The EMBO Journal Vol.17 No.12 pp.3372–3384, 1998

PRAK, a novel protein kinase regulated by the p38 MAP kinase

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We have identified and cloned a novel serine/threonine kinase, p38-regulated/activated protein kinase (PRAK). PRAK is a 471 amino acid protein with 20–30% sequence identity to the known MAP kinase-regulated protein kinases RSK1/2/3, MNK1/2 and MAPKAP-K2/3. PRAK was found to be expressed in all human tissues and cell lines examined. In HeLa cells, PRAK was activated in response to cellular stress and proinflammatory cytokines. PRAK activity was regulated by p38α and p38β both *in vitro* and *in vivo* and Thr182 was shown to be the regulatory phosphorylation site. Activated PRAK in turn phosphorylated small heat shock protein 27 (HSP27) at the physiologically relevant sites. An in-gel kinase assay demonstrated that PRAK is a major stress-activated kinase that can phosphorylate small heat shock protein, suggesting a potential role for PRAK in mediating stress-induced HSP27 phosphorylation *in vivo*.

Keywords: HSP27/MAP kinase/p38/PRAK/protein kinase

Introduction

A variety of extracellular stimuli produce cellular responses via activation of the mitogen-activated protein (MAP) kinase cascades (Davis, 1993; Waskiewicz and Cooper, 1995; Su and Karin, 1996; Fanger *et al.*, 1997; Robinson and Cobb, 1997). The specificity of the cellular response is determined by the activation of a particular MAP kinase pathway in response to a given stimulus and by the activation of downstream targets by a given MAP kinase. MAP kinase family members have been found to regulate diverse biological functions by phosphorylation of specific target molecules found within the cell membrane, cytoplasm and nucleus, and thereby participate in the regulation of a variety of cellular processes including cell proliferation, differentiation and immune responses (Blenis, 1993; Marshall, 1994; Cano and Mahadevan, 1995; Seger and Krebs, 1995).

p38 (or p38α, also known as CSBP and RK) is a MAP kinase superfamily member which was first identified and cloned as an intracellular protein rapidly tyrosine phosphorylated upon treatment of macrophages with lipopolysaccharides (Han *et al.*, 1993, 1994). p38α was also cloned as a specific target of pyridinyl imidazole derivatives such as SB203580 which inhibit the production of proinflammatory cytokines by monocytes (Lee *et al.*, 1994). Three closely related kinases of p38 subsequently were cloned and characterized: p38β (Jiang *et al.*, 1996), p38γ (also known as ERK6 or SAPK3) (Lechner *et al.*, 1996; Li *et al.*, 1996; Mertens *et al.*, 1996) and p38δ (also known as SAPK4) (Goedert *et al.*, 1997; Jiang *et al.*, 1997; Wang *et al.*, 1997). p38α and p38β are sensitive to SB203580 inhibition, but the activities of p38γ and p38δ are unaffected (Goedert *et al.*, 1997). The entire family of p38 MAP kinases can be activated by osmotic changes in the extracellular environment. Other stimuli, such as UV light, oxidation and proinflammatory cytokines, are potent activators of specific p38 family members (Raingeaud *et al.*, 1995; Jiang *et al.*, 1997). In addition, some growth factors, such as erythropoietin and interleukin-3 (IL-3), are activators of p38α in certain cell types (Foltz *et al.*, 1997; Nagata *et al.*, 1997). All four p38 family members are activated by MAP kinase kinase (MKK) 3 or MKK6 via phosphorylation of the TGY motif (Derijard *et al.*, 1995). Several proteins have been identified as substrates of p38α including transcription factors (Price *et al.*, 1996; Wang and Ron, 1996; Han *et al.*, 1997; Janknecht and Hunter, 1997; Whitmarsh *et al.*, 1997) and protein kinases (Rouse *et al.*, 1994; Ludwig *et al.*, 1996; McLaughlin *et al.*, 1996; Fukunaga and Hunter, 1997; Waskiewicz *et al.*, 1997).

Protein kinase substrates of MAP kinases are believed to play an important role in amplifying and diversifying MAP kinase signals. Three groups of kinases have been shown to be activated by MAP kinases, ribosomal S6 kinase (RSK also known as MAPKAP-K1), MAP kinase-activated protein kinase (MAPKAP-K) and MAP kinase-interacting kinase or MAP kinase signal-integrating kinase (MNK) (Rouse *et al.*, 1994; Zhao *et al.*, 1996; Fukunaga and Hunter, 1997; Waskiewicz *et al.*, 1997). The activity of RSK proteins is regulated by the ERK kinase pathway, and these proteins play important roles in a variety of processes including cell proliferation (Jones *et al.*, 1988; Blenis, 1993; Zhao *et al.*, 1996). The two known MAPKAP-K family members, MAPKAP-K2 and MAPKAP-K3, can be activated by p38α *in vitro* and their activation can be inhibited *in vivo* by SB203580, a specific inhibitor of p38α and p38β (McLaughlin *et al.*, 1996). In turn, activated MAPKAP-K2/3 can phosphorylate small heat shock protein 27 (HSP27) (Stokoe *et al.*, 1992b), lymphocyte-specific protein 1 (LSP1) (Huang *et al.*, 1997a), cAMP response element-binding protein (CREB), ATF1 (Tan *et al.*, 1996) and tyrosine hydroxylase (Thomas *et al.*, 1997). A third group of MAP kinase-regulated protein kinases, MNK, was identified recently (Fukunaga and

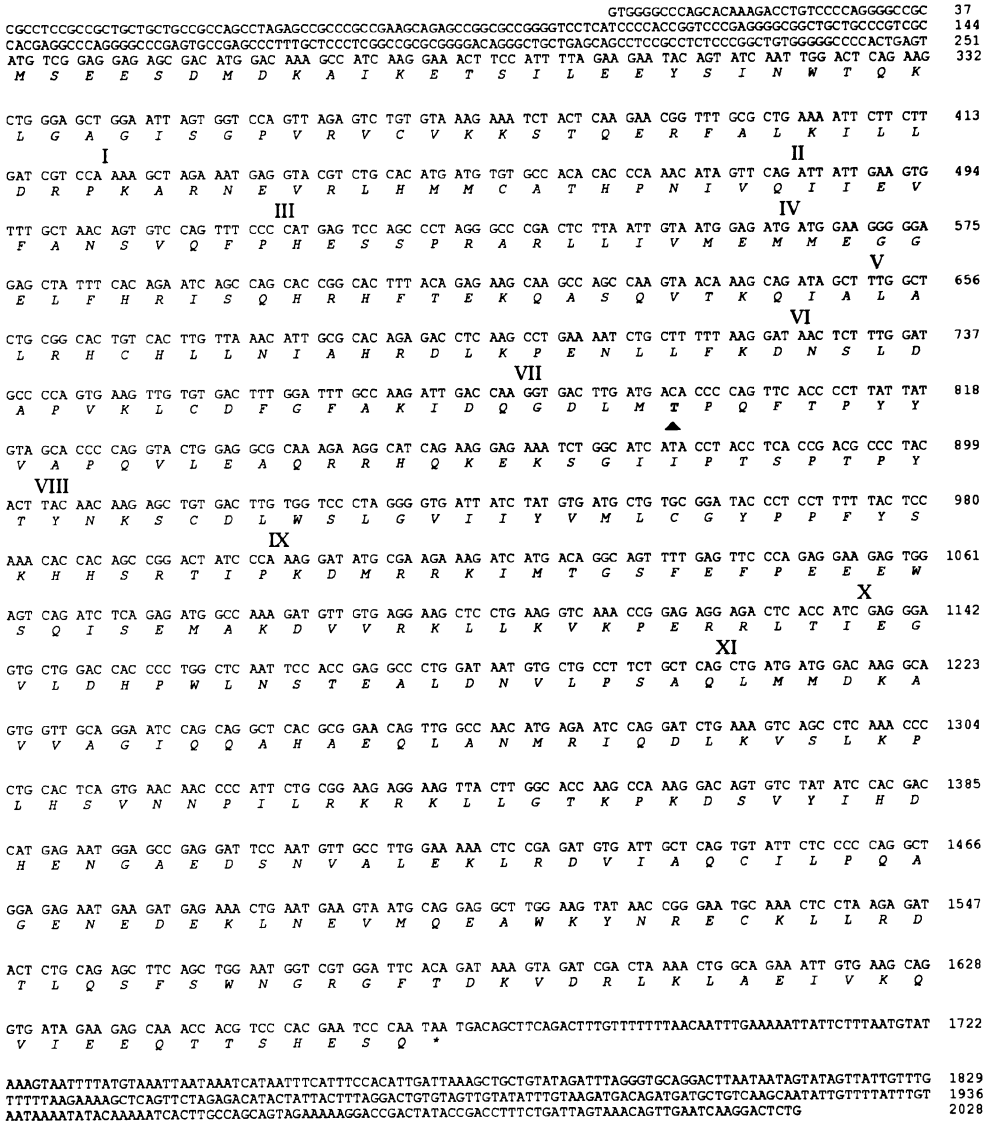

Fig. 1. The deduced amino acid and nucleotide sequence of human PRAK. The 11 protein kinase subdomains are indicated under the protein sequence with Roman numerals. The potential phosphorylation site (Thr182) of PRAK is marked by a solid triangle. The asterisk indicates a stop codon. The cDNA sequence of PRAK has been deposited in the DDBJ/EMBL/GenBank database under the accession number AF032437.

Hunter, 1997; Waskiewicz *et al.*, 1997). These kinases are regulated by p38 and ERK, but not by JNK. MNK1/2 can phosphorylate eukaryotic initiation factor-4E (eIF-4E) *in vitro*, which suggests an important link between MAP kinase activation and translational initiation (Waskiewicz *et al.*, 1997).

Despite the identification of several protein kinases as substrates for p38, there is evidence of additional downstream protein kinases. For example, Iordanov *et al.* reported that short wavelength UV irradiation (UVC) elicits p38-dependent CREB phosphorylation, and that the phosphorylation was mediated by an unknown p38-activated protein kinase (Iordanov *et al.*, 1997). To understand better the regulation and function of the p38 pathways, it is necessary to identify and characterize substrates of p38. Here, we describe the structure, function and regulation of a new protein kinase, p38-regulated/activated kinase (PRAK). This enzyme is activated by stress-related extracellular stimuli. The regulatory phosphorylation site of PRAK is Thr182 and this residue is phosphorylated by p38α and p38β *in vitro*. Activated PRAK specifically phosphorylates HSP27 at its physiologically relevant sites *in vitro*. In-gel kinase assays indicated that PRAK is one of the major stress-activated HSP27 kinases in whole-cell extracts from HeLa cells, suggesting that PRAK has a potential role in mediating stress-induced small heat shock protein phosphorylation *in vivo*.

Results

Molecular cloning of human PRAK

Although the RSK and MAPKAP-K share only 35% amino acid sequence identity, a common feature of these MAP kinase-regulated kinases was identified. Each contains the phosphorylation site LX*TP located within the

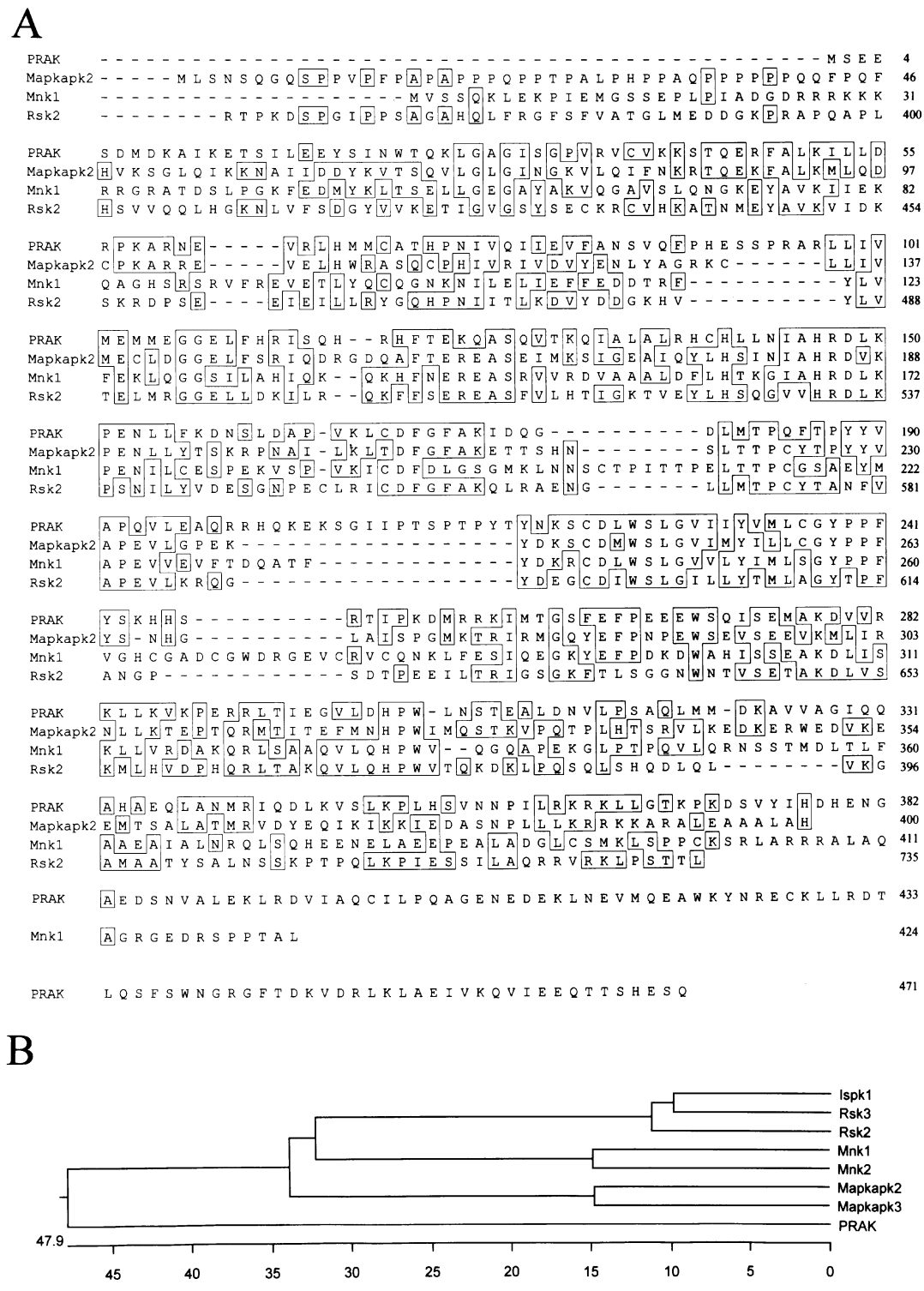

Fig. 2. (**A**) Sequence comparison of human PRAK, MAPKAP-K2, MNK1 and the C-terminal kinase domain of RSK2 using the Lasergene Megaline program (DNAStar, Madison, WI). Residues conserved between two or more protein kinases are boxed. (**B**) The ancestral relationships among human PRAK, human MAPKAP-K2, human MAPKAP-K3, human MNK1, murine MNK2, human RSK1(ISPK1), human RSK2, and human RSK3 are presented as a phylogenetic tree created by the Lasergene Megaline program. The scale beneath the tree measures the distance between sequences, and units indicate substitution events.

T-loop (also called Loop 12) between kinase domains VII and VIII. The sequences adjacent to this phosphorylation site are also conserved within the RSK and MAPKAP-K proteins. We hypothesized that there may be additional MAP kinase-regulated protein kinases that contain conserved LXTP sites and distinct flanking sequences. The peptide sequences LXTPCYTPYYVAP and LXTPCYTANFVAP from MAPKAP-K and RSK respectively were used to search the dbest database using the tblastn program. Seven expressed sequence tag (EST) clones were identified

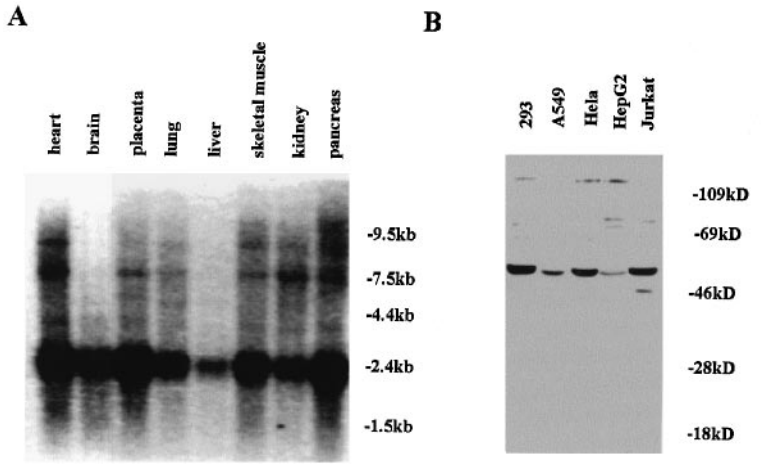

Fig. 3. Expression of PRAK. (**A**) A blot containing 2 μg of poly(A)$^+$ RNA isolated from various human tissues was hybridized with a probe specific to PRAK. (**B**) Equal amounts of cell-free lysates from 293, A549, HeLa, HepG2 and Jurkat cells were separated by 12% SDS–PAGE and transferred onto a nitrocellulose membrane. PRAK was detected using a specific polyclonal antibody.

that contained the LXTP site and novel flanking sequences. The sequence LTTPCGSAEYMAP was present in four EST clones (DDBJ/EMBL/GenBank accession Nos AA080236, W24753, AA083972 and AA157440). The sequence LXTPQFTPYYVAP was found in a single EST clone (DDBJ/EMBL/GenBank accession No. R68329) and the sequence LKTPCFTLHYAAP was present in two clones (DDBJ/EMBL/GenBank accession Nos N57096 and H09985). Additional sequences of these EST clones indicated that they are cDNAs for novel protein kinases. We hypothesized that these clones represent three new protein kinase groups that are regulated by MAP kinases. The EST clones of DDBJ/EMBL/GenBank accession Nos AA157440, R68329 and N57096 were sequenced completely and used to screen a human placental cDNA library to isolate full-length cDNA clones. While this study was in progress, two new protein kinases were reported by Waskiewicz *et al.* (1997) and Fukunaga and Hunter (1997), termed MNK1 and 2, that contained the sequence LXTPCGSAEYMAP. These protein kinases appear to belong to a new protein kinase group because they have only 30% identity to the RSK and MAPKAP-K. Importantly, this group of protein kinases is regulated by MAP kinases as our hypothesis would predict (Fukunaga and Hunter, 1997; Waskiewicz *et al.*, 1997). Because the available evidence suggests our approach may identify new kinase substrates of MAP kinases, we focused our efforts on the cDNA clones isolated from the human placental cDNA library that encode a LXTPQFTPYYVAP sequence. Complete sequencing of the three isolated clones revealed that they all encoded a single protein. The nucleotide sequence of the longest clone is shown in Figure 1. No in-frame stop codon was found at the 5′ end of the clone. 5′-RACE was performed but no additional 5′ sequence was obtained. Therefore, we tentatively assigned the first ATG as the start codon. The cDNA has an open reading frame encoding a protein of 471 amino acids with a calculated molecular mass of 54 kDa (Figure 1). This is consistent with the molecular mass of the endogenous protein on Western blots (Figure 3, see below). Since this protein kinase is regulated/activated specifically by p38 (see below), we named this kinase p38-regulated/activated kinase (PRAK). PRAK contains the conserved

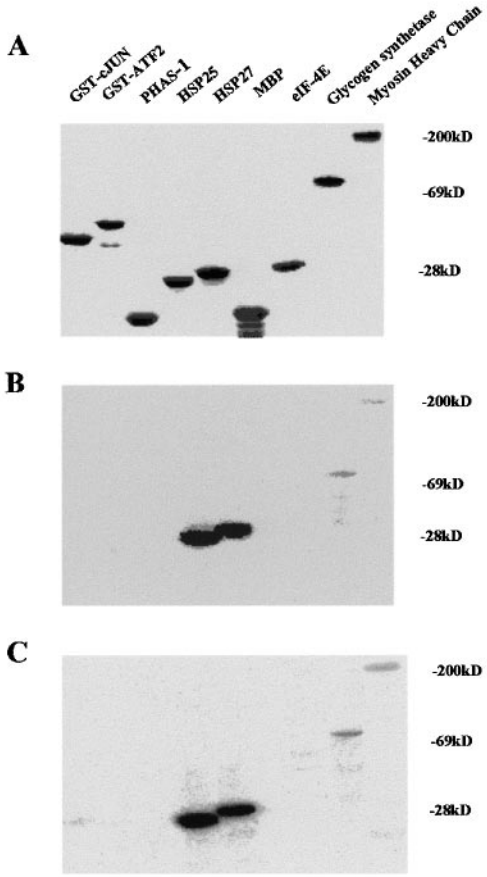

Fig. 4. HSP25 and HSP27 are preferred substrates for PRAK *in vitro*. (**A**) The Coomassie Blue stain of the protein substrates used in the kinase assays. Approximately 10 μg of GST–c-Jun (1–93), GST–ATF2 (1–109), PHAS-1, HSP25, HSP27, MBP, eIF-4E, glycogen synthetase and myosin heavy chain were used as substrates, and 0.5 μg of fully activated PRAK (**B**) or MAPKAP-K2 (**C**) were used as kinases for *in vitro* kinase assays. The kinase reactions were stopped by adding SDS sample buffer, and reaction products were analyzed by SDS–PAGE. Substrate phosphorylation was detected by autoradiography, and was quantified by phosphoimaging. Comparable results were obtained in two independent experiments.

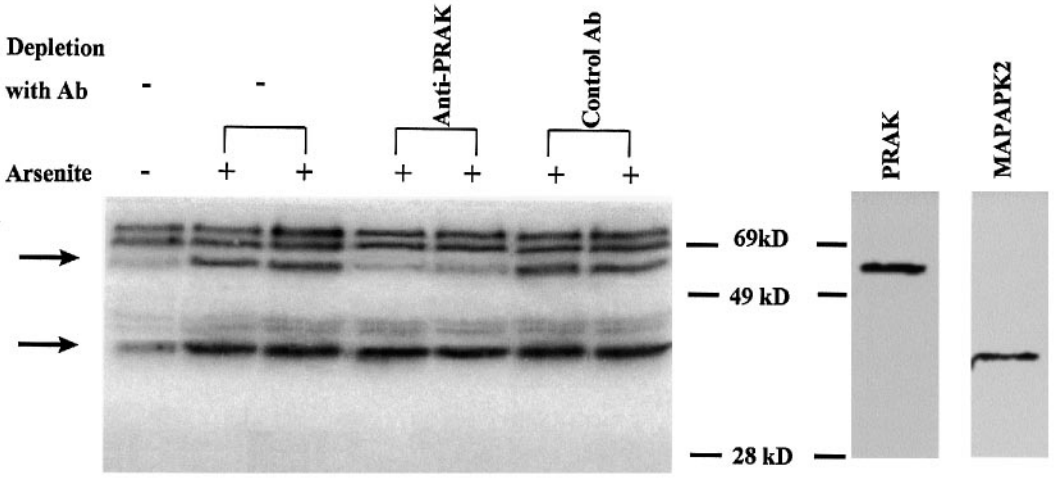

Fig. 5. PRAK is a HSP27 kinase activated by arsenite treatment in HeLa cells. In-gel kinase assay using HSP27 polymerized inside SDS–PAGE. The cell lysates of HeLa cells treated with or without 200 μM arsenite for 30 min were analyzed. Depletion of PRAK in the cell lysate was achieved by using anti-PRAK polyclonal antibody. The positions of PRAK and MAPKAP-K2 were determined by Western blotting as shown in the right hand panel.

protein kinase domains I–XI which are characteristic of all protein kinases (Figure 1). A DDBJ/EMBL/GenBank database search revealed that PRAK is most closely related to the MAPKAP-K, MNK and RSK kinases. A sequence alignment of PRAK with a member of the RSK group, MAPKAP-K group and MNK group proteins is shown in Figure 2A. PRAK is 32.8, 33.1, 22.8, 25.4, 22.7, 20.0 and 20.1% identical to MAPKAP-K2, MAPKAP-K3, MNK1, MNK2, ISPK1 (RSK1), RSK2 and RSK3, respectively. A computer-generated diagram showing the relatedness of these protein kinases is shown in Figure 2B. The relatively low homology of this protein to the known MAP kinase-regulated protein kinases supports our prediction that the protein containing LXTPQFTPYYVAP may belong to a new kinase group. The tissue distribution of PRAK mRNA was assessed by Northern blot hybridization (Figure 3A). The PRAK transcript is ~2.4 kb and is present in all eight tissues examined (Figure 3A). PRAK protein expression was analyzed in five different human cell lines by Western blot using an anti-PRAK antibody. The protein was detected in all of the cell lines as a 54 kDa protein, consistent with the predicted molecular weight (Figure 3B).

HSP27 is a preferred substrate for PRAK in vitro

To identify the substrate of PRAK, we tested a panel of proteins which are known substrates of PRAK-related protein kinases. As shown in Figure 4B, HSP27 and HSP25 are good substrates of PRAK *in vitro*. Phosphorylation of HSP27 and HSP25 by PRAK is similar in magnitude to that seen with MAPKAP-K2 when equal amounts of fully activated kinase were used in *in vitro* kinase assays (compare Figure 4B and C). Both PRAK and MAPKAP-K2 can phosphorylate glycogen synthetase, and myosin heavy chain to a lesser extent. PHAS-1, MBP, eIF-4E and the N-terminal portions of c-Jun (1–93) and ATF2 (1–109) were poor substrates for PRAK and MAPKAP-K2. To determine if PRAK is a major kinase for HSP27 in stress-activated cells, an in-gel kinase assay using recombinant HSP27 as substrate was performed (Figure 5, right panel). There are four major kinases in HeLa cell lysates that phosphorylate HSP27. Stimulation of HeLa cells with arsenite resulted in activation of the 45 and 54 kDa HSP27 kinases. Depletion of PRAK in cell lysates with an anti-PRAK polyclonal antibody specifically removed the kinase activity associated with the 54 kDa protein, while the other HSP27 kinases were unaffected. These data strongly suggested that PRAK was a major stress-activated kinase that can phosphorylate HSP27. Western blot analysis (Figure 5, left panel), revealed that the stress-activated 45 kDa HSP27 kinase in HeLa cell lysates is MAPKAP-K2. MAPKAP-K2 has been reported to phosphorylate serines 15, 78 and 82 of HSP27 in response to stress or mitogens *in vivo* (Stokoe *et al.*, 1992b; Knauf *et al.*, 1994). Therefore, we performed an experiment to determine whether or not PRAK phosphorylates HSP27 at the same sites as MAPKAP-K2. Recombinant HSP27 treated with PRAK or MAPKAP-K2 in the presence of [γ-^{32}P]ATP was used for tryptic phosphopeptide mapping. As shown in Figure 6, PRAK and MAPKAP-K2 phosphorylated the same peptides. Thus PRAK, like MAPKAP-K2, phosphorylates HSP27 at the functionally relevant sites. The overlapping specificity of the two kinases, MAPKAP-K2 and PRAK, is not without precedent (Whitmarsh *et al.*, 1995; Price *et al.*, 1996; Janknecht and Hunter, 1997). However, the physiological relevance of this overlap requires further investigation.

PRAK is activated by environmental stress and proinflammatory cytokines

A variety of agonists were tested for their ability to activate endogenous PRAK in HeLa cells. PRAK activity was measured using an immunokinase assay with HSP27 as the substrate. A Western blot demonstrated that equal amounts of PRAK were used in each assay (data not shown). As shown in Figure 7A, PRAK was strongly activated by stress stimuli such as arsenite, anisomycin and H_2O_2, and by the proinflammatory cytokine tumor necrosis factor-α (TNF-α). Phorbol-12-myristate-13-acetate (PMA) and calcium ionophore A23187 also activated PRAK but to a lesser extent. In contrast, IL-6 had no effect on PRAK activation in HeLa cells. Growth-

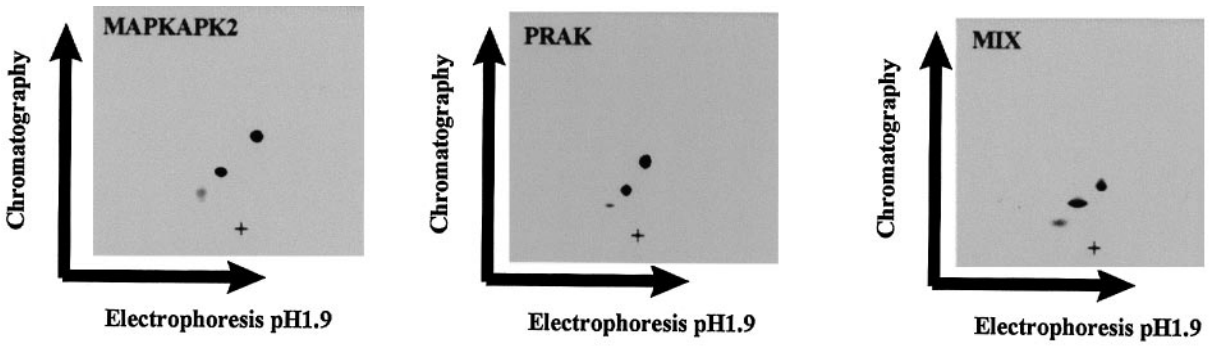

Fig. 6. Tryptic phosphopeptide map of HSP27 phosphorylated by activated GST–PRAK and GST–MAPKAP-K2 *in vitro*. The panel marked as MIX indicates a mixture of equal amounts of HSP27 proteins phosphorylated by GST–PRAK and GST–MAPKAP-K2 prior to tryptic phosphopeptide mapping. The + sign on each panel indicates the original sample loading site. The perpendicular arrowheads indicate two-dimensional separations of phosphopeptides by electrophoresis and chromatography.

related stimuli epidermal growth factor (EGF), fibroblast growth factor (FGF), platelet-derived growth factor (PDGF) and serum had no or only modest effects on PRAK activity in HeLa cells (Figure 7B). A similar activation profile was obtained when transiently expressed hemagglutinin (HA)-tagged PRAK was assayed by immunokinase assay using anti-HA monoclonal antibody 12CA5 (data not shown).

PRAK is a physiological substrate for p38α and p38β

PRAK was cloned because it contained a putative phosphorylation site for MAP kinases. The data presented above demonstrate that PRAK is activated by a panel of stimuli that are known to activate p38 and c-Jun N-terminal kinase [JNK also known as stress-activated protein kinase (SAPK)]. We therefore hypothesized that PRAK is a downstream target of the p38 or JNK signaling pathways. To test this hypothesis, we performed an *in vitro* kinase assay using recombinant PRAK as the substrate and extracellular signal-regulated kinase 2 (ERK2), JNK2, p38α, p38β, p38γ or p38δ as the kinase (Figure 8). Equal amounts of each recombinant MAP kinase, activated by the appropriate upstream kinase (see Materials and methods), were used (Figure 8A). p38α, p38β, p38γ and p38δ showed higher activity toward PRAK in comparison with JNK2 and ERK2 (Figure 8B). Coupled kinase assays were performed to address whether the phosphorylation of PRAK by these MAP kinases influenced intrinsic PRAK activity using HSP27 as the substrate. PRAK activity was dramatically increased when it was phosphorylated by p38α or p38β (Figure 8C). In contrast, the enhancement of PRAK activity by p38γ, p38δ, JNK2 or ERK2 was not significant. Interestingly, p38δ efficiently phosphorylated PRAK (Figure 8B), but did not lead to activation of PRAK activity (Figure 8C). The inability of p38δ to activate PRAK is probably due to its failure to phosphorylate the regulatory site(s) of PRAK (see below).

Next, we determined if PRAK was downstream of the p38 pathway in intact cells. Substitution of the phosphorylation sites of MKK with glutamic acid (E) or aspartic acid (D) results in constitutively active forms of MKKs. Previously, we reported that expression of constitutively active MKK by recombinant adenovirus could activate a given MAP kinase pathway in intact cells (Huang *et al.*, 1997b). Here, the same strategy was used independently to activate the p38, JNK or ERK pathway by expression of MKK1(E), MKK6(E) or MKK7(D). Twenty four hours after cells were infected with recombinant viruses, endogenous PRAK activity was measured using an immunokinase assay (Figure 9). Expression of MKK6(E), which activates the p38 signaling pathway, caused endogenous p38α/β and PRAK activation, supporting the concept that PRAK is regulated by the p38 pathway. In contrast, MKK7(D) expression activated JNK1 but not PRAK, and expression of MKK1(E) enhanced ERK2 activity but had

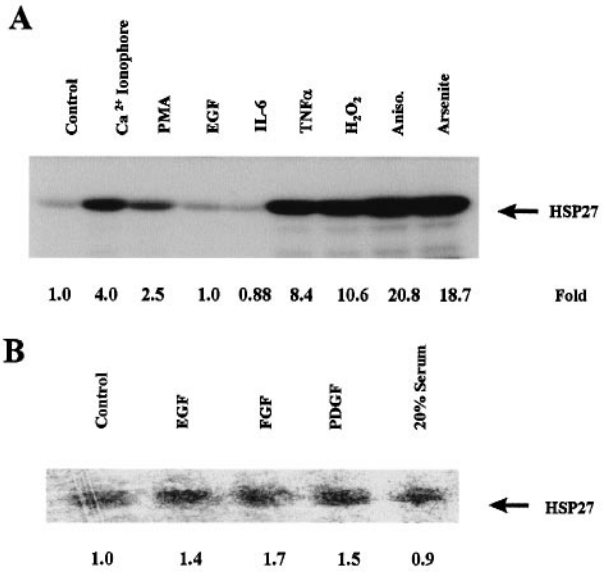

Fig. 7. Activation of endogenous PRAK by various stimuli. HeLa cells were cultured in DMEM with 10% FBS. Approximately 10^6 cells (per sample) were treated with the Ca^{2+} ionophore A23187 (5 μM), PMA (100 nM), EGF (1 ng/ml), IL-6 (250 U/ml), TNF-α (100 ng/ml), H_2O_2 (400 μM), anisomycin (50 ng/ml), arsenite (200 μM) (**A**), or EGF (1 ng/ml), bFGF (10 ng/ml), PDGF-BB (10 ng/ml) and 20% serum (**B**) for 20 min at 37°C. Cells were serum starved for 5 h before the stimulation with growth factors. The kinase activity of PRAK isolated by immunoprecipitation with anti-PRAK antiserum was measured using HSP27 as the substrate. The fold activation by the different stimuli was calculated by dividing the radioactive intensity of phosphorylated HSP27 for each treatment by the untreated control and is shown under each lane. Similar results were obtained in two experiments.

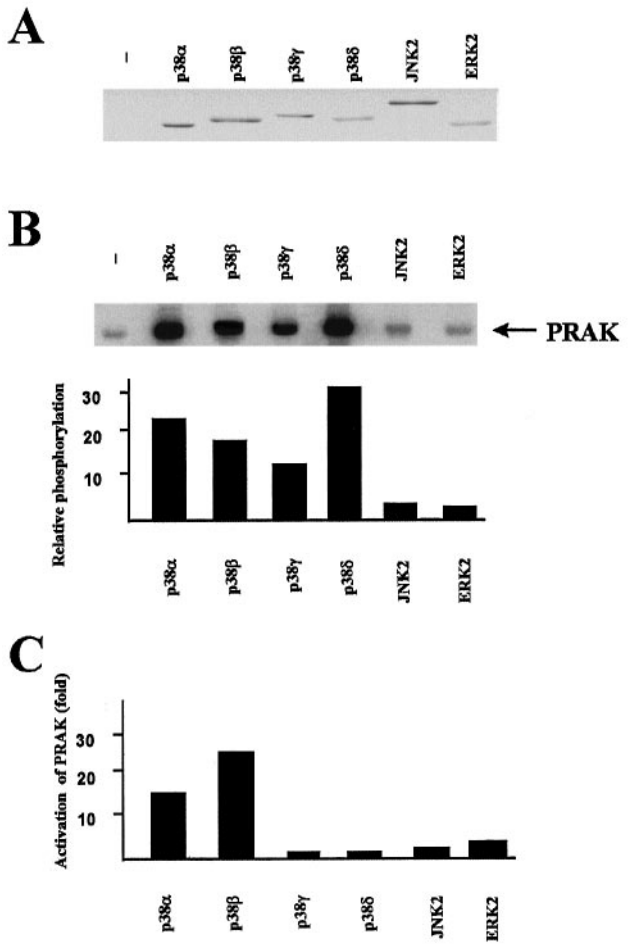

Fig. 8. Phosphorylation and activation of PRAK by different MAP kinases *in vitro*. (**A**) Coomassie Blue stain of recombinant MAP kinases used in the experiments. (**B**) Phosphorylation of PRAK (5 μg) by different MAP kinases (0.5 μg) that were activated *in vitro* by MKKs as described in Materials and methods. The phosphorylated products were resolved by SDS–PAGE and quantified by phosphoimaging. The relative phosphorylation of PRAK by each MAP kinase was calculated by subtracting the intensity of PRAK autophosphorylation from that of each MAP kinase-treated PRAK. The result is shown as a bar graph. (**C**) Activation of PRAK by different MAP kinases was assessed by *in vitro* coupled kinase assays. Several paired kinase reactions, containing equal amounts of each MAP kinase (0.5 μg) and 10 μg of HSP27 in the presence or absence of 2 μg of GST–PRAK, were performed. The phosphorylated products were resolved by SDS–PAGE and quantified by phosphoimaging. The fold activation of PRAK by p38α, p38β, p38γ, p38δ, JNK2 and ERK2 was determined by the formula: [(radioactive intensity of HSP27 phosphorylated by a given MAP kinase activated PRAK and this MAP kinase) – (radioactive intensity of HSP27 phosphorylated by the given MAP kinase alone)]/(radioactive intensity of HSP27 phosphorylated by untreated PRAK). Comparable results were obtained in two independent experiments.

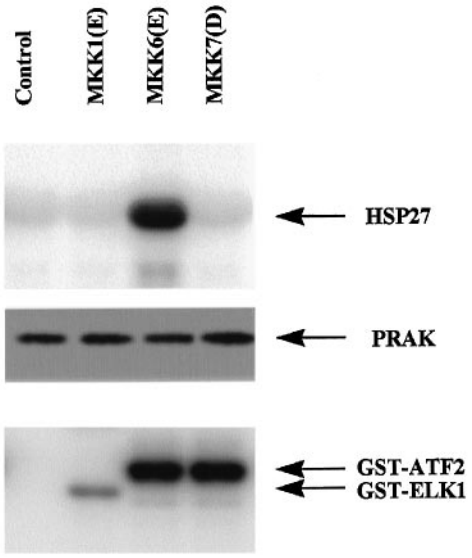

Fig. 9. Effect of individual MAP kinase pathways on PRAK activity in intact cells. The constitutively active form of MAP kinase kinase, MKK1(E), MKK6(E) or MKK7(D) was expressed in HeLa cells (~10^6 per sample) using an adenovirus-mediated gene delivery system. The control infection was done with an adenovirus encoding β-galactosidase. An m.o.i. of 10 was used for each of the recombinant viruses, because nearly 100% infection efficiency was achieved at this titer. After 24 h, the cells were lysed and PRAK was immunoprecipitated using an anti-PRAK antibody. The lysates subsequently were used to immunoprecipitate p38α/β, ERK2, p38α/β or JNK1 (corresponding to the lanes in the figure) from the cells expressing β-galactosidase, MKK1(E), MKK6(E) or MKK7(D), respectively. The upper panel shows endogenous PRAK activity from different cell lysates measured by immunokinase assays using HSP27 as the substrate. The middle panel shows that equal amounts of PRAK determined by Western blotting were used for the immunoprecipitation. The lower panel shows endogenous activities of p38α/β, ERK2, p38α/β or JNK2 (corresponding to the lanes in figure) of the cells infected by the control virus, ad-MKK1(E), ad-MM6(E) or ad-MKK7(D). GST–ELK1 was used as the ERK substrate, GST–ATF2 was used as the substrate for p38 and JNK. Comparable results were obtained in duplicate experiments.

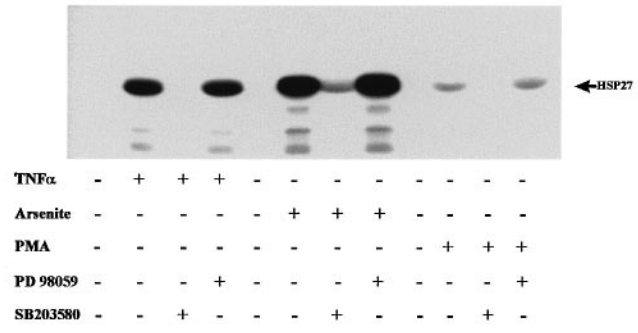

Fig. 10. Effects of SB203580 and PD98059 on endogenous PRAK activity in the cells treated with TNF-α, arsenite or PMA. HeLa cells (~10^6 per sample) were pre-treated with PD98059 (10 μM) or SB203580 (10 μM) for 30 min, and then stimulated with TNF-α (100 ng/ml), arsenite (200 μM) or PMA (100 nM) for 20 min. PRAK activities from these different treatments were measured by immunokinase assays with HSP27 as the substrate. The + or – under each lane indicates the presence or absence of a specific treatment. Comparable results were obtained in three experiments.

no influence on PRAK activity. Therefore, JNK1 and ERK2 are unable to activate PRAK *in vivo*. Since MKK1 and MKK7 activate all ERK and JNK isoforms (Cowley *et al.*, 1994; Marshall, 1994; Holland *et al.*, 1997; Lu *et al.*, 1997; Tournier *et al.*, 1997), these data suggest that ERK1 and JNK2/3 are also unable to activate PRAK. Thus, PRAK is regulated specifically by the p38 pathway in intact cells.

To test further whether PRAK is regulated by p38α/β under physiological conditions, arsenite, TNF-α and PMA were used to stimulate HeLa cells that had been pre-treated with a specific inhibitor for p38α/β, SB203580 (Figure 10). Blocking of p38α/β signaling dramatically inhibited arsenite- and TNF-α-, as well as PMA-induced

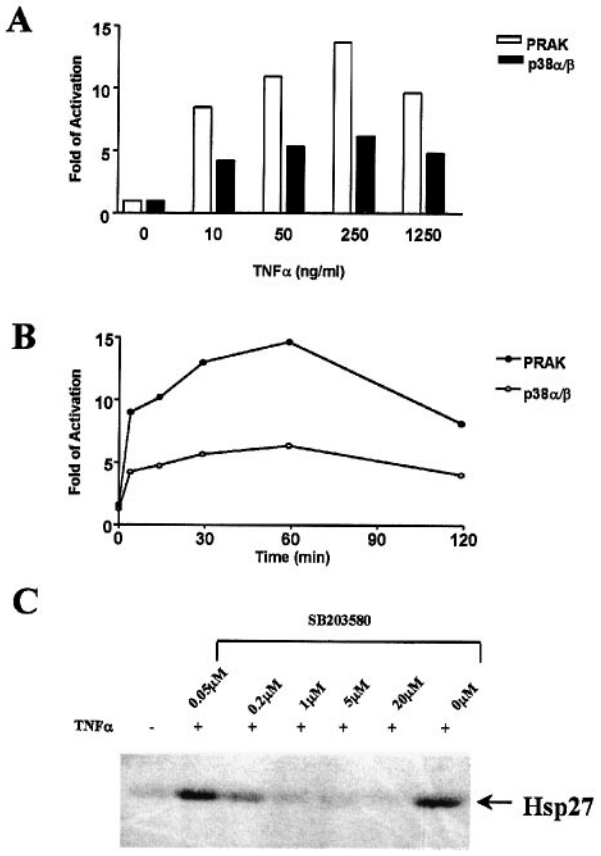

Fig. 11. Correlation of p38 and PRAK activation in HeLa cells stimulated with TNF-α. (**A**) HeLa cells (~10^6 per sample) were incubated for 20 min with different concentrations of TNF-α at 37°C. (**B**) HeLa cells were treated with 50 ng/ml TNF-α for different times at 37°C. (**C**) HeLa cells were pre-treated with different doses of SB203085 for 30 min and then stimulated with 50 ng/ml TNF for 20 min. p38α/β and/or PRAK protein kinase activities were measured by immunokinase assays with GST–ATF2 and HSP27 as substrates, respectively. The fold activation was determined by dividing each stimulated activity by that of the unstimulated activity. Comparable results were obtained in duplicate experiments.

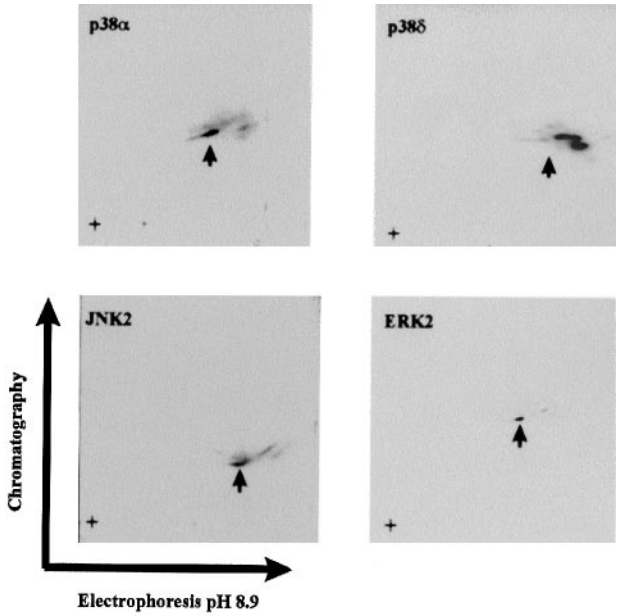

Fig. 12. Peptide mapping of PRAK phosphorylated by p38α, p38δ, JNK2 and ERK2. GST–PRAK phosphorylated by p38α, p38δ, JNK2, and ERK2 was resolved on SDS–PAGE and transferred onto nitrocellulose. The phosphorylated PRAK was excised from the membrane and processed to tryptic peptide mapping using the standard method. The + on each panel indicates the original sample loading site. The perpendicular arrowheads indicate two-dimensional separations of the phosphopeptide by electrophoresis and chromatography. The small arrows inside each panel point to the position of the tryptic peptide of PRAK containing the putative regulatory site.

PRAK activation. In contrast, treatment of cells with PD98059 at a concentration (10 μM) that inhibits PMA-induced ERK activation by 50% in HeLa cells (data not shown) had no effect on stress-induced PRAK activation, indicating that the ERK pathways does not play a role in regulating PRAK activity in HeLa cells. Taken together, these data indicate that p38α and p38β are *in vivo* regulators of PRAK.

To evaluate further the role of p38α and p38β in stress-induced PRAK activation, a series of experiments was performed to determine if p38 activation correlated with PRAK activation. HeLa cells were stimulated with increasing doses of TNF-α for 20 min or treated with 50 ng/ml of TNF-α for different periods of time. The activities of PRAK and p38α/β were measured by immunokinase assays. As shown in Figure 11A and B, there is a good correlation between TNF-α-induced activation of both PRAK and p38. Moreover, TNF-induced PRAK activation was inhibited in a dose-dependent manner by the p38α/β inhibitor SB203085, with an IC_{50} of 0.5 μM (Figure 11C). A similar IC_{50} for arsenite-induced PRAK activation was found (data not shown). These data also suggest that PRAK and p38 are in the same signal transduction pathway.

Since no antibody is available to precipitate p38α and p38β differentially, we were unable to address their independent roles in PRAK activation in intact cells.

PRAK is regulated by the phosphorylation on Thr182

To analyze the phosphorylation site of PRAK by MAP kinases, phosphopeptide mapping was performed. Although differences were observed in the extent of phosphorylation of PRAK by p38α, JNK2 and ERK2 (Figure 8A), the phosphopeptide maps of PRAK phosphorylated by these three MAP kinases appeared very similar (Figure 12). By calculating the R_f of the major phosphorylated peptides, we concluded that p38α, JNK2 and ERK2 preferentially phosphorylated the same site in PRAK. p38δ strongly phosphorylated PRAK but had no significant effect on PRAK activity (Figure 8). Phosphopeptide mapping of p38δ-phosphorylated PRAK demonstrated that p38δ phosphorylated a different site(s) in PRAK than p38α. We conclude that p38δ did not phosphorylate the regulatory site of PRAK.

PRAK was identified because it contained a conserved LMTP site in the T-loop. We suspected that this site was the regulatory phosphorylation site. To determine if the LMTP site was phosphorylated by p38α/β, we prepared recombinant PRAK protein in which the LMTP sequence was mutated to LMDP [PRAK (182D)] and LMAP [PRAK (182A)]. Wild-type PRAK, PRAK (182D) and PRAK (182A) were treated with p38β in the presence of [γ-^{32}P]ATP. ^{32}P-Labeled PRAK (wt), PRAK (182D) and PRAK (182A) were isolated and analyzed by peptide

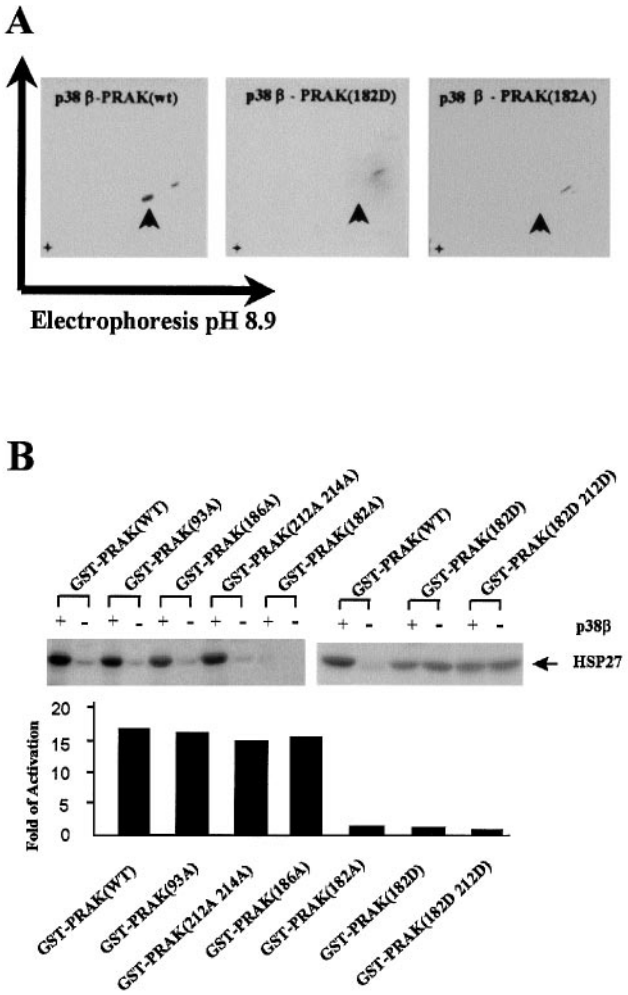

Fig. 13. Determination of the regulatory phosphorylation site of PRAK. (**A**) Wild-type GST–PRAK (wt) and two GST–PRAK mutants, PRAK (182A) and PRAK (182D), were treated with p38β *in vitro* and subjected to phosphopeptide mapping using the same experimental conditions as in Figure 12. The small arrows inside each panel indicate the position of the PRAK peptide containing the putative regulatory phosphorylation site. This phosphorylation site disappeared when Thr182 was mutated to alanine or aspartic acid. (**B**) Each of the proline-directed serine or threonine residues in PRAK was substituted with alanine, and some of them also were converted to aspartic acid. The recombinant GST fusion proteins of these mutants were prepared. A coupled kinase assay was performed as described in Figure 8C using 10 μg of HSP27 as the substrate and 2 μg of each mutant or wild-type PRAK in the presence (+) or absence (–) of 0.2 μg of p38β. Kinase activities of GST–PRAK (wt), GST–PRAK (93A), GST–PRAK (182A), GST–PRAK (186A), GST–PRAK (212A, 214A), GST–PRAK (182D) and GST–PRAK (182D,212D) were determined by quantifying the radioactive intensity of phosphorylated HSP27. The fold activation was calculated as described in Figure 8C and is shown as a bar graph. Mutation of Thr182 abolished activation of PRAK by p38β while the other potential proline-directed phosphorylation sites had no role in PRAK activation. Comparable results were obtained in three experiments.

mapping. As shown in Figure 13A, p38β phosphorylated the same peptide as p38α (Figure 12). Mutation of Thr182 abolished the major phosphorylation site of PRAK. Edman degradation of this major phosphopeptide released free phosphate at the 8th cycle (data not shown), which is consistent with the position of Thr182 in the tryptic peptide. There are five proline-directed serine or threonine residues in PRAK that could be the sites for MAP kinase phosphorylation. To confirm that Thr182 is the only regulatory site of PRAK, we created additional mutants that had Ser93, Thr186, Ser212 and Thr214 changed to Ala to prevent phosphorylation at each of these sites. The activity of wild-type or mutated PRAK proteins, before and after p38α or p38β phosphorylation, was determined by *in vitro* kinase assay. As shown in the left panel of Figure 13B, all mutants except PRAK (182A) were activated by p38β to a similar extent, suggesting that the phosphorylation of these sites is not required for PRAK activation. Similar results were obtained with p38α (data not shown). Mutation of Thr182 to Ala completely abolished the induction of PRAK by p38β, demonstrating that Thr182 is indeed the regulatory site. Moreover, changing Thr182 to a negatively charged aspartic acid partially mimicked phosphorylation of PRAK, the mutant having ~50% of the fully activated PRAK activity (Figure13B, right panel). Since there is a weak phosphorylated peptide that was phosphorylated by p38β (Figure 13A), we sought to address whether this phosphorylation has any effect on PRAK activity. We have determined that the phosphorylation site is a serine residue by phosphoamino acid analysis and determined that it is Ser 212 by phosphopeptide mapping of PRAK(93A) and PRAK(212A, 214A) (data not shown). Since mutation of Ser212 to Ala does not have any effect on PRAK activation by p38 (Figure 13B, left panel), it is unlikely that phosphorylation at this site has a positive effect on PRAK activity. This was confirmed further by double mutation of Thr182 and Ser212 to aspartic acid. The additional aspartic acid mutation at Ser212 does not result in an enhancement of PRAK(182D) activity, supporting the idea that residue 182 is the sole regulation site of PRAK.

Discussion

PRAK is a new class of MAP kinase-regulated protein kinase and is regulated specifically by the p38 pathway

We have identified a new serine/threonine kinase, termed PRAK, which is strictly regulated by p38α/β *in vitro* and *in vivo*. Like all known MAP kinase-regulated protein kinases, PRAK has a MAP kinase phosphorylation site (ΨX[S/T]P) located in the T-loop between kinase subdomain VII and VIII. The overall sequence identity of PRAK to the known MAP kinase-regulated protein kinases is ~20–30%, which suggests that PRAK does not belong to any known group of MAP kinase-regulated protein kinases. We suggest that PRAK may represent a new protein kinase group regulated by MAP kinases.

In addition to the relatively low homology of PRAK to the other known MAP kinase-regulated protein kinases, PRAK has structural and functional characteristics distinct from each of the known MAP kinase-regulated protein kinase groups. Structurally, PRAK is different from RSK members in that it lacks a second N-terminal kinase domain (Blenis, 1993). PRAK is regulated specifically by p38α and p38β, while RSKs are believed to be regulated strictly by the ERK pathway (Zhao *et al.*, 1996). The difference in downstream target(s) between PRAK and RSKs suggests that activation of RSKs and PRAK leads to different biological effects (Blenis, 1993). PRAK is distinct from MNK1/2 because MNKs are able to interact

with p38α as well as ERK1/2 (Fukunaga and Hunter, 1997; Waskiewicz *et al.*, 1997). MNK can phosphorylate eIF-4E while PRAK cannot, suggesting a difference in substrate specificity and biological function (Waskiewicz *et al.*, 1997). MAPKAP-K2 is probably the most closely related kinase to PRAK in terms of regulation and substrate specificity (Rouse *et al.*, 1994). Several features differentiate PRAK from MAPKAP-K2 and its isoforms. First, although PRAK and MAPKAP-K2 share a common feature, namely a regulatory phosphorylation site LXTP, the other phosphorylation sites found in MAPKAP-K2 are not conserved in PRAK (see protein sequence alignment shown in Figure 2). Since the multiple phosphorylation characteristic of MAPKAP-K (Ben-Levy *et al.*, 1995; Ludwig *et al.*, 1996) does not exist in PRAK, the structural basis for full activation of these kinase is probably different. Secondly, *in vitro* ERK is an equally potent activator of MAPKAP- K2 as p38α (Stokoe *et al.*, 1992a; Ben-Levy *et al.*, 1995; Ludwig *et al.*, 1996) while PRAK is activated selectively by p38α/β. This suggests that different structural features of PRAK and MAPKAP-K2 are required for upstream activator recognition. PRAK and MAPKAP-K2 appear to have a similar *in vivo* activation profile (Rouse *et al.*, 1994), but the mechanism of activation may be different. The inability of ERK to activate MAPKAP-K2 *in vivo* is likely to be due to the N-terminal proline-rich domain of MAPKAP-K2, which suppresses activating phosphorylation by ERK but not by p38 (Ben-Levy *et al.*, 1995). Since PRAK does not contain a proline-rich domain, the selectivity for p38 *in vivo* and *in vitro* may be determined by a different mechanism.

PRAK is a potential HSP27 kinase

HSP27 is phosphorylated in response to stimulation by cytokines, growth factors and chemical/physical stress. Two proteins, with molecular mass of 45 and 54 kDa, were found to be major HSP27 kinases in hamster and human cells (Huot *et al.*, 1995, 1997). Based on the (re)activation *in vitro* by ERK, antigenic properties and substrate specificity, at least one of the HSP27 kinases was believed to be MAPKAP-K2 or one of its isoforms (Huot *et al.*, 1995). The data presented here suggest that there are four major kinases that can phosphorylate HSP27 in HeLa cell lysates. Two of these kinases, with molecular masses of ~45 and 54 kDa respectively, are activated by the stress stimulus arsenite (Figure 5). It is unclear whether these two proteins correspond to the reported major HSP27 kinase activities in hamster CCL39 cells and human umbilical vein endothelial cells (HUVECs) (Huot *et al.*, 1995, 1997). Our data demonstrated that PRAK is one of the inducible HSP27 kinases in HeLa cells. The following evidence is consistent with the idea that PRAK may be a physiological HSP27 kinase: (i) PRAK phosphorylates HSP27 at its physiologically relevant sites; (ii) PRAK activation is correlated with the activation of p38α which has been shown to be correlated with HSP27 phosphorylation in response to stress (Huot *et al.*, 1997); (iii) activation of PRAK in intact cells is blocked by SB203580 which also blocks HSP27 phosphorylation (Guay *et al.*, 1997); and (iv) depletion of PRAK from stress-stimulated HeLa cell lysates removed the 54 kDa HSP27 kinase. MAPKAP-K2 has been reported to migrate on SDS–PAGE at a quite different molecular mass and often with multiple bands depending on the species (Cano *et al.*, 1996). However, we only detected a 45 kDa protein in HeLa cell lysates using an anti-MAPKAP-K2-specific antibody (Figure 5). Since the PRAK antibody we generated cannot detect and immunoprecipitate recombinant MAPKAP-K2 protein (data not shown) and did not deplete the 45 kDa HSP27 kinase corresponding to MAPKAP-K2 (Figure 5), the 54 kDa HSP27 kinase in HeLa cells is PRAK. Further study is needed to address the relationship between PRAK and the previously identified major HSP27 kinases. MAPKAP-K2 was identified as a major HSP25 kinase because it co-purified with HSP25 kinase activity in rabbit skeletal muscle throughout its purification. It is not known whether PRAK co-purifies with the major HSP25 kinase or not. If not, it is possible that PRAK is a minor HSP27 kinase activity in cell extracts, but appears as a more major activity using in-gel kinase assays because it can be reactivated from SDS denaturation more readily than MAPKAP-K2. Whether PRAK is a physiologically relevant kinase for small heat shock proteins awaits further investigation.

The function of PRAK in the p38 pathway

The p38 MAP kinase pathway has been implicated in many biological process such as stress- and growth stimuli-induced gene expression (Lee *et al.*, 1994; Perregaux *et al.*, 1995), cytoskeletal reorganization (Huot *et al.*, 1997; Zechner *et al.*, 1997), cell proliferation and apoptosis (Xia *et al.*, 1995; Kawasaki *et al.*, 1997). The biological consequences of these responses are associated with many pathological changes that occur in the course of inflammatory/immunologic and cardiovascular diseases such as septic shock and cardiac hypertrophy (Bogoyevitch *et al.*, 1996; Yin *et al.*, 1997; Zechner *et al.*, 1997). As a downstream kinase, PRAK should amplifying and diversifying p38's signals and thereby participate in one or more p38-mediated cellular responses. Since PRAK can phosphorylate HSP27 *in vitro* and phosphorylation of HSP27 has been suggested to play a role in actin reorganization, PRAK may mediate the downstream signal of the p38 pathway in regulating cytoskeletal organization. Two lines of evidence indicate that the p38 pathway plays an important role in regulating cytoskeletal organization: (i) activation of the p38 pathway is sufficient to confer sarcomeric organization in cardiac myocytes, and inhibition of p38α/β by SB203580 blocked phenylephrine, an α1-adrenergic receptor agonist, induced sarcomeric unit formation (Zechner *et al.*, 1997); and (ii) in HUVECs, oxidative stress-induced stress fiber formation was inhibited by SB203580 treatment (Huot *et al.*, 1997). In addition, overexpression of an inactive PRAK mutant in cardiac myocytes blocked p38 activation-induced hypertrophy characterized by sarcomeric unit formation and cell enlargement. However, expression of a constitutively active mutant of PRAK did not confer morphological changes observed by activation of the p38 pathway in cultured cardiac myocytes (Y.Wang, personal communication). Thus, PRAK may contribute to p38-mediated cytoskeletal reorganization in cardiac myocytes. Whether PRAK operates through phosphorylation of HSP27 to mediate p38 induced cytoskeletal changes awaits further investigation. Identification of other physiologically relev-

ant substrate(s) for PRAK is clearly the next important step in understanding the function of PRAK activation.

In summary, we have identified a new protein kinase regulated by the p38 MAP kinase pathway. The newly identified PRAK may represent a new group of MAP kinase-regulated protein kinases comparable with the RSK, MAPKAP-K and MNK groups. We have demonstrated that PRAK is strictly regulated by p38α/β *in vitro* and *in vivo*. PRAK is a major stress-activated kinase in HeLa cells that can phosphorylate HSP27. Identification of PRAK provides more information regarding the biological function of the p38 signaling pathway. The biological consequence of PRAK activation deserves further investigation.

Materials and methods

cDNA cloning

Two peptide sequences, LXTPCYTPYYAP and LXTPCTANFVAP from MAPKAP-K2/3 and RSK2/3, were used to search for new homologies of human MAP kinase-regulated protein kinases in the dbest DDBJ/EMBL/GenBank database using the tblastn program. Clones containing the LXTP sequence flanked by unknown sequences were considered as novel clones. Seven clones with DDBJ/EMBL/GenBank accession Nos AA08236, W24753, AA08397, AA157440, R68329, N57069 and H09985 were found by this search encoding three different cDNAs. Clones, AA157440, R68392 and N57096 were purchased from Research Genetics (Huntsville, AL), and were analyzed further by complete DNA sequencing (The Scripps Research Institute core facility, La Jolla, CA). A total of 5×10^5 phages of a human placental cDNA library (Stratagene, La Jolla, CA) were screened using R68392 DNA as probe. Three positive phage clones were purified after the secondary screen and used for phagemid rescue. The three isolated plasmids contained inserts of 1.2, 1.6 and 2.0 kb. DNA sequencing of each clone indicated that all three clones were from the same gene. The clone containing the longest insert (containing the full-length PRAK cDNA) was sequenced completely on both strands.

5′-RACE

The Marathon™ cDNA amplification kit (ClonTech, San Francisco, CA) and Marathon-ready human placental cDNA (ClonTech) were used for 5′-RACE as described by the manufacturer.

cDNA constructs and expression plasmids

Four PRAK single mutants, PRAK (93A), PRAK (182D), PRAK (182A) and PRAK (186A), and two PRAK double mutants, PRAK (212A, 214A) and PRAK (182D, 212D), were created by substituting S93 with A, T182 with D and A, T186 with A, both S212 and T214 with A and both T182 and S212 with D in the PRAK cDNA using a PCR-based method (Innis *et al.*, 1990). Oligonucleotide sequences used in the PCRs are available upon request. Full-length PCR products containing the different mutations, wild-type PRAK and MAPKAP-K2 (Zu *et al.*, 1994) were cloned downstream and in-frame to glutathione *S*-transferase (GST) in the vector pGEX-KG (Guan and Dixon, 1991). Each mutant clone was characterized by DNA sequencing.

Bacterial expression constructs of His_6-tagged MKK6(E), MKK7(D), p38α, p38β, p38γ, p38δ, ERK2, JNK2 and HSP27 were constructed as described previously (Jiang *et al.*, 1996, 1997). The spliced form of p38β with 364 amino acids (also known as $p38\beta_2$ DDBJ/EMBL/GenBank # AF001008) was used in the experiments described in this study. The HA epitope-tagged wild-type PRAK and mutant PRAK (182D) were cloned into the mammalian expression vector pcDNA3 (Invitrogen, San Diego, CA) by a PCR recombination method as described (Jiang *et al.*, 1996).

Recombinant proteins

GST fusion proteins of ATF2 (1–109) (Han *et al.*, 1996), c-Jun (1–93) (Derijard *et al.*, 1994), ELK1 (307–428) (Marais *et al.*, 1993) and those described above were expressed in the bacterial strain BL21 and purified using glutathione–Sepharose 4B beads (Pharmacia Biotech, Uppsala, Sweden) as described by the manufacturer. All of the His_6-tagged recombinant proteins were expressed in the BL21(DE3) strain and purified using the Ni-NTA purification system (Qiagen). PHAS-I was purchased from Stratagene (La Jolla, CA). Myelin basic protein (MBP) was from Sigma (St. Louis, MO). The eIF-4E expression plasmid was obtained from Dr C.G.Proud (University of Kent at Canterbury) and the recombinant eIF-4E was purified as described (Edery *et al.*, 1988). Activated MEK1 protein was a gift from Dr Kunliang Guan (University of Michigan Medical School).

Full activation of recombinant p38α, p38β, p38γ or p38δ *in vitro* was achieved by incubation with recombinant MKK6(E) at a 5:1 molar ratio at 37°C for 15 min in the presence of ATP. Full activation of recombinant ERK2 and JNK2 was achieved by incubation with MEK1(E) or MKK7(D) respectively. The different dose of MKKs and time of incubation have been tested to optimize the conditions for full activation of these recombinant MAPKs (data not shown). PRAK or MAPKAP-K2 were activated by incubating GST–PRAK or GST–MAPKAP-K2 fusion proteins coupled to glutathione–Sepharose beads with p38α and ATP at 37°C for 40 min with shaking. After incubation, the beads were washed three times with phosphate-buffered saline (PBS) to remove p38α and free ATP. Activated GST fusion proteins were then eluted as described above.

Northern blot analysis

A multiple human tissue mRNA blot containing 2 μg of poly(A)$^+$ RNA from various tissues was purchased from Clontech (San Francisco, CA). The blot was hybridized as described (Jiang *et al.*, 1996) to a probe prepared by labeling the coding region of the PRAK cDNA fragment with [α-^{32}P]dCTP using random priming (Han *et al.*, 1991).

Preparation of anti-PRAK antibody

Two rabbits were used to generate anti-PRAK polyclonal antibody using recombinant PRAK protein as described (Ausubel *et al.*, 1995). The specificity of the antibodies was examined by Western blot analysis and by immunoprecipitation using recombinant PRAK and MAPKAP-K2. The antibody raised in both rabbits can specifically detect PRAK, but not MAPKAP-K2 (data not shown). No detectable MAPKAP-K2 can be precipitated by anti-PRAK antibodies (data not shown).

Western blot analysis

Total cell lysates were prepared using lysis buffer A: 20 mM Tris–HCl, pH 7.5, 120 mM NaCl, 10% glycerol, 1 mM Na_3VO_4, 2 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 1% Triton X-100. Equal protein loading of cell extracts in SDS–PAGE was determined by Bio-Rad protein assay solution (Bio-Rad, Hercules, CA) and by staining the transferred nitrocellulose membrane with Ponceau S solution (Sigma, St Louis, MO). Rabbit polyclonal antibodies raised against bacterially expressed recombinant GST–PRAK and goat polyclonal antibody against MAPKAP-K2-specific peptide (RVDYEQIKIKKIEDASN) (Santa Cruz Biotech. Santa Cruz, CA) were used for Western blots. Standard Western blot methods were used (Han *et al.*, 1993).

Protein kinase assays

In vitro kinase assays were performed using standard experimental conditions as described (Jiang *et al.*, 1996). Prior to MAP kinase and MAP kinase-regulated protein kinase-coupled assays, the recombinant MAP kinases were activated by bacterially expressed constitutively active MKKs as described above. Kinase assays were carried out at 37°C for 30 min in a reaction volume of 40 μl, containing equal amount of each MAP kinase (0.5 μg) determined by Coomassie Blue staining, ~2 μg of GST–PRAK or GST–MAPKAP-K2, 10 μg of substrate, 250 μM ATP and 10 μCi of [γ-^{32}P]ATP. Reactions were terminated by addition of Laemmli sample buffer. Reaction products were resolved by SDS–PAGE and the extent of protein phosphorylation was visualized by autoradiography or phosphoimaging.

For the kinase assays with immunoprecipitated endogenous protein kinase or transiently expressed HA-tagged protein, ~10^6 cells grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) were used for each sample. The cells, with or without pre-treatment with SB203580 (Calbiochem, San Diego, CA) or PD98059 (New England Biolabs. Beverly, MA), were treated with different stimuli for the times specified in the figure legends or text. A total of 10^6 cells were lysed using 300 μl of lysis buffer containing 1% Triton X-100 as described (Jiang *et al.*, 1996). Endogenous PRAK or transiently expressed HA-tagged PRAK was precipitated by adding 10 μl of an anti-PRAK antibody or 2 μg of anti-HA monoclonal antibody 12CA5 to the nuclei-free cell lysates respectively and incubating for 2 h at 4°C, and then adding 10 μl of protein G–agarose beads and incubating with moderate stirring at 4°C for a further 1 h. The beads were washed five times with 1 ml of lysis buffer and subsequently used for kinase

assays using HSP27 as a substrate. p38α/β were precipitated by antibody against recombinant p38α which has cross-reactivity to p38β. ERK2 and JNK1 were immunoprecipitated using anti-ERK2 (UBI) and anti-JNK1 polyclonal antibodies (obtained from Dr M.Karin, University of California at San Diego) respectively. GST–ELK1 was used as substrate for ERK2. GST–ATF2 was used for JNK2 and p38α/β.

In-gel kinase assay

Ten μg of HeLa cell lysate with or without arsenite treatment was resolved by SDS–PAGE using a gel polymerized with 0.5 mg/ml HSP27. The separated proteins were denatured and renatured as described (Wang and Erikson, 1992). The kinase reaction was performed in 5 ml of the kinase buffer described above containing 50 μM ATP and 50 μCi of [γ-^{32}P]ATP for 1 h at 30°C. The reaction was terminated by washing the gel with a fixing solution containing 10 mM sodium pyrophosphate and 5% trichloroacetic acid. The gel was dried and subjected to autoradiography.

In vitro binding of PRAK and p38

GST–PRAK bound to glutathione–agarose beads and His-tagged p38α, p38β, p38γ, p38δ, ERK2 and JNK2 were used to examine the *in vitro* interaction of PRAK with each of these MAP kinases as described (Han *et al.*, 1997).

Construction and preparation of recombinant adenovirus

The recombinant adenovirus encoding MKK1(E) [also known as MEK1(E)] or MKK6(E) was constructed and prepared as previously described (Huang *et al.*, 1997b). The spliced form of MKK6 with 334 amino acids (also known as MKK6b) was used in the experiment. MKK7D expression virus was created using the same method (Huang *et al.*, 1997b). We established that nearly 100% infection of HeLa cells can be achieved at an m.o.i. of 10.

Phosphoamino acid analysis, phosphopeptide mapping and Edman degradation

These experiments were performed as described by Boyle *et al*. (1991).

Cell transfections

HeLa cells were plated in 6-well plates with 50% confluence in DMEM supplemented with 10% FBS. After 24 h, the cells was transfected with expression vectors containing HA-tagged PRAK or empty vector pcDNA3 using lipofectamine (Life Technologies, Inc., Grand Island, NY). After 36 h, the cells were treated with the different stimuli for 30 min and the kinase activity of HA-tagged PRAK was assayed as described (Han *et al.*, 1996).

Acknowledgements

We thank Dr Christopher G.Proud for eIF-4E expression plasmid, Dr Hermann Gram for JNK2 protein, and B.Chastain for secretarial assistance. This work was supported by National Institutes of Health Grants GM51417, AI41637 and American Heart Association Grant-In-Aid 95007690. J.H. is an established investigator of the American Heart Association. This is publication number 11296-IMM from the Department of Immunology of The Scripps Research Institute.

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Received November 27, 1997; revised and accepted April 7, 1998