations of anisomycin in KB cells, but not the phosphorylation of Ser-366 or Ser-396

We used phospho-specific antibodies to examine the effect of TNF-α and low concentrations of anisomycin on the phosphorylation of Ser-359, Ser-366, Ser-377 and Ser-396 (Figure 7). In these experiments, serum and amino acids were withdrawn from the culture medium for 4 h (as in Figure 6, lane E) and then stimulated with anisomycin or TNF-α. Both agonists stimulated the phosphorylation of Ser-359 and Ser-377 and the phosphorylation of both sites was largely suppressed by SB 203580. The phosphorylation of Ser-396 was high in unstimulated cells and was not increased by stimulation with either anisomycin or TNF-α, nor did SB 203580 suppress the phosphorylation of this site. The phosphorylation of Ser-366 was also not increased by stimulation with anisomycin or TNF-α but was slightly decreased by pre-treatment with SB 203580 (Figure 7A).

SB 203580 does not inhibit the phosphorylation of Ser-359 or Ser-377 by high concentrations of anisomycin

We have reported previously that SB 203580 does not suppress the phosphorylation of Ser-359 induced by high (10 μg/ml)

concentrations of anisomycin [9] and similar results were obtained in the present study. Interestingly, SB 203580 also had little effect on the phosphorylation of Ser-377 induced by this concentration of anisomycin, whereas the phosphorylation of Ser-377 observed in the absence of anisomycin, or induced by low concentrations of anisomycin, was largely suppressed by SB 203580 (Figure 7B).

DISCUSSION

We have reported previously [9] that SAPK4/p38δ phosphorylates eEF2 kinase at Ser-359 and Ser-396 inhibiting its activity by 80% *in vitro*. We also showed that high concentrations of anisomycin induce the phosphorylation of eEF2 kinase at Ser-359 in human KB cells via an SB 203580-insensitive pathway, and that phosphorylation is prevented by the overexpression of a catalytically inactive mutant of SAPK4/p38δ. These findings are consistent with phosphorylation of Ser-359 being mediated by SAPK4/p38δ under these conditions [9]. In this paper we confirmed these results, but also made the unexpected observation that the phosphorylation of eEF2 kinase at Ser-359 induced by either low concentrations of anisomycin or TNFα is prevented by SB 203580 (Figure 7), an inhibitor of SAPK2a/p38 and SAPK2b/p38β2 that does not inhibit SAPK4/p38δ [11,12]. However, SAPK2a/p38 and SAPK2b/p38β2 phosphorylate eEF2 kinase very slowly [9] and phosphorylation occurs exclusively at Ser-396 (Figure 2). The SB 203580-sensitive pathway that leads to the phosphorylation of eEF2 kinase at Ser-359 is therefore unclear. It could be mediated by another SB 203580-sensitive protein kinase that has not yet been identified, or by the SAPK2a/p38 and/or SAPK2b/p38β2 catalysed inhibition of a Ser-359 phosphatase. It is also conceivable that another post-translational modification, or the interaction of eEF2 kinase with another protein, might induce a conformational change that allows SAPK2a/p38 and/or SAPK2b/p38β2 to phosphorylate Ser-359 in cells. We have excluded two other possibilities, namely that SAPK2a/p38 phosphorylates and activates SAPK4/p38δ directly and that phosphorylation of Ser-377 by MAPKAP-K2 accelerates the rate at which Ser-359 is phosphorylated by SAPK4/p38δ *in vitro* (A. Knebel, unpublished work). However, another possibility that cannot be excluded is that SAPK2a/p38 and/or SAPK2b/p38β2 enhances the MKK3/MKK6-catalysed activation of SAPK4/p38δ by an unknown mechanism.

In the present study, we demonstrated that eEF2 kinase is phosphorylated at Ser-396 in KB cells. Like Ser-359, Ser-396 is followed by proline and can be phosphorylated *in vitro* by SAPK2a/p38, SAPK2b/p38β2 and SAPK4/p38δ, causing a modest 25% decrease in activity (Figure 2D). Ser-396 is also phosphorylated in KB cells but, in contrast to the phosphorylation of Ser-359, the level of phosphorylation of Ser-396 does not change in response to anisomycin or TNF-α, or in response to the removal or addition of serum and amino acids from the medium. The phosphorylation of Ser-396 in cells may therefore be catalysed by a constitutively active SB 203580-insensitive proline-directed protein kinase that has yet to be identified. Alternatively, Ser-396 may be resistant to dephosphorylation, and become maximally phosphorylated as a consequence of phosphorylation by one or more SAPK isoforms.

Like the phosphorylation of Ser-359, the phosphorylation of Ser-377 is induced by anisomycin or TNFα. (Figure 7). Moreover, as for Ser-359, the phosphorylation of Ser-377 induced by high concentrations of anisomycin is insensitive to SB 203580, while phosphorylation triggered by TNFα or low concentrations of anisomycin is largely suppressed by SB 203580. Ser-377 is not followed by a proline residue and is not phosphorylated by any proline-directed protein kinase that we have studied. It does, however, lie in a consensus sequence for phosphorylation by MAPKAP-K2 (Hyd-Xaa-Arg-Xaa-Xaa-Ser-, where Hyd represents a bulky hydrophobic residue and Xaa represents any amino acid) [24], a protein kinase that is activated by SAPK2a/p38 in cells. We found that MAPKAP-K2, the closely related MAPKAP-K3, and MAPKAP-K5 all phosphorylate Ser-377 specifically *in vitro*, and MAPKAP-K2 phosphorylated eEF2 kinase at a similar rate to HSP27 (Figure 3), one of its physiological substrates [19]. We therefore conclude that the SB 203580-sensitive phosphorylation of eEF2 kinase at Ser- 377 is catalysed by one or more protein kinases that are activated by SAPK2a/p38, namely MAPKAP-K2, MAPKAP-K3 and/or MAPKAP-K5. However, the identity of the SB 203580-insensitive Ser-377 kinase activated at high concentrations of anisomycin is unknown. In addition, the role of Ser-377 phos-

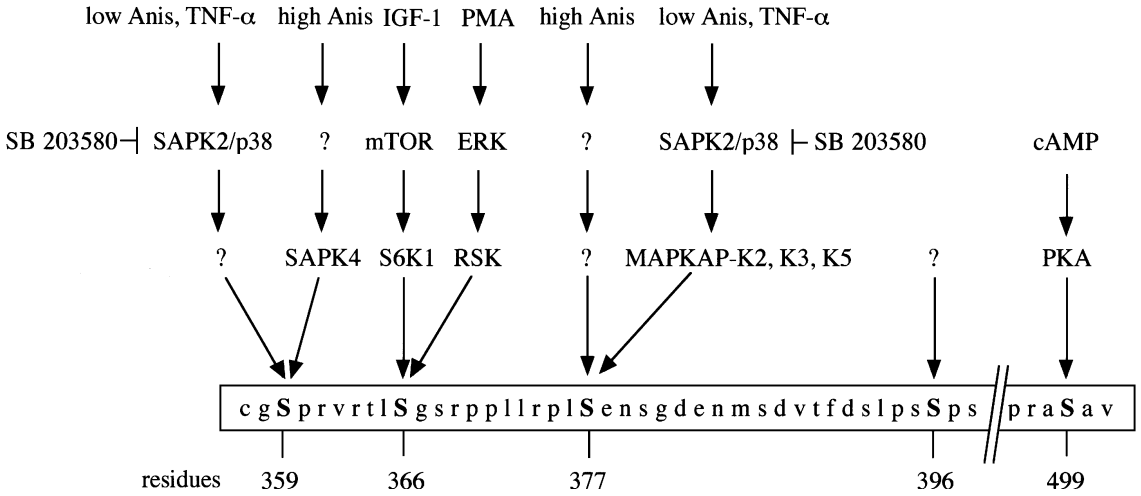

Figure 8 The signalling pathways acting on the five phosphorylation sites of eEF2 kinase

The sequences surrounding the phosphorylation sites on human eEF2 kinase are shown using the single letter code for amino acids, with the phosphorylated serines in bold capital letters. Abbreviations: Anis, anisomycin; mTOR, mammalian target of rapamycin; ERK, extracellular signal regulated kinase; PKA, cAMP dependent protein kinase; PMA, phorbol 12-myristate 13-acetate.

phorylation is unclear, because its phosphorylation did not affect eEF2 kinase activity *in vitro* whether measured in the presence (Figure 5) or absence (results not shown) of Ca/CaM. The combined phosphorylation of Ser-396 and Ser-377 did not cause any inhibition further than that observed after phosphorylation of Ser-396 alone (Figure 5).

Anisomycin or TNFα induced the dephosphorylation of eEF2 at Thr-56. Moreover, the dephosphorylation of eEF2 mirrored the phosphorylation of eEF2 kinase at Ser-359 and Ser-377, being insensitive to SB 203580 at high concentrations of anisomycin and sensitive to SB 203580 at low concentrations of anisomycin or after stimulation with TNF-α (Figure 1). However, the phosphorylation of Ser-377 did not alter the activity of eEF2 kinase *in vitro* under the conditions studied, whereas the phosphorylation of Ser-359 decreased activity by 80%. We therefore conclude that it is the phosphorylation of eEF2 kinase at Ser-359 that is likely to underlie the dephosphorylation and activation of eEF2 by anisomycin or TNFα. The phosphorylation of Ser-366 and Ser-396 did not change significantly in response to anisomycin or TNFα and was only inhibited slightly (Ser-366) or unaffected (Ser-396) by SB 203580.

In summary, it is clear that, in addition to its allosteric activation by Ca/CaM, eEF2 kinase is regulated by multisite phosphorylation. Although much work remains to be done, it is evident that eEF2 kinase is phosphorylated on at least five different serine residues, probably mediated by seven or more protein kinases (Figure 8). Moreover, the presence of additional phosphorylation sites cannot be excluded. Protein synthesis is presumably of such fundamental importance to cell viability and function that the rate at which nascent polypeptides are elongated has to respond to a great many extracellular signals, as well as to the availability of nutrients and other factors.

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