Activation of the MAP kinase homologue RK requires the phosphorylation of Thr-180 and Tyr-182 and both residues are phosphorylated in chemically stressed KB cells

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Abstract A MAP kinase homologue, termed the reactivating kinase (RK), lies in a signalling pathway which mediates cellular responses to stress. Here we demonstrate that the stress-induced activation of the RK in human KB cells is accompanied by the phosphorylation of Thr-180 and Tyr-182, and that the phosphorylation of both residues is required for the activation of this enzyme.

Key words: MAP kinase; Stress; Arsenite; Protein kinase; Phosphotyrosine

1. Introduction

The p42 and p44 isoforms of mitogen-activated protein (MAP) kinase (p42/p44 MAPK) are central components of a signalling pathway which is activated by mitogens and growth factors and whose sustained activation is not only required, but sufficient to trigger the proliferation or differentiation of many cells (reviewed in [1]). Recently a homologue of p42/p44 MAPK, termed the reactivating kinase (RK), was identified in mammalian cells which is activated in response to cellular stresses [2], bacterial endotoxin (LPS) [3] and the cytokine interleukin-1 (IL-1) [4]. One of the downstream targets of the RK is MAP kinase-activated protein (MAPKAP) kinase-2 [2,5,6] which phosphorylates heat-shock protein HSP27 [5,6]. There is evidence that HSP27 phosphorylation promotes the polymerisation of actin, and so counteracts the disruptive effects of stress on actin microfilament structure, thereby aiding cell survival [7].

The RK is inhibited specifically by a novel class of pyridinyl imidazoles, termed CSAIDs, which inhibit the activation of MAPKAP kinase-2 and the phosphorylation of HSP27 in vivo in response to cellular stresses, LPS and IL-1 [6]. CSAIDs were originally developed as inhibitors of the LPS-induced synthesis of IL-1 and tumour necrosis factor (TNF) in monocytes [8], implicating proteins 'downstream' of the RK in the synthesis of these cytokines. For these reasons CSAIDs may be efficaceous in the treatment of inflammatory diseases, such as rheumatoid arthritis.

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The p42/p44 MAPKs are activated by phosphorylation of a threonine and a tyrosine residue located in a TEY sequence (Thr-183 and Tyr-185 of p42 MAPK) just N-terminal to the APE motif found between subdomains VII and VIII of nearly all protein kinases [9]. The activation of p42/p44 MAPKs is catalysed by the 'dual specificity' enzyme MAP kinase kinase (MAPKK) which phosphorylates both residues [10], while inactivation is catalysed by protein phosphatase 2A (PP2A) which dephosphorylates Thr-183 specifically [9,11], by protein tyrosine phosphatases which dephosphorylate Tyr-185 [9,11], and by CL100, a 'dual specificity' enzyme which dephosphorylates both residues [12,13].

The RK is most similar to HOG1, an *S. cerevisiae* MAP kinase homologue that lies in a signalling pathway which restores the osmotic gradient across the yeast cell membrane in response to high external osmolarity [14]. The RK and HOG1 both contain the sequence TGY (residues 180–182 in mammalian RK) located in the equivalent position to the TEY sequence of p42 and p44 MAPKs [2,3]. The RK, like p42/44 MAPKs is inactivated in vitro by PP2A, protein tyrosine phosphatase (PTP)-1B or CL100 [2]. However, whether Thr-180 and Tyr-182 are the key phosphorylation sites in vivo, or whether activation results from the phosphorylation of alternative/additional residues is unknown. In this paper we identify the sites on the RK whose phosphorylation is induced by cellular stress in human KB cells and elucidate their role in the activation and inactivation of this enzyme.

2. Materials and methods

2.1. Materials

Radiochemicals were purchased from Amersham International (Amersham, UK), protein A-Sepharose and reduced Triton X-100 from Sigma (Poole, UK), glutathione-Sepharose and protein G-Sepharose from Pharmacia (Milton Keynes, UK) and sequence grade modified trypsin from Promega (Southampton, UK). Antibodies against the C-terminal 14 residues of XMpk2, a *Xenopus* RK homologue, which immunoprecipitates RK from rat PC12 cells (hereafter termed anti-RK antibodies) were raised in rabbits [2]. The catalytic subunit of PP2A was purified from bovine heart as for rabbit muscle PP2A [15] and provided by Dr. R.W. MacKintosh at Dundee. Bacterially expressed PTP-1B and GST-MAPKAP kinase-2 were gifts from Dr. D. Barford (University of Oxford, UK) and Dr. R. Ben-Levi and Professor C.J. Marshall (Institute of Cancer Research, London), respectively.

2.2. Preparation of a constitutively activated mutant of Xenopus MEK2

XMEK2 cDNA, a generous gift from Dr. B. Errede (University of North Carolina, USA) was amplified by PCR with oligonucleotides designed to create an *Nco*I site at the first ATG codon and an *Xho*I site in front of the TGA stop codon. The PCR product was digested with *Nco*I and *Xho*I, and ligated into the *Nco*I *Xho*I site of the vector

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pET21-GST [2]. The GST-XMEK2(E286, E290) mutant was prepared essentially as in [16], using as a template the plasmid pET21-GST-XMEK2 and two overlapping oligonucleotides designed to change both codons 286 (TCC) and 290 (ACC) to GAG. The expression plasmids were transformed into *E. coli* BL21(DE3) and after induction with 0.5 mM IPTG for 5 h at 20°C, the GST-XMEK2 proteins were purified by affinity chromatography on glutathione-Sepharose.

2.3. Expression of MalE fusion proteins of Xenopus *Mpk1 and Mpk2*

The XMpk2 cDNA [2] was subcloned into the *Xmn*I–*Bam*HI site of the vector pMAL-c2 (New England Biolabs, MA, USA) and the expression plasmid for MalE-XMpk1 was prepared as described [2]. The sequences of the MalE fusion proteins after the Factor Xa cleavage site (IEGR) are ISEFGS followed by amino acids 4–361 of XMpk1 and ST followed by amino acids 4–361 of XMpk2. The plasmids were transformed into *E. coli* TG-1 and after induction with 0.2 mM IPTG for 6 h at 20°C, the MalE-XMpk1 and MalE-XMpk2 proteins were purified by affinity chromatography on amylose beads as recommended by the supplier.

2.4. Phosphorylation and dephosphorylation of the RK in vitro

4 μg GST-XMEK2-(E286, E290) and 20 μg MalE-XMpk2 were incubated for up to 16 h at 30°C in 0.1 ml of 50 mM Tris-HCl, pH 7.5, 10 mM magnesium-acetate, 1 mM DTT, 0.1 μM okadaic acid, 0.2 mM sodium orthovanadate (buffer A) and 0.1 mM ATP (60 μCi [γ-^{32}P]ATP) and the reaction mixture was passed through a 5 ml column of Sephadex G-50 Superfine equilibrated in 50 mM Tris-HCl, pH 7.0, 0.1 mM EDTA, 0.03% (w/v) Brij 35 and 0.1% 2-mercaptoethanol to remove ATP. Dephosphorylation was then carried out for 30 min at 30°C with 2.5 μg/ml PP2A or 50 μg/ml PTP-1B. In measuring the phosphorylation stoichiometry, the molecular mass of MalE-XMpk2 was taken as 84 kDa and protein was estimated by the method of Bradford.

2.5. Labelling of KB cells in vivo

KB cells were cultured in 6 cm dishes in a 95% air/5% CO_2 atmosphere as described [6]. When the cells had reached confluence, each dish was incubated for 5–7 h in 3 ml of phosphate-free DMEM medium (ICN, Oxon, UK) supplemented with 10% foetal calf serum (dialysed against 20 mM HEPES, pH 7.5, to remove phosphate) in the presence of 1–2.5 mCi/ml carrier-free [^{32}P]orthophosphate. The cells were stimulated for 15 min with 0.5 mM sodium arsenite, lysed in 0.4 ml of 20 mM Tris-acetate, pH 7.0, 0.27 M sucrose, 1 mM EDTA, 1 mM EGTA, 1% (w/v) Triton X-100, 10 mM sodium β-glycerophosphate, 50 mM sodium fluoride, 5 mM sodium pyrophosphate, 1 mM sodium orthovanadate, 0.1% (v/v) 2-mercaptoethanol, 0.2 mM phenylmethylsulphonyl fluoride (termed lysis buffer), centrifuged for 10 min at 13,000 × *g* (4°C) and the supernatant (termed KB cell extract) removed and used in the immunoprecipitation experiments described below.

2.6. Immunoprecipitation of the RK from KB cell lysates

An aliquot of anti-RK antiserum (25 μl) was incubated for 30 min at 4°C on a shaker with 10 μl protein A-Sepharose, and after washing the antibody-protein A-Sepharose conjugate three times with phosphate-buffered saline, KB cell extract from one dish of cells was added. After incubation for 1 h at 4°C on a shaker, the antibody-antigen-protein A-Sepharose was washed three times with lysis buffer containing 0.5 M NaCl and once with lysis buffer alone. The supernatant was removed, and the antibody–antigen complex resuspended in 10 μl 1% SDS, heated for 2 min at 100°C and subjected to electrophoresis on a 12.5% polyacrylamide gel.

2.7. Digestion of the RK with trypsin

The band corresponding to in vivo phosphorylated RK was excised from the polyacrylamide gel, and the gel piece homogenised in 5 vols. of 25 mM *N*-ethylmorpholine-HCl, pH 7.7, containing 0.1% (w/v) SDS and 0.5% (v/v) 2-mercaptoethanol. The suspension was incubated for 12 h at 37°C on a shaker, then centrifuged for 5 min at 13,000 × *g*. The supernatant (containing 60–80% of the ^{32}P radioactivity in the gel slice) was removed, 2 μl of 10 mg/ml bovine serum albumin (BSA) added and 0.2 vols. of 100% (w/v) trichloroacetic acid (TCA) added. After centrifugation for 2 min at 13,000 × *g*, the TCA pellet was washed 3 times with 20% TCA, 3 times with ice-cold acetone, dried and incubated for 1 h at 4°C with 0.1 ml of freshly prepared performic acid. After dilution to 0.5 ml with water and lyophilisation, 10 μl of 1% reduced Triton X-100, 2.5 μl of 2 M Tris-HCl, pH 8.0, 2 μl of 1 mg/ml trypsin and 85.5 μl water were added, and the suspension incubated for 12 h at 30°C. The digest was centrifuged for 5 min at 13,000 × *g* and the supernatant, containing 97% of the ^{32}P radioactivity, chromatographed on a Vydac C_{18} column (see section 3). In vitro phosphorylated RK was precipitated with TCA and digested with trypsin in an identical manner except that BSA was omitted.

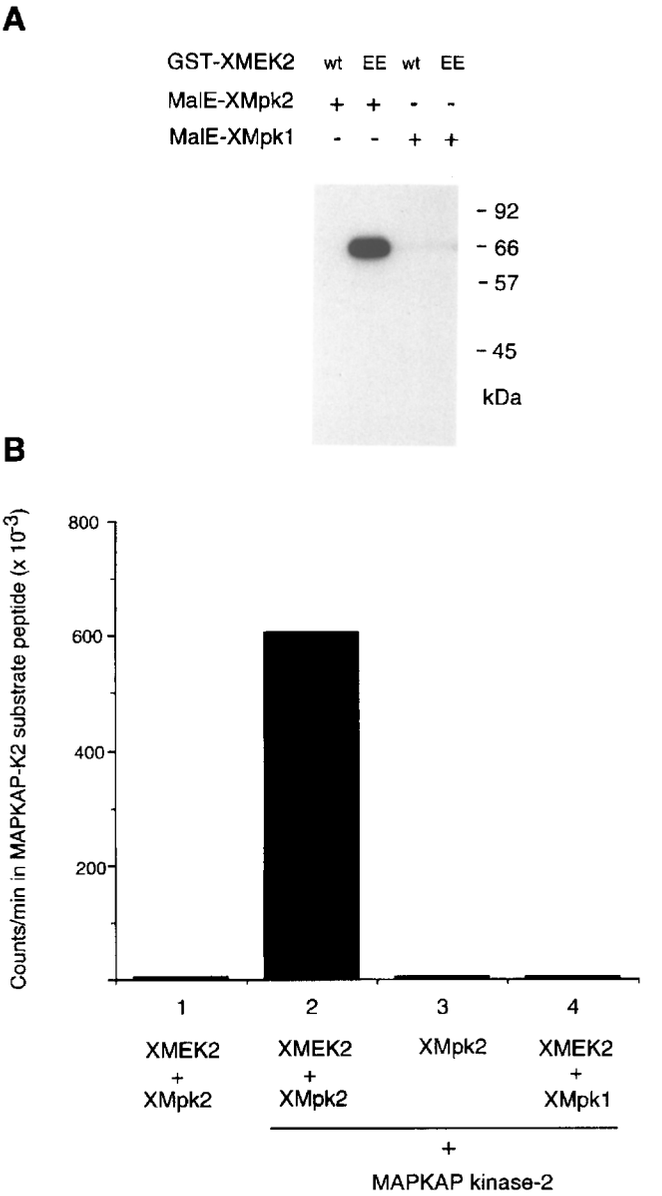

Fig. 1. XMEK2(E286, E290) phosphorylates and activates XMpk2 in vitro. (A) Bacterially produced wild-type GST-XMEK2 or GST-XMEK2(E286, E290) proteins (0.5 μg) were incubated with MalE-XMpk1 or MalE-XMpk2 (1 μg) in 12 μl of buffer A containing 10 μM ATP and 3 μCi [γ-^{32}P]ATP. After 30 min at 30°C, reaction mixtures were heated for 3 min in the presence of SDS, subjected to electrophoresis on a 12% polyacrylamide gel and autoradiographed. (B) Recombinant GST-XMEK2(E286, E290) (0.8 μg) was incubated for 20 min at 30°C with MalE-XMpk1 or MalE-XMpk2 (1.5 μg) in 12 μl of buffer A containing 0.2 mM ATP. An aliquot (2 μl) was then incubated with GST-MAPKAP kinase-2 in 12 μl of buffer containing 0.2 mM ATP. After 10 min at 22°C and 15 min at 30°C, a 5 μl aliquot was assayed for MAPKAP kinase-2 activity with the substrate peptide KKLNRTLSVA and 0.1 mM [γ-^{32}P]ATP (4 μCi) as described [22].

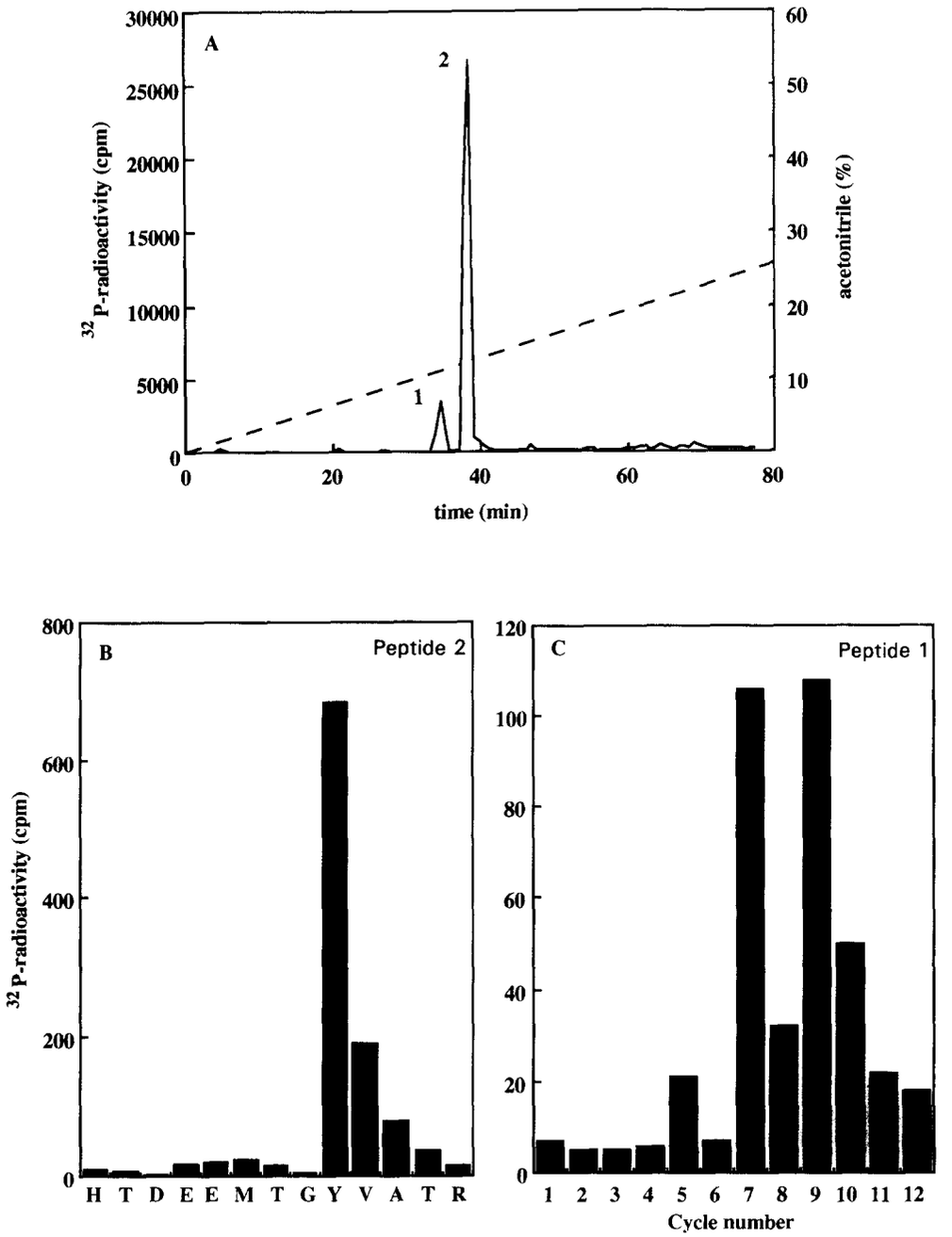

Fig. 2. Identification of tryptic phosphopeptides on XMpk2 phosphorylated by XMEK2. (A) ^{32}P-Labelled XMpk2 containing 0.23 mol phosphate per mol protein, was digested with trypsin and chromatographed on a Vydac C_{18} column equilibrated in 0.1% (v/v) trifluoroacetic acid (TFA). The column was developed with a linear acetonitrile gradient in TFA (broken line) with an increase in acetonitrile concentration of 0.33% per min and fractions of 0.5 ml were collected. The minor and major phosphopeptides referred to in the text are marked 1 and 2, respectively. (B) The major phosphopeptide from A (peptide 2) was first sequenced conventionally on an Applied Biosystems 476A Sequencer to identify amino acid residues, and then on an Applied Biosystems 470A Sequencer by solid-phase sequencing after coupling the peptide (5000 cpm) covalently to a Sequelon arylamine membrane via its carboxyl group [22]. The figure shows ^{32}P radioactivity released and amino acid residues (single letter code) identified after each cycle of Edman degradation. (C) The minor phosphopeptide-1 from A (1000 cpm) was analysed by solid-phase sequencing as in B.

3. Results

3.1. *Phosphorylation of the* Xenopus *RK homologue (XMpk2) in vitro*

A *Xenopus* cDNA has been identified that encodes a MAPKK homologue (XMEK2) more homologous to the upstream activator of *S. cerevisiae* HOG1 (PBS2) than the activator of p42/p44 MAPKs (MAPKK-1) [17]. Activation of MAPKK-1 is accompanied by the phosphorylation of Ser-217 and Ser-221, and changing these residues to Glu produces a MAPKK with a low level of constitutive activity [18]. These two serine residues are conserved in all MAPKKs (although in some cases the second Ser is substituted for Thr), suggesting that all members of this protein kinase subfamily are activated

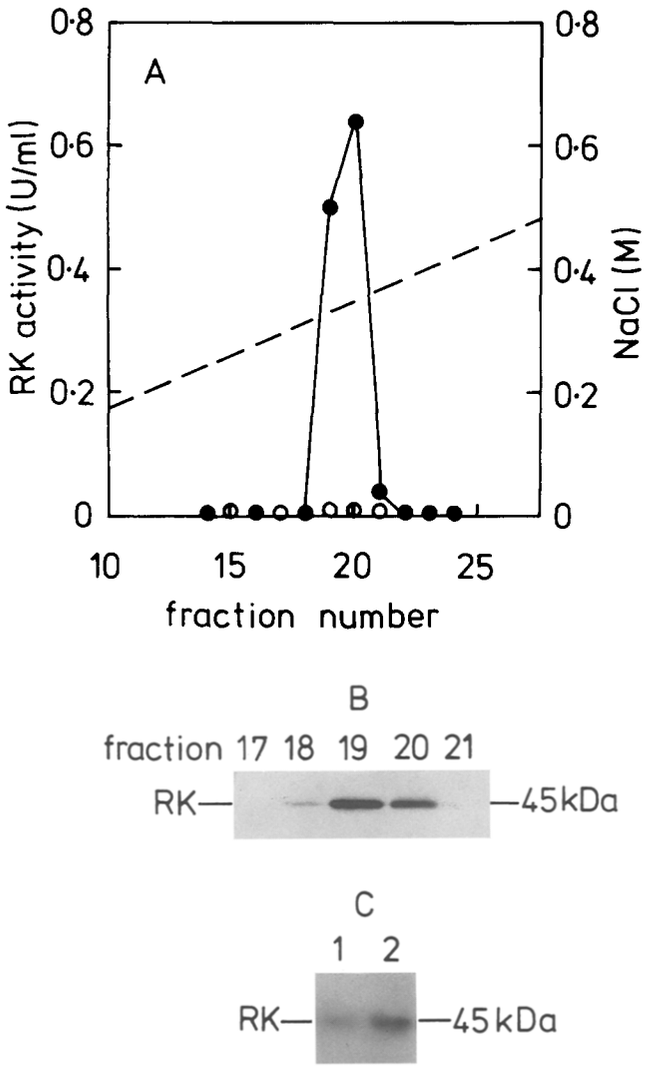

Fig. 3. Activation and phosphorylation of the RK in human KB cells. (A) Cell lysates from KB cells (0.3 mg protein) were chromatographed on Mono Q as in [2] except that a Pharmacia Smart system was used. The column was 5 × 0.16 cm, the gradient was 4 ml and the fractions (0.1 ml) were assayed for RK activity as in [2]. One unit of RK activity was that amount which increased the activity of MAPKAP kinase-2 by 1 U/min. The closed circles show results from arsenite-stimulated cells, the open circles results from unstimulated cells, and the broken line the NaCl gradient. (B) Mono Q fractions obtained by chromatography of an extract from arsenite-stimulated cells [2] were electrophoresed on 12.5% SDS-polyacrylamide gels and immunoblotted with anti-RK antibody as in [2]. The RK migrated slightly slower than ovalbumin (43 kDa) corresponding to an apparent molecular mass of 45 kDa. (C) The RK was immunoprecipitated from the lysates of ^{32}P-labelled KB cells, subjected to electrophoresis as in B and autoradiographed to reveal the 45 kDa RK protein. Lane 1, RK from unstimulated cells; lane 2, RK from cells stimulated for 15 min with 0.5 mM sodium arsenite. No other ^{32}P-labelled band was immunoprecipitated by the anti-RK antibody.

by a common mechanism. In order to produce an XMEK2 that might be capable of activating the RK, we therefore changed Ser-286 and Thr-290 in the activation domain of XMEK2 to Glu. GST-XMEK2(E286, E290), but not the wild-type enzyme, phosphorylated MalE-XMpk2 (Fig. 1A). As a consequence of phosphorylation by GST-XMEK2, MalE-XMpk2 was activated as shown by its ability to activate recombinant GST-MAPKAP kinase-2, the downstream target of XMpk2 (Fig. 1B). In contrast, recombinant MalE-XMpk1, the other known *Xenopus* MAP kinase homologue, which is more closely related to the mammalian p42/p44 MAPKs, was not activated by GST-XMEK2(E286, E290) (Fig. 1B).

After incubation with MgATP and GST-XMEK2(E286, E290) for 16 h as in section 2.4, MalE-XMpk2 was phosphorylated to 0.23 mol phosphate per mol protein and its specific activity reached 575 RK U/ml [2]. This phosphorylated MalE-XMpk2 was precipitated with TCA and subsequent tryptic digestion solubilised 97% of the ^{32}P radioactivity. Chromatography on a C_{18} column at pH 1.9 revealed two phosphopeptides eluting at 35 min and 39 min, termed phosphopeptides 1 and 2, respectively (Fig. 2A). Phosphopeptide 1 accounted for 7% and phosphopeptide 2 for 51% of the ^{32}P radioactivity applied to the column. Phosphopeptide-2 was pure, and its sequence HTDEEMTGYVATR corresponded to the tryptic peptide containing Thr-181 and Tyr-183 of XMpk2 (Fig. 2B). However, a burst of counts occurred only after the ninth cycle of Edman degradation, but not after the seventh, indicating that Tyr-183 was phosphorylated but that Thr-181 was not. This was confirmed by phosphoamino acid analysis which revealed phosphotyrosine, but no phosphothreonine (data not shown).

3.2. Dephosphorylation and inactivation of XMpk2

RK activation is induced in rat PC12 cells by incubation with sodium arsenite, a compound which mimics the effects of heat shock on gene expression and HSP27 phosphorylation [2], and the arsenite-activated RK is inactivated by incubation with either PP2A or PTP-1B [2]. In the present work, we found that the MalE-XMpk2 which had been activated by GST-XMEK2(E286, E290) could be completely inactivated by incubation with either PP2A or PTP-1B. Inactivation by PP2A, which was prevented by the specific inhibitor okadaic acid, was accompanied by only 11% dephosphorylation of MalE-XMpk2 and resulted in the disappearance of phosphopeptide-1, with no effect on phosphopeptide 2 (data not shown). Phosphopeptide-1 contained phosphothreonine and phosphotyrosine (data not shown), and when subjected to sequence analysis a burst of ^{32}P radioactivity occurred after the seventh and ninth cycles of Edman degradation (Fig. 2C), demonstrating that it corresponds to the doubly phosphorylated derivative of the peptide 175–187, labelled at both Thr-181 and Tyr-183. Inactivation by PTP-1B was accompanied by the total loss of phosphotyrosine from MalE-XMpk2 (data not shown). Taken together, the results demonstrate that the activation of MalE-XMpk2 requires the phosphorylation of Thr-181 as well as Tyr-183. However $<15\%$ of the phosphate introduced into XMpk2 under these conditions is located at Thr-181.

3.3. Activation and phosphorylation of the RK in human KB cells

The RK was inactive in unstimulated KB cells, but became active when the cells were incubated with sodium arsenite (Fig. 3A). As observed previously in rat PC12 and human HeLa cells [2], RK activity was eluted from Mono Q as a sharp peak at 0.35 M NaCl mainly in fractions 19 and 20. The anti-RK antiserum recognised a 45 kDa protein in immunoblotting experiments which co-eluted with the RK activity from Mono Q (Fig. 3B). The antibody also immunodepleted RK activity from the Mono Q fractions (data not shown), and immunoprecipi-

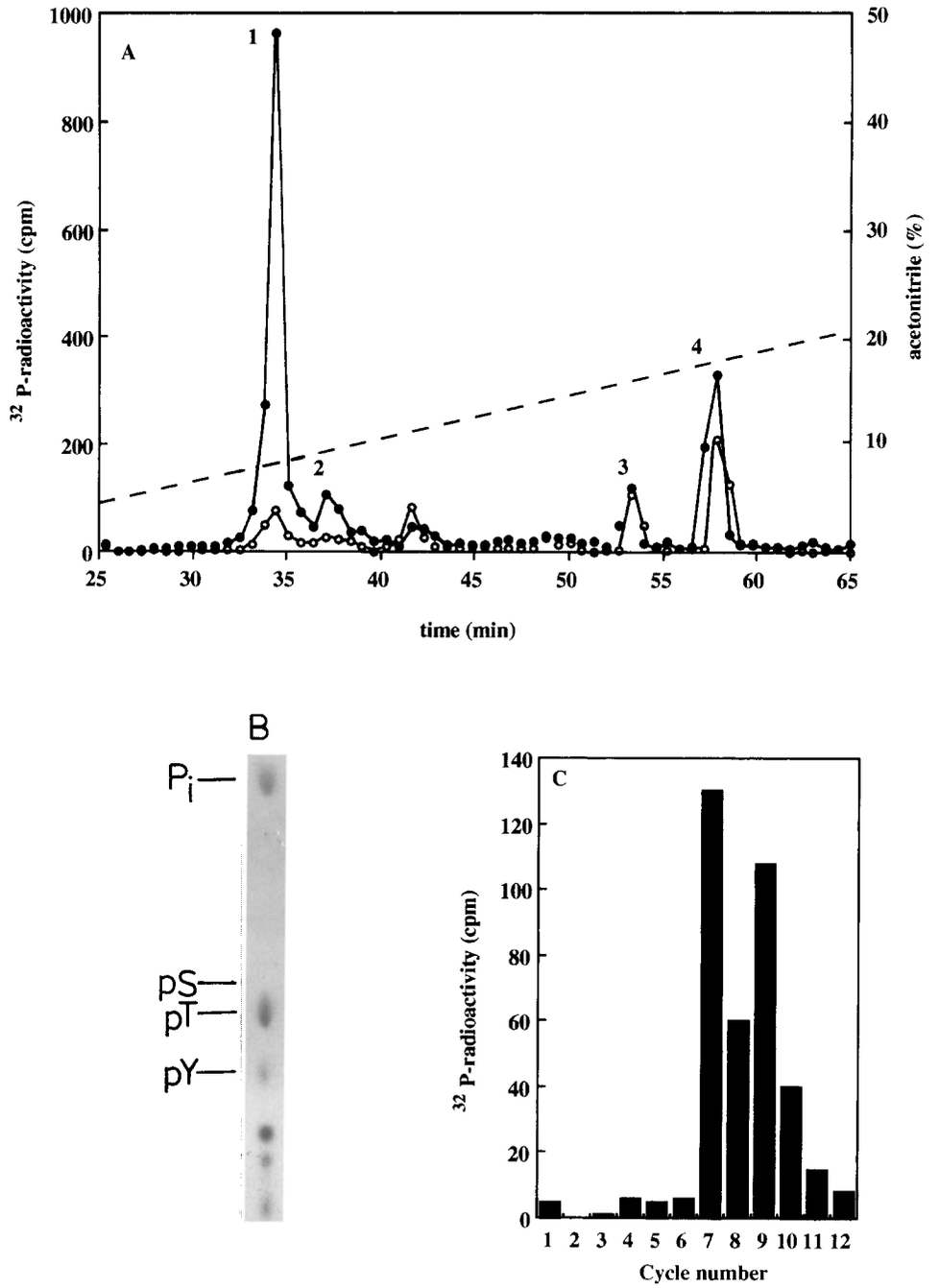

Fig. 4. Identification and analysis of ^{32}P-labeled phosphopeptides obtained by digestion of in vivo phosphorylated RK with trypsin. (A) ^{32}P-Labelled RK from arsenite-treated KB cells in Fig. 3C was digested with trypsin and chromatographed on a Vydac C_{18} column as in Fig. 2A. The open circles show the RK phosphopeptides from unstimulated cells, the closed circles phosphopeptides from arsenite-stimulated cells, and the broken line the acetonitrile gradient. (B) Phosphoamino acid analysis of phosphopeptide 1 from A. The phosphopeptide from arsenite-stimulated cells was incubated for 90 min at 110°C in 6 M HCl and electrophoresed on thin-layer cellulose at pH 3.5 to resolve orthophosphate (P_i), phosphoserine (pS), phosphothreonine (pT) and phosphotyrosine (pY) and autoradiographed. The other ^{32}P-labelled spots represent partially hydrolysed peptides. (C) The sites of phosphorylation in phosphopeptide-1 from arsenite-stimulated cells in A were identified by solid-phase sequencing as in Fig. 2B. 1000 cpm was coupled to the arylamine membrane, and the figure shows the ^{32}P radioactivity released after each cycle of Edman degradation.

tated a single 45 kDa ^{32}P-labelled protein from extracts of KB cells which had been incubated with [^{32}P]orthophosphate (Fig. 3C). The 45 kDa protein was phosphorylated in unstimulated cells, but phosphorylation increased by 90 ± 40% (±S.D., three experiments) after stimulation for 15 min with 0.5 mM arsenite (Fig. 3C). The RK bands from unstimulated and arsenite-stimulated cells were digested with trypsin and chromatographed on a C_{18} column. The RK from unstimulated cells showed four tryptic phosphopeptides, and phosphopeptide 1 eluting at 35 min was increased dramatically after stimulation with arsenite (Fig. 4A). Phosphopeptide-1 contained phosphothreonine and phosphotyrosine (Fig. 4B), co-eluted

with the diphosphorylated derivative of the peptide 175–187 from MalE-XMpk2 phosphorylated in vitro by GST-XMEK2 (Fig. 2A), and was phosphorylated at the seventh and ninth residues (Fig. 4C) as expected. The sequence of the peptide 175–187 from MalE-XMpk2 [2] only differs from human RK [8] in having Glu at residue 178 instead of Asp, which does not affect its elution from the C_{18} column significantly. The minor phosphopeptide-2 from arsenite-treated cells, eluting at 38/39 min largely contained phosphotyrosine (data not shown) and presumably represents monophosphorylated derivatives of the peptide 175–187 (mainly labelled at Tyr-183 only, see Fig. 2). Phosphopeptides 3 and 4 eluting at 54 and 59 min were unaffected by stimulating cells with arsenite (Fig. 4A) and contained phosphoserine only (data not shown).

4. Discussion

Here, we have established that the RK, like p42/p44 MAPKs, is only active if phosphorylated at both the Thr and the Tyr residue in the Thr-Xaa-Tyr motif (Thr-180 and Tyr-182 of human RK; Thr-181 and Tyr-183 of XMpk2), and inactivated when phosphate is removed from either residue. Since Thr-180 and Tyr-182 are both phosphorylated to a similar extent after stimulation of human KB cells for 15 min with arsenite, and no other residues are phosphorylated in response to this stimulus (Fig. 4C), the phosphorylation of these residues clearly underlies the stress-induced activation of RK. The reason why activation of RK and p42/p44 MAPKs involves such a dual Thr/Tyr phosphorylation is still unclear, although one possibility is that it allows dephosphorylation and inactivation to be controlled in a complex and sophisticated manner by three types of protein phosphatase (see section 1 and [11]).

MAPKK-1 phosphorylates Tyr-185 of p42 MAPK preferentially [19], and the present work shows that GST-XMEK2(E286, E290) also phosphorylates the *Xenopus* RK homologue XMpk2 much more rapidly at Tyr-183 than at Thr-181 in vitro. Indeed, we cannot be certain that GST-XMEK2 phosphorylates Thr-181 at all, because phosphorylation of Tyr-183 by XMEK2 may then permit MalE-XMpk2 to slowly phosphorylate itself at Thr-181. However, GST-XMEK2(E286, E290) phosphorylates MalE-XMpk2 poorly because, even after incubation for 16 h at a molar ratio GST-XMEK2/MalE-XMpk2 of 1:5, Tyr-183 of XMPK2 was phosphorylated to only 0.20 mol/mol, while Thr-181 was phosphorylated to 0.03 mol/mol. After incubation for much shorter times (0.5–2 h) the phosphorylation of Thr-181 was far lower, but phosphorylation of Tyr-183 was only reduced to a small extent.

While our work was in progress, the sequence of a mammalian MAPKK homologue was reported, termed SEK1 [20] or MKK4 [21], whose catalytic domain is >90% identical to XMEK2. MKK4 was reported to phosphorylate and activate the RK in vitro (termed p38 MAP kinase by these investigators [21]) and SEK1 to phosphorylate another stress and cytokine-activated MAP kinase homologue, termed c-Jun kinase (JNK) or stress-activated protein kinase (SAPK) [20]. JNK/SAPK was reported to be phosphorylated on Tyr and not Thr in vitro by SEK1 recovered from anisomycin-treated cells, but the effect of PP2A and PTPases on JNK/SAPK which had been activated by MKK4/SEK1 was not reported. Further work is needed to find out whether MKK4/SEK1/XMEK2, or other enzymes, are responsible for activating the RK and/or JNK/SAPK in vivo.

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