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The interleukin-1-stimulated protein kinase that phosphorylates heat shock protein hsp27 is activated by MAP kinase

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Abstract

In KB cells, interleukin-1 (IL-1), epidermal growth factor and phorbol ester transiently activated both MAP kinase and a serine kinase which phosphorylated the heat shock protein hsp27. Extracts made from IL-1-stimulated KB cells phosphorylated recombinant hsp27, in vitro, on serine residues 78 and 82 which are contained within Arg-X-X-Ser motifs similar to those phosphorylated by the ribosomal protein S6 kinases. Upon size exclusion chromatography, however, hsp27 kinase eluted as a single peak of activity at 50–60 kDa, clearly separated from ribosomal protein S6 kinases. Treatment of partially purified hsp27 kinase with protein phosphatase-2a reduced its activity by 80%. De-phosphorylated hsp27 kinase could be approximately 50% reactivated by a factor present in IL-1-treated cell extracts in the presence of ATP. This factor co-eluted with MAP kinase after partial purification by DEAE-cellulose, phenyl Sepharose, and size exclusion chromatography. Purified sea star $p44^{mpk}$ and recombinant ERK2 MAP kinases were also capable of re-activating hsp27 kinase to a similar extent. These data suggest that hsp27 kinase is downstream from, and probably a direct target of MAP kinase.

Key words: Protein kinase; Interleukin-1; Signal transduction; Protein phosphorylation

1. Introduction

Interleukin-1 (IL-1) is a cytokine capable of inducing many pro-inflammatory and immunologic responses [1]. The signal transduction pathways linking cell surface IL-1 receptors to gene activation are unclear [2], but one of the earliest cellular responses to IL-1 is the activation of protein serine/threonine kinases. We recently identified [3] one class of these enzymes as mitogen-activated protein kinases (MAP kinases, also called extracellular-signal regulated kinases, reviewed in [4]). MAP kinases are activated by many different extracellular stimuli which may associate with cellular receptors having integral or associated tyrosine kinases, or which couple with GTP-binding proteins [5]. As well as stimulating the activity of MAP kinase, IL-1 has been shown to cause the rapid phosphorylation of the small heat shock protein hsp27 in fibroblasts, endothelial cells, HepG2 hepatoma cells and U937 cells [6–8]. The synthesis of hsp27 is elevated in response to various cellular insults and it may play a role in the development of tolerance to heat shock [9] or other stresses. Although the precise cellular function of this protein is not known, it inhibits the polymerization of actin [10]. As is the case for MAP kinase activation, many other agents besides IL-1 have been reported to cause increased hsp27 phosphorylation in various cells. These include tumor necrosis factor [6], bradykinin [8], fibroblast growth factor, ATP [8], serum, PMA [11], and leukemia inhibitory factor [12]. Recently, the major sites of phosphorylation of human hsp27 [13] and its murine homolog [14] were found to reside in the conserved motif Arg-X-X-Ser, a motif that is also recognized by ribosomal protein S6 kinase II (RSK). RSK is phosphorylated and activated by MAP kinase [15]. The aim of the present study was to determine if the IL-1-stimulated hsp27 kinase activity is, like RSK, potentially dependent upon upstream activation of MAP kinase.

2. Materials and methods

2.1. Materials

KB (ATCC CCL17) human epidermoid carcinoma cells were maintained in Dulbecco's modified medium (DMEM) supplemented with 10% fetal bovine serum (Intergen, Purchase NY) and were used within 24 h of attaining confluence. Cells were stimulated with IL-1α (20 ng/ml), PMA (100 ng/ml, added in DMSO; 0.01% final DMSO concentration) of EGF (50 ng/ml) by the direct addition of these agents to the maintainance medium, typically for 15 min, before cell lysis as de-

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Abbreviations: EGF, epidermal growth factor; hsp27, 27 kilodalton heat shock protein; IL-1, interleukin-1α; MAP kinase, mitogen-activated protein kinase; MOPS, 3-(*N*-morpholino)propanesulfonic acid; PMA, phorbol 12-myristate 13-acetate; PP-2a, protein phosphatase, type-2a; RSK, ribosomal protein S6 kinase II.

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scribed below. Human recombinant IL-1 was prepared as described previously [3], EGF was obtained from Sigma. Recombinant human hsp27, expressed in *E. coli*, and a monoclonal antibody reactive against hsp27 were purchased from StressGen Biotechnologies Corp., (Victoria B.C., Canada). MAP kinase substrate peptide T669 (RRREL-VEPLTPSGE) and peptide S82 (RRRALSRQLSSGVSEIA) corresponding, respectively, to amino acid residues 663–673 of the human EGF receptor [16] plus three additional N-terminal arginine residues and to amino acids 75–88 of hsp27 [17] including two additional N-terminal arginines were synthesized on an Applied Biosystems 430A peptide synthesizer and were purified by hplc. Identity and purity of each peptide was confirmed by ^{252}Cf mass spectrometry. The peptide RRRLSSLRA corresponding to amino acids 231–239 of human 40S ribosomal protein S6 [18] was purchased from UBI. (Lake Placid, NY). The pseudosubstrate inhibitor of protein kinase C (RFARKGALRQ-KNV) and protein kinase A inhibitor (TTYADFIASGRTGRRNAI-HD) were synthesized as described above. Protein phosphatase-2a, and MAP kinase $p44^{mpk}$ from *Pisaster ochraceus* were purchased from UBI. Recombinant rat ERK-2 containing a 6 histidine N-terminal extension was expressed in *E. coli* and activated with highly purified rabbit skeletal muscle MAP kinase kinase and was the kind gift of Dr. Lee Graves (University of Washington, Seattle, WA). Okadaic acid was obtained from Gibco BRL (Grand Island, NY)

2.2 Protein kinase assays

KB cell monolayers were treated with IL-1 or other agents for various times, washed with ice-cold PBS containing 0.2 mM sodium orthovanadate and then scraped into a lysis buffer (1 ml per 10^7 cells) containing 30 mM MOPS, pH 7.0/10% (w/v) glycerol/80 mM β-glycerophosphate/20 mM EGTA/0.1 mM sodium orthovanadate/10 mM $MgCl_2$/0.1% Triton X-100/5 mM dithiothreitol/2 mM phenylmethylsulfonyl fluoride/10 μM pepstatin A/10 μM leupeptin. Cells were lysed by passage through 25-gauge hypodermic needles, followded by centrifugation at 13,000 × *g* for 30 min at 4°C. Unfractionated lysates, or column fractions (5–10 μl) were incubated for 15 min at 30°C in a final volume of 30 μl with either recombinant hsp27 (0.5–1 μg) or peptide S82 (0.5 mM). Reactions also contained 100 μM [γ-^{32}P]ATP (0.5 μCi/nmol), 30 mM *p*-nitrophenyl-phosphate, 5 μM protein kinase C inhibitor, 20 μM protein kinase A inhibitor, 10 mM MOPS, pH 7.0 and 10 mM $MgCl_2$. Reactions containing hsp27 were stopped by adding 10 μl of a 4 × concentrated SDS-PAGE sample buffer and boiling. The samples were electrophoresed on 8–16% polyacrylamide gels [19] which were then stained with Coomassie brilliant blue R, dried and exposed to Kodak storage phosphor screens. Incorporation of radiolabeled phosphate into hsp27 was visualized and quantitated using a phosphorimager (Molecular Dynamics, Sunnyvale, CA). Incorporation of [^{32}P]phosphate into S82 peptide or S6 peptide was measured by acidifying the reaction mixtures with formic acid (25% final concentration) and spotting aliquots onto P81 phosphocellulose paper discs. The discs were washed with 75 mM phosphoric acid, dried, and Cerenkov counted. Preparation of cell lysates containing MAP kinase and assay of MAP kinase using T669 peptide substrate have been described in detail elsewhere [3].

2.3 Peptide mapping

KB cells (1×10^7) were metabollically labelled for 2 h in medium supplemented with 0.5 mCi/ml [^{32}P]orthophosphate before addition of IL-1 or diluent. After a further 15 min, the cells were washed twice with cold PBS, lysed in 1 ml of RIPA buffer (20 mM Tris-HCl pH 7.5/150 mM NaCl/1% NP-40/1% sodium deoxycholate/0.1% SDS/15 mM *p*-nitrophenylphosphate/10 mM NaF/0.2 mM sodium orthovanadate) and clarified by microcentrifugation for 10 min at 4°C. Aliquots of the labelled cell lysates were incubated at 4°C for 2 h with 20 μg of anti-hsp27 antibody and 10 μl of Pansorbin (Calbiochem). Immunoprecipitates were washed extensively, eluted by boiling in SDS-PAGE sample buffer and resolved by SDS-PAGE. The gels were subjected to autoradiography to localize labelled hsp27 which was excised from the gels, eluted, oxidized and digested with TPCK-treated trypsin as described [20]. S82 peptide was phosphorylated in the presence of [γ-^{32}P]ATP and a lysate made from IL-1-treated cells as described above. The peptide was separeated from unreacted ATP by electrophoresis on a cellulose TLC plate in a pH 3.5 buffer (pyridine/acetic acid/water, 10:100:1890), eluted with formic acid and digested with TPCK-trypsin. Radiolabelled tryptic fragments of hsp27 and S82 peptide were compared by two-dimensional mapping on cellulose TLC plates. The first dimension consisted of electrophoresis at either pH 3.5 (1.4 kV, 30 min) or pH 8.9 (1% ammonium bicarbonate buffer, 1 kV for 25 min). In the second dimension, the plates were developed by ascending chromatography in *n*-butanol/acetic acid/pyridine/water (15:3:12:10). The plates were analysed using a Phosphorimager. Sequence analysis of ^{32}P-labeled S82 peptide was performed using a modified ABI model 475A liquid-pulsed sequencer. Following every cycle of automated NH_2-terminal sequence analysis 15% of each PTH-amino acid was identified and quantitated; the remaining 85% was collected and counted by liquid scintillation spectrometry.

2.4. Partial purification of hsp27 kinase and MAP kinase

Cell extracts, prepared as described above, were loaded onto a Mono Q HR 5/5 column (Pharmacia) equilibrated with buffer A (25 mM HEPES, pH 7.4, 2 mM EDTA, 1 mM DTT, 80 mM β-glycerophosphate, 0.2 mM PMSF, 2 μM leupeptin, 2 μM pepstatin A, 25 mM *p*-nitrophenyl phosphate and 10% ethylene glycol). All chromatographic steps were performed at 4°C. After loading, the column was washed with five column volumes of buffer A, then eluted with a linear salt gradient from 0–750 mM NaCl in buffer A. Selected fractions were assayed for kinase activity using hsp27, S82 peptide and S6 peptide as substrates, the remainder of each fraction was frozen and stored at −80°C. Fractions containing the major IL-1-stimulated hsp27 kinase activity were thawed and pooled and a portion chromatographed on a Superose 12 HR 10/30 column (Pharmacia) equilibrated with buffer A. Aliquots were removed from selected fractions for kinase assays then all fractions were frozen and stored at −80°C. MAP kinase was purified from large-scale cultures of IL-1-stimulated KB cells by sequential chromatography on DEAE-cellulose, Phenyl-Sepharose and Superose 12 exactly as described [3].

2.5. Dephosphorylation and reactivation of hsp27 kinase

Hsp27 kinase, partially purified as described above, was divided into two portions. One half was concentrated fivefold using a Centriprep 10 concentrator (Amicon), diluted back to the original volume with buffer A (containing phosphatase inhibitors) and reconcentrated to one-fifth of the original volume. The retentate was than desalted using Bio-Gel P-6DG spin columns equilibrated in buffer A. The other half of the material was subjected to the same procedure except that the buffers used for desalting and concentration lacked EGTA and the phosphatase inhibitors β glycerophosphate and *p*-nitrophenyl phosphate. One hundred microliters of the preparation containing phosphatase inhibitors was mixed with 2 μl of 2.5 μM okadaic acid, and an equal volume of the sample lacking inhibitors was mixed with 2 μl of 10% DMSO vehicle. Each mixture was then treated with 0.5 units of phosphatase-2a for 30 min at 30°C. Okadaic acid was then added to the sample lacking phosphatase inhibitors ('inactive hsp27 kinase'), and vehicle to the other sample ('active hsp27 kinase'). Both preparations were mixed with 50 μl of a reactivation buffer containing 30 mM MOPS pH 7.5, 90 mM *p*-nitrophenyl phosphate, 30 mM β-glycerophosphate, 0.3 mM Na_3VO_4, 0.3 mM Na_2MoO_4, 30 mM $MgCl_2$, 30 mM NaF, 2 mM dithiothreitol, 100 μM ATP, 1 mM PMSF, 10 μM leupeptin and 10 μM pepstatin A. Aliquots (25 μl) were mixed with 5 μl of either partially purified MAP kinase (see above, specific activity 0.464 pmol phosphate transferred to T669 peptide per minute per μl), purified p44mpk (0.422 pmol/min/μl), recombinant ERK-2 (0.108 pmol/min/μl), or buffer control. After a further 60 min incubation at 30°C, 1 μg of hsp27 and 1 μCi of [γ-^{32}P]ATP were added to each tube in 10 μl of 20 mM MOPS pH 7.5, 10 mM $MgCl_2$, and the reactions were allowed to proceed for a further 20 min. The incorporation of [^{32}P]phosphate into hsp27 was analysed as described above.

3. Results

Extracts prepared from IL-1-treated KB cells were incubated with recombinant hsp27 and [γ-^{32}P]ATP. The reaction products were separated by SDS-PAGE and visualized by phosphorimaging (Fig. 1). IL-1 caused a greater than 10-fold increase in the incorporation of

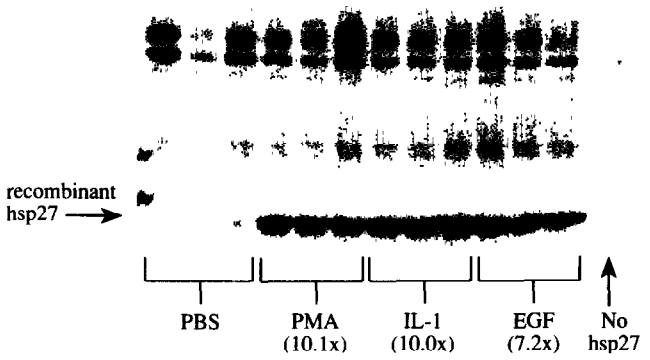

Fig. 1. In vitro phosphorylation of recombinant human hsp27 by cytoplasmic extracts from KB cells. Groups of three dishes of KB cells were treated with 100 ng/ml PMA (lanes 4–6), 20 ng/ml IL-1 (lanes 7–9), 50 ng/ml EGF (lanes 10–12) or phosphate-buffered saline (lanes 1–3 and 13) for 15 min. Aliquots of cell lysates (10 μl) were incubated with [γ-^{32}P]ATP and hsp27 (0.5 μg, lanes 1–12) or diluent (lane 13) in an in vitro kinase assay as described in section 2. Phosphorylated proteins were separated by SDS-PAGE. Incorporation of [^{32}P]phosphate into hsp27 (arrowed) was quantitated using a phosphorimager. A grey-scale image is reproduced here, and the increase in hsp27 phosphorylation with respect to the resting level (mean fold-induction) is indicated for each treatment.

phosphate into hsp27, consistent with the activation of one or more protein kinases. PMA and EGF, which cause comparable increases in MAP kinase activity in KB cells, also stimulated hsp27 kinase activity to a similar extent. It was important to determine if the kinase(s) stimulated by IL-1 phosphorylated hsp27 on the same sites that have been previously reported for heat shock-, mitogen-activated, or endogenously-active kinases from various cell lines [13,14]. We accordingly synthesized a peptide which corresponded to the amino acids flanking these sites. This peptide, S82, was indeed phosphorylated to high stoichiometry by extracts from IL-1-treated KB cells, at a rate 1.5-3 times that obtained using extracts from untreated cells (data not shown). Because the peptide was also a substrate for the catalytic subunit of protein kinase A (data not shown) we routinely included a specific inhibitor of this enzyme in our in vitro assays. Endogenous hsp27 was immuno-precipitated from resting and IL-1-stimulated KB cells metabolically labelled with [^{32}P]phosphate. This material, comprising a mixture of all phosphorylated isoforms, together with phosphorylated peptide S82, was digested with trypsin and 2D-phosphopeptide maps were prepared (Fig. 2). IL-1 treatment of intact cells caused an increase in the intensity of a major phosphopeptide (peptide 1 in the figure) and the appearence of two new phosphopeptides (4 and 5) when the first-dimension electrophoresis was carried out at pH 8.9. All of these peptides, and a faint additional peptide (number 2) were detectable in digests of phosphorylated peptide S82. If electrophoresis was performed at pH 3.5, two major and one minor peptide were detected in extracts from IL-1-stimulated cells, these were superimposible with peptides derived from peptide S82 (not shown).

We next compared the time courses for IL-1-stimulated phosphorylation of hsp27, peptide S82, and a peptide based upon the RSK phosphorylation site in ribosomal protein S6, with the time course of MAP kinase activation (Fig.3). Although the basal and maximally stimulated levels of the various activities differed considerably, all displayed similar transient kinetics, with the peak of hsp27 kinase activity being just slightly later than the peak of MAP kinase activity. Phosphorylation of S82 and S6 peptides was particularly well correlated.

In order to characterise the IL-1-stimulated hsp27 kinase further, we fractionated extracts from resting and IL-1-stimulated KB cells by ion-exchange chromatography. A major peak (peak II) and minor peak (peak III) of hsp27 kinase activity were found in untreated cell extracts eluting at 230 mM and 320 mM NaCl, respectively (Fig. 4a). Peak III was unchanged in IL-1-stimulated cells and was not characterized further. Extracts from IL-1-stimulated cells contained a new peak (peak I) which eluted at 180 mM NaCl, but which also contained a shoulder coinciding with the peak II present in control cells. Whether peak I represents a kinase not active in resting cells or arises as a result of an activating modification of peak II hsp27 kinase is not clear at this time. Column fractions were also assayed for RSK activity using the S6 peptide substrate. More variable results were obtained, but usually at least four poorly-resolved

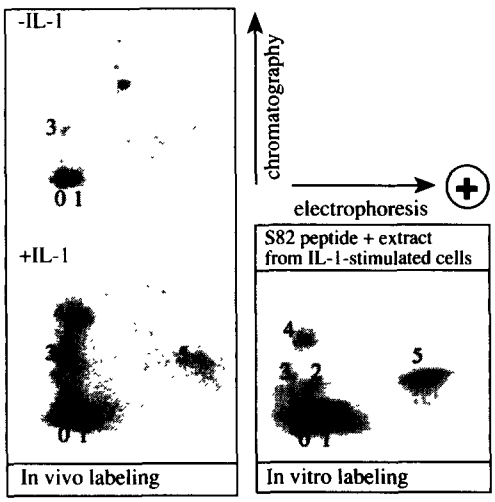

Fig. 2. Two-dimensional phosphopeptide mapping of hsp27. KB cells were metabolically labeled with [^{32}P]orthophosphate then treated with IL-1 or vehicle for 15 min. Hsp27 was immunoprecipitated from lysates of the cells and purified by SDS-PAGE, and digested with TLCK-treated trypsin. Peptide S82 (36 nmol) was phosphorylated in vitro using hsp27 kinase from IL-1-treated KB cells. Radiolabeled peptide was purified by thin-layer electrophoresis and also digested with TLCK-trypsin under identical conditions. Samples of the hsp27 peptides from resting cells (70 Cerenkov counts/min, top left), IL-1-treated cells (100 cpm, bottom left), and S82 peptide (170 cpm, bottom right) were spotted onto TLC plates (0 = origin) and subjected to two dimentional mapping as described in section 2.

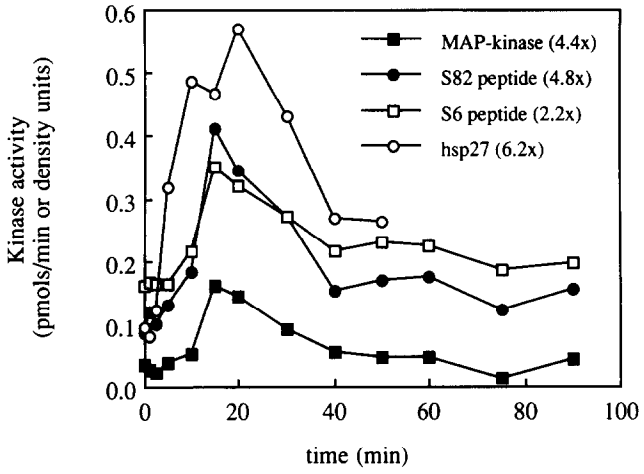

Fig. 3. Kinetics of activation of protein kinase activities by IL-1. KB cells were stimulated for various times with IL-1 (20 ng/ml) or were left unstimulated; lysates were prepared and assayed for their ability to phosphorylate hsp27 (600 nM in the kinase assay) or peptides T669 (1 mM, MAP kinase activity), S82 (0.5 mM) and S6 (0.5 mM). The maximum fold-increase of each kinase activity is indicated in parentheses.

peaks of activity were observed from both stimulated and unstimulated extracts (Fig. 4b). There was a superficial similarity in the activity profiles of peptide S6 kinase and the hsp27 kinase in the region of peaks I and II, though the difference between stimulated and unstimulated levels was not as great as with hsp27 kinase. The other kinases detected using S6 peptide as substrate were apparently incapable of phosphorylating hsp27. Mono Q fractions were also assayed for kinase activity using peptide S82 as substrate and the activity profile correlated precisely with that obtained using S6 peptide (data not shown). Peak I hsp27 kinase was further fractionated by size exclusion chromatography on Superose 12 (Fig. 4c). Three partially resolved peaks of activity were found that were capable of phosphorylating peptide S82; eluting at positions expected for proteins of molecular mass around 150 kDa, a broad shoulder at 45–60 kDa, and at 25–30 kDa, respectively (data not shown). However, only a single peak of hsp27 kinase was detected, at a position corresponding to the 45–60 kDa peak of S82 kinase activity. More importantly, S6 kinase activity was clearly resolved from hsp27 kinase, and comprised a major peak eluting just within the included volume of the column, and a smaller peak which eluted later than hsp27 kinase (Fig. 4c). Some of the hsp27 kinase peak was used to phosphorylate peptide S82 in the presence of [γ-^{32}P]ATP. During radiosequence analysis of the phosphorylated peptide, radioactivity was recovered after six and ten degradative cycles indicating that the phosphorylated residues coresponded to Ser^{78} and Ser^{82}, respectively (data not shown).

Some of the protein serine/threonine kinases thought to be involved in the response of cells to mitogens are known to be regulated by phosphorylation catalysed by upstream regulatory kinases [4,15]. We attemped to determine if the same was true for hsp27 kinase. A sample of partially-purified hsp27 kinase was subjected to a two-stage procedure designed to dephosphorylate potentially regulatory phosphoaminoacids in hsp27 kinase. The first step involved the removal of phosphatase inhibitors. The second step consisted of treating this material with purified protein phosphatase-2a (PP-2a), which selectively dephosphorylates phosphoserine and phosphothreonine residues. Fig. 5 shows that hsp27 kinase treated with PP-2a and then with okadaic acid (a selective and extremely potent inhibitor of this enzyme) contained only 18% of the activity of hsp27 kinase treated with okadaic acid prior to PP-2a. (compare the left-hand lanes in panels 2 and 3, respectively). In initial experiments (not

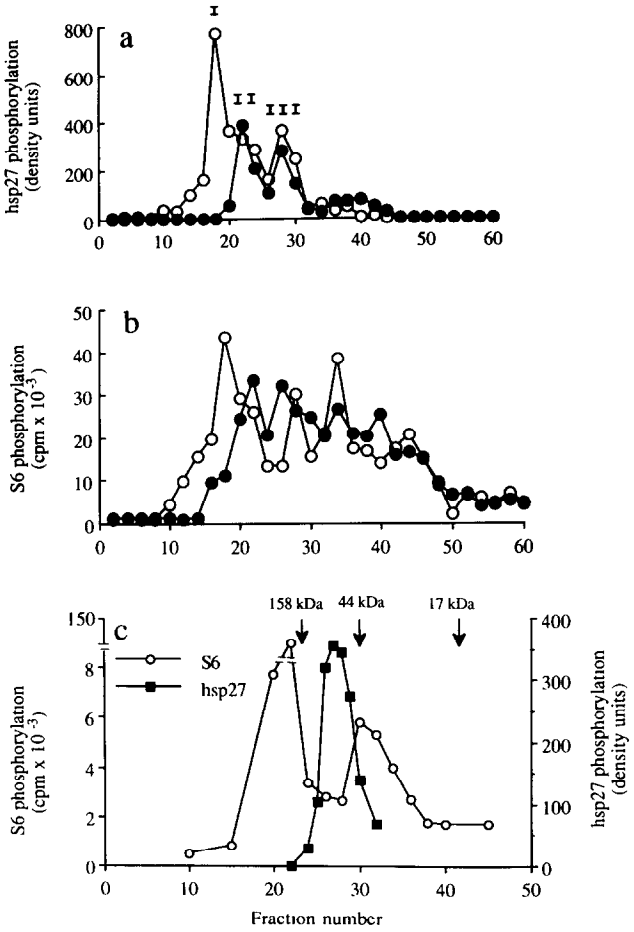

Fig. 4. Chromatographic characterization of IL-1-stimulated hsp27 kinase. (a) Lysates prepared from four confluent 175 cm^2 flasks of IL-1-stimulated KB cells (○) or resting cells (●) were applied to a Mono Q column. The column was developed as described in section 2. Aliquots of selected fractions were assayed for their ability to phosphorylate hsp27. In (b) the same fractions were assayed for peptide S6 kinase activity. (c) IL-1-stimulated fractions 17–19 from the Mono Q column were pooled, concentrated and chromatographed on a Superose 12 column as described in section 2. Selected fractions were assayed for kinase activity using either hsp27 (■) or peptide S6 (○) as substrate. Molecular mass standards used to calibrate the column were bovine γ-globulin (158 kDa), chicken ovalbumin (44 kDa), and equine myoglobin (17 kDa).

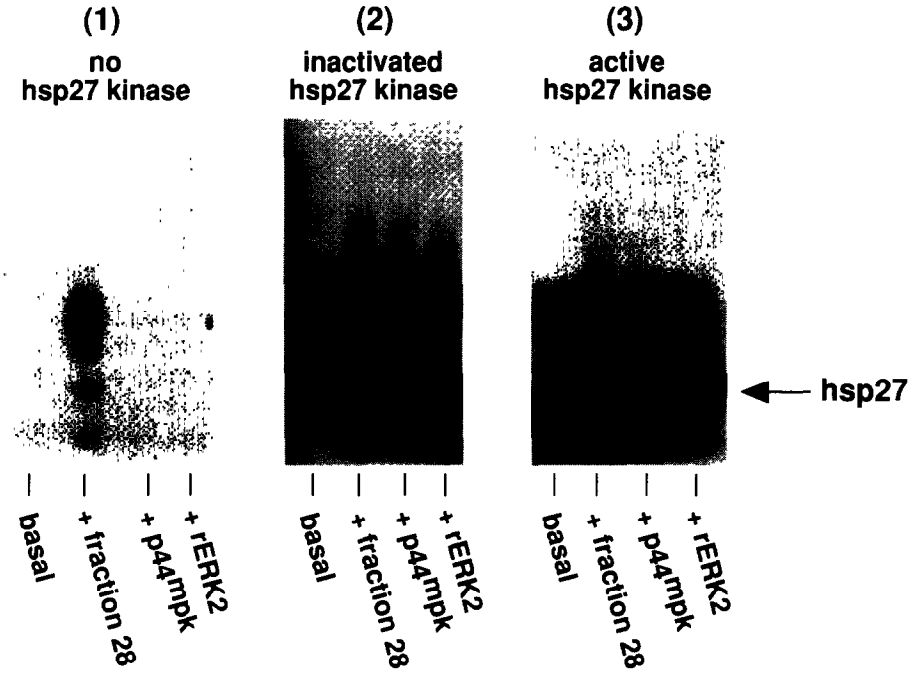

Fig. 5. Inactivation of hsp27 kinase by de-phosphorylation and re-activation by MAP kinase. Partially-purified hsp27 kinasewas de-phosphorylated by treatment with PP-2a followed by okadaic acid (panel 2) or maintained in a phosphorylated active state by addition of okadaic acid prior to PP-2a (panel 3). Aliquots of each sample were incubated in the presence of ATP, magnesium, and either a buffer control (basal), partially-purified MAP kinase (fraction 28), p44mpk, or recombinant ERK-2, as indicated. Subsequently, [γ-^{32}P]ATP and hsp27 were added. The MAP kinases were also incubated with hsp27 in the absence of hsp27 kinase (panel 1). Incorporation of labeled phosphate into hsp27 (arrowed) was determined as described in section 2.

shown) cell lysates were depleted of MAP kinase by passage over DEAE Sephacel. Hsp27 kinase in these crude preparations was inactivated by de-phosphorylation and could be fully re-activated when mixed with a small amount of unfractionated cell extract in the presence of $MgCl_2$ and ATP, the re-activating activity was retained by DEAE-Sephacel and phenyl sepharose columns and co-chromatographed with MAP kinase on an analytical size exclusion chromatography column. We therefore tested various MAP kinase preparations for their ability to re-activate the partially purified de-phosphorylated hsp27 kinase (Fig. 5, panel 2). The peak fraction of IL-1-stimulated MAP-kinase from Superose 12 (fraction 28 in Fig. 5) re-activated hsp27 kinase to about 50% of the control activity, sea star $p44^{mpk}$, with the same specific activity against T669 peptide also re-activated hsp27 kinase to a similar extent. Recombinant ERK-2, activated in vitro with MAP kinase kinase caused a lesser activation, but this preparation also contained less T669-phosphorylating activity. None of the MAP kinase preparations enhanced the activity of 'active' hsp27 kinase (Fig. 5, panel 3). In the absence of added hsp27 kinase, neither $p44^{mpk}$ nor ERK-2 were capable of detectably phosphorylating hsp27. A very low level of endogenous hsp27 phosphorylation was detected in partially purified IL-1-stimulated MAP kinase attributable to the small amount of hsp27 kinase which binds to DEAE-cellulose and co-elutes with MAP kinase upon phenyl Sepharose chromatography (GDV and TAB, unpublished); this activity was too small to acount for the observed re-activation, however.

4. Discussion

Here we provide an initial characterization of the IL-1-stimulated protein serine kinase that phosphorylates hsp27. An earlier study by Guesdon and Saklatvala [21] demonstrated that cytosolic extracts of IL-1-stimulated MRC-5 fibroblasts phosphorylated hsp27 at a greater rate than unstimulated cells to produce the same set of phosphorylated hsp27 isoforms as were seen in intact cells metabolically labeled with [^{32}P]phosphate. Landry and co-workers [13] reported that heat shock and mitogens (serum, thrombin, and fibroblast growth factor) co-induced kinases that were able to phosphorylate hsp27 and ribosomal protein S6 in vitro. Similarly, the murine homolog of hsp27, hsp25, was phosphorylated in vitro by an endogenous kinase from Ehrlich ascites cells [14]. No biochemical characterization of the activated kinase(s) was reported in these studies. In our study we found a single major peak of IL-1-stimulatable hsp27 kinase activity (peak I) which was also capable of phosphorylating a peptide substrate. However, there are clearly a number of other kinases present in both IL-1-treated and resting cells which will phosphorylate the

peptide substrates but not hsp27. Upon size exclusion chromatography, peak I hsp27 kinase eluted as a 45–60 kDa protein, distinguishing it from previously described S6 kinases of 70 kDa and 90 kDa [22]. Notably, only one of three S82 peptide kinases resolved by size exclusion chromatography phosphorylated recombinant hsp27. Taken together, the chromatographic data suggest that the phosphorylation site present in the folded hsp27 polypeptide may be accessible to hsp27 kinase but not to these other kinases.

The reversible inactivation of hsp27 kinase by PP-2a strongly suggests that its activity is positively modulated by phosphorylation. Although the data shown in Fig. 5 strongly suggest that MAP kinases are good candidates for activators of hsp27 kinase, we were unable to achieve complete re-activation of partially-purified hsp27 kinase with any of the enzymes used. There may be technical reasons for this, or additional factors may be required for full activation. The latter possibility is supported by our observation that cruder preparations of hsp27 kinase can be completely re-activated by the addition of MAP kinase, therefore we cannot exclude the possibility that other hsp27 kinase activators are present in unfractionated hsp27 kinase preparations. Other potential activators may exist which are not detectable using our methods.

Interleukin-1, PMA, and EGF activate cells in different ways; the IL-1-receptor does not possess intrinsic tyrosine kinase activity, not does it activate protein kinase C [2]. Arguably, the only signal that all three agents generate in common is MAP kinase activation. Furthermore, the similar kinetics of activation of MAP kinase and hsp27 kinase is consistent with their being closely coupled. Confirmation that MAP kinase is neccessary for the activation of hsp27 kinase in intact cells will require the specific inactivation of the former, perhaps by the use of antisense strategy or dominant negative mutants of MAP kinase. Stokoe et al. [23] have recently identified MAP kinase activated protein kinase 2 (MAPKAP kinase 2) as the major enzyme responsible for phosphorylation of the small heat shock proteins of mammalian cells. Rabbit skeletal muscle MAPKAP kinase 2 was recently purified as two related isoforms of 60 and 53 kDa which are dependent upon phosphorylation by p44 or p42 MAP kinases for activity [24]. Like the enzyme we describe, MAPKAP kinase 2 preferentially phosphorylates human hsp27 at Ser^{82} and Ser^{78}. It is therefore possible that the IL-1-activated hsp27 kinase present in KB cells is a human homolog of rabbit MAPKAP kinase 2.

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