Dephosphorylation of the Small Heat Shock Protein Hsp27 in Vivo by Protein Phosphatase 2A*

(Received for publication, September 14, 1993, and in revised form, November 22, 1993)

Jennifer Cairns, Shixin Qin, Robin Philp, Y. H. Tan, and Graeme R. Guy†

From the Signal Transduction Laboratory, Institute of Molecular and Cell Biology, National University of Singapore, 10 Kent Ridge Crescent, Singapore 0511

The phosphorylation of the Hsp27 complex is rapidly altered in MRC-5 cells when they are exposed to mitogens, cytokines, stress, or serine/threonine protein phosphatase inhibitors. Here we performed experiments to identify which cellular protein phosphatase (PP1, PP2A, or PP2B) is responsible for the in vivo phosphorylation/dephosphorylation of Hsp27. In their purified forms, PP2A dephosphorylates Hsp27 more effectively than PP2B, whereas PP1 is weakly active. Measurements of enzyme activity oflysates derived from inhibitor-treated cells indicated that Hsp27 phosphatase activity is equally sensitive to okadaic acid (PP1/PP2A inhibitor) and cyclosporin (PP2B inhibitor) and that both okadaic acid and cyclosporin treatment inhibited Hsp27 phosphatase activity in vitro. Together the in vitro data suggest that both PP2A and PP2B can dephosphorylate Hsp27. However, the phosphorylation of Hsp27 in vivo is only affected when cells are treated with PP1 and PP2A inhibitors (okadaic acid, calyculin A) or cantharidin (PP2A inhibitor), but not the PP2B inhibitor, cyclosporin A, suggesting PP2A to be the main enzyme dephosphorylating Hsp27 in the cells. Purification and immuno blotting of Hsp27 phosphatase from MRC-5 cells also suggest it to be PP2A and not PP1 or PP2B. The ability of PP2A to dephosphorylate Hsp27 is shown to be regulated by the phosphorylation state of PP2A itself.

Treatment of eukaryotic/prokaryotic cells by higher than normal growth temperature, anoxia, glucose deprivation, or free radicals is associated with an increased synthesis of a family of proteins known as the heat shock proteins (Hsps) (1–5). They include proteins which are the small Hsps, such as the heat shock protein Drosophila, and the human endometrial and cervical tumors. The phosphorylation of the Hsp27 complex is rapidly altered in MRC-5 cells when they are exposed to mitogens, cytokines, stress, or serine/threonine protein phosphatase inhibitors. Here we performed experiments to identify which cellular protein phosphatase (PP1, PP2A, or PP2B) is responsible for the in vivo phosphorylation/dephosphorylation of Hsp27. In their purified forms, PP2A dephosphorylates Hsp27 more effectively than PP2B, whereas PP1 is weakly active. Measurements of enzyme activity of lysates derived from inhibitor-treated cells indicated that Hsp27 phosphatase activity is equally sensitive to okadaic acid (PP1/PP2A inhibitor) and cyclosporin (PP2B inhibitor) and that both okadaic acid and cyclosporin treatment inhibited Hsp27 phosphatase activity in vitro. Together the in vitro data suggest that both PP2A and PP2B can dephosphorylate Hsp27. However, the phosphorylation of Hsp27 in vivo is only affected when cells are treated with PP1 and PP2A inhibitors (okadaic acid, calyculin A) or cantharidin (PP2A inhibitor), but not the PP2B inhibitor, cyclosporin A, suggesting PP2A to be the main enzyme dephosphorylating Hsp27 in the cells. Purification and immunoblotting of Hsp27 phosphatase from MRC-5 cells also suggest it to be PP2A and not PP1 or PP2B. The ability of PP2A to dephosphorylate Hsp27 is shown to be regulated by the phosphorylation state of PP2A itself.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† To whom correspondence should be addressed.

* The abbreviations used are: Hsp, heat shock protein(s); Hsp27 and Hsp25, 27- and 25-kDa heat shock protein, respectively; TNF, tumor necrosis factor; IL, interleukin; PP, protein phosphatase; OA, okadaic acid; DOA, docosahexaenoic acid; MAP, mitogen-activated protein; MAPKAP, mitogen-activated protein kinase; MAPKAP kinase, mitogen-activated protein kinase-activated protein kinase; PAG, polyacrylamide gel electrophoresis; HPLC, high performance liquid chromatography; cat, catalytic subunit.

** The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† To whom correspondence should be addressed.

* The abbreviations used are: Hsp(s), heat shock protein(s); Hsp27 and Hsp25, 27- and 25-kDa heat shock protein, respectively; TNF, tumor necrosis factor; IL, interleukin; PP, protein phosphatase; OA, okadaic acid; DOA, docosahexaenoic acid; MAP, mitogen-activated protein; MAPKAP, mitogen-activated protein kinase; MAPKAP kinase, mitogen-activated protein kinase-activated protein kinase; PAG, polyacrylamide gel electrophoresis; HPLC, high performance liquid chromatography; cat, catalytic subunit.

† To whom correspondence should be addressed.

* The abbreviations used are: Hsp(s), heat shock protein(s); Hsp27 and Hsp25, 27- and 25-kDa heat shock protein, respectively; TNF, tumor necrosis factor; IL, interleukin; PP, protein phosphatase; OA, okadaic acid; DOA, docosahexaenoic acid; MAP, mitogen-activated protein; MAPKAP, mitogen-activated protein kinase; MAPKAP kinase, mitogen-activated protein kinase-activated protein kinase; PAG, polyacrylamide gel electrophoresis; HPLC, high performance liquid chromatography; cat, catalytic subunit.
phosphorylation (42). In the event, phosphorylation of small molecular weight Hsps are potentially regulated by two pathways: the MAP kinase and the calcium/calmodulin/calcineurin pathway.

In this paper, we compare the in vitro and the in vivo phosphorylation/dephosphorylation of Hsp27 by PP1, PP2A, and PP2B and their respective inhibitors. Clearly the in vitro data show that PP2A as well as PP2B dephosphorylate Hsp27. However, the in vivo data suggest PP2A to be the only enzyme that is primarily responsible for the dephosphorylation/phosphorylation of Hsp27 in MRC-5 cells. Furthermore, when the PP2A catalytic subunit is phosphorylated on tyrosine, it no longer dephosphorylates Hsp27. Together, the data suggest that PP2A is the cellular phosphatase for Hsp27 and that the phosphorylation of Hsp27 is controlled by the opposing action of specific kinases and PP2A. The present data are consistent with the hypothesis that a protein phosphatase recognizing cellular Hsp27 and other phosphoproteins is inactivated during IL-1/TNFα signal transduction (29, 40, 43, 44, 46).

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—MRC-5 fibroblasts (ATCC, Bethesda, MD) were maintained in modified Eagle's medium supplemented with 2% glutamine, 100 units/ml each of penicillin and streptomycin, and 10% fetal calf serum (rvine, UT). Clone Laboratories, Logan, UT, in a humidified atmosphere of 95% air and 5% CO₂ at 37°C. Confluent cells were used for all experiments.

**Reagents**—A 21-amino acid peptide containing two of the known sites of phosphorylation of the full-length Hsp27 (Ser²² and Ser²³) was synthesized at the Institute of Molecular and Cell Biology, National University of Singapore. Recombinant Hsp27 and the monoclonal antibody to Hsp27 were purchased from StressGen Biotechnologies Corp, Victoria, B. C., Canada. The following reagents were from Sigma: streptavidin-g-agarose, protein A-Sepharose, calcineurin, calmodulin, and 3,5-diaminobenzidine tetrahydrochloride and cantharidin. Okadaic acid was from Bio1m (Plymouth Meeting, PA). Heparin-Sepharose was purchased from Pharmacia LKB Biotechnology, Inc. The hydroxyureacinidine ester of biotin was purchased from Pierce Chemical Co. CAM-dependent protein kinase A was from Promega (Madison, WI), and 1-2,4-bis(2,4,6-trimethylphenyl)imidazole was a generous gift from Pharmacia (Piscataway, NJ). The following reagents were from Bachem (Pleasanton, CA, and Biotin (Plymouth Meeting, PA). Bifunctional reagents were used to immobilize proteins. In some experiments, the purified phosphatase catalytic subunits of PP1 and PP2A were used to dephosphorylate the labeled substrates. Dephosphorylation reactions were conducted for 10 min at 30°C in phosphate assay buffer (5 mM Tris-HCl, pH 7.0, 0.1 mM EDTA, 0.1% 2-mercaptoethanol, and 1 mg/ml bovine serum albumin). The reactions were terminated with 1 ml of 6 M HCl and immediately spinning the beads in a microcentrifuge. An aliquot of 50 ml of the supernatant was then removed and counted for ³²P released. Inhibition of phosphatase activity by okadaic acid and its derivatives was determined by incubating the indicated compound with the enzyme for 15 min prior to the addition of labeled substrate. Where recombinant Hsp27 was used as the phosphopeptide substrate, the dephosphorylated Hsp27 was then eluted off the beads by sonicating briefly in sample loading buffer (detailed above) to analyzed by two-dimensional gel electrophoresis as described above. The elution protocol was rigorously tested to avoid any artifacts by comparing the isomers of ³²P-labeled Hsp27 that was eluted from immunobeads with that from cytosolic extracts. No differences were observed in any of the multiple Hsp27 isoforms using this protocol.

**Metabolic Labeling and Lysis of MRC-5 Cells**—Cells were plated onto 90-mm plastic tissue culture dishes and grown to confluence prior to labeling with ³¹P-orthophosphate as described previously (17). Briefly, the cells were washed with serum-free medium (10 mM Tris, pH 7.4, containing 150 mM NaCl, 5 mM MgCl₂, 5 mM KCl, 1.6 mM CaCl₂, 0.5% glucose) before incubation with 0.5 mM [³¹P]orthophosphate for 3 h at 37°C. In some experiments, agents were added for the last 15 min of the incubation. The cells were then washed with ice-cold phosphate-buffered saline and lysed in 200 ml of solubilization buffer (10 mM Tris-HCl, pH 7.4, containing 50 mM EDTA, 50 mM NaCl, 30 mM sodium pyrophosphate, 50 mM NaF, 100 mM sodium orthovanadate, 0.65% Nonidet P-40, 2 mM leupeptin, and 2 mM phenylmethylsulfonyl fluoride). The lysates were centrifuged for 5 min at 14,000 rpm, and the soluble fraction was lyophilized prior to addition of sample loading buffer (59.7 ml of buffer in 44.9 ml of water containing 5.5 ml of 0.5% amphotolys (pH range 3–10), 1.45 ml of dithiothreitol, and 300 ml of 1% bromphenol blue) for first-dimension separation. The protein content of the extract was assayed using a BCA assay kit (Pierce).

**Two-dimensional Gel Electrophoresis**—12 μg of the cell extract was subjected to isoelectric focusing for 18,000 Vh with pH 3–10 amphotolys using the Millipore Investigator two-dimensional electrophoresis system (Bedford, MA). The gels were separated on the second dimension of 12.5% SDS-polyacrylamide gels using constant voltage. The gels were then dried, and ³¹P polyphosphates were located by autoradiography at –80°C. The phosphoproteins were analyzed on a Visage 2000 Image system (BioImage Products, Ann Arbor, MI).

**Preparation of Substrate Phosphoproteins**—The Hsp27 peptide containing the sequence AAPYSLRSQRLSSVGSEIR was biotinylated as follows. One mg of the peptide was dissolved in 1 ml of 0.1 mM NaHCO₃ buffer, pH 8.4, before the addition of 150 μM biotin in dimethyl sulfoxide. The mixture was then incubated for 2 h at room temperature before separating the free biotin from the peptide on a Sephadex G-15 column. The biotinylated peptide was eluted into 0.5-ml fractions using 50 mM potassium phosphate, pH 6.7, containing 6 mM MgCl₂, and the fractions with the highest optical density readings at 280 nm were pooled for column fractionation. The biotinylated peptide was phosphorylated for 24 h with 25 mCi of [γ³²P]ATP in the presence of 400 units of cAMP-dependent protein kinase A catalytic subunit. A preliminary experiment indicated that maximum phosphorylation of the peptide is achieved at 24 h. Streptavidin-agarose beads were then added to attach to the biotinylated peptide, and the complex was washed with cold phosphate-buffered saline until the counts in the wash were less than 100 cpm. The immobilized substrate was then stored at 4°C for the phosphatase assay.

The full-length recombinant Hsp27 was also phosphorylated as above except that the protein was immunoprecipitated with specific antibodies and protein A-Sepharose beads. The beads were washed and stored as described above for the Hsp27 peptide.

**Determination of PP1 and PP2A Activity**—Phosphatase activity against the Hsp27 phosphopeptide and Hsp27 phosphoprotein was assayed by the liberation of ³²P. Assays were conducted by adding 65 μl of total cell lysate or 20 μg of the immobilized substrate or 150 μl of the immobilized beads. In some experiments, the purified phosphatase catalytic subunits of PP1 and PP2A were used to dephosphorylate the labeled substrates. Dephosphorylation reactions were conducted for 10 min at 30°C in phosphate assay buffer (5 mM Tris-HCl, pH 7.0, 0.1 mM EDTA, 0.1% 2-mercaptoethanol, and 1 mg/ml bovine serum albumin). The reactions were terminated with 1 ml of 6 M HCl and immediately spinning the beads in a microcentrifuge. An aliquot of 50 μl of the supernatant was then removed and counted for ³²P released. Inhibition of phosphatase activity by okadaic acid and its derivatives was determined by incubating the indicated compound with the enzyme for 15 min prior to the addition of labeled substrate. Where recombinant Hsp27 was used as the phosphopeptide substrate, the dephosphorylated Hsp27 was then eluted off the beads by sonicating briefly in sample loading buffer (detailed above) and analyzed by two-dimensional gel electrophoresis as described above. The elution protocol was rigorously tested to avoid any artifacts by comparing the isomers of ³²P-labeled Hsp27 that was eluted from immunobeads with that from cytosolic extracts. No differences were observed in any of the multiple Hsp27 isoforms using this protocol.

The activity of the purified PP1 and PP2A was also determined using a kit from Life Technologies, Inc. The following the manufacturer's instructions, phosphatase A was labeled with ¹²⁵I and used as the substrate. Purified PP1 or PP2A was then added to the radiolabeled substrate in the absence or presence of either okadaic acid or its derivatives and incubated at 30°C for 10 min. The reaction was terminated by the addition of 20% trichloroacetic acid. After centrifugation, the supernatants containing released ³²P were counted in a Betacounter (LKB, Wallac, Finland).

**Determination of Calciuminulin Activity**—A 19-amino acid peptide with the sequence DLLVPPGFRD)RRVSAE-NH₂ was synthesized (Multi- Peptide Systems) and used as the substrate for the assay. The peptide was biotinylated and phosphorylated using protein kinase A as described above for the Hsp27 peptide. After phosphorylation, the biotinylated peptide was precipitated with streptavidin-agarose beads, and the immobilized peptide was then used as the substrate to assay for calcineurin activity.

In each experiment, identical volumes of phosphorylated substrate immobilized on beads were aliquoted and counted prior to the assay to ensure that the same amount of phosphorylated substrate was used for each reaction. In all dephosphorylation reactions, the measurements were done during the linear phase of labeled phosphate release. Confluent MRC-5 cells were either treated or treated with 5 mM okadaic acid and 1 μM calcineurin A for 1 h at 37°C. The cells were then lysed in 400 μl of 0.2 mM Tris-HCl buffer, pH 8.0, containing 100 mM NaCl, 6 mM MgCl₂, 0.5 mM dithiothreitol, 1.0 mM CaCl₂, and 0.1% Nonidet P-40, and the lysates were cleared of insoluble material by centrifugation at 14,000 rpm. To analyze phosphorylation, the immobilized substrate in the presence of 25 units of calmodulin and incubated for 10 min at 30°C, after which the reaction was terminated as before, and an
aliquote of 50 μl of the supernatant was removed to count for 32P released. In some assays, 500 nm okadaic acid was added to the cyclophilin A-treated cell lysates for 10 min prior to adding the mixture to the peptide substrate to initiate the reaction.

*In Vivo Effect of Cyclophilin A on Dephosphorylation of Hsp27*—MRC-5 cells, preheated with 3M OA or 3M DOA (both 30 min at 37 °C), were treated with OA or DOA for 30 min or 60 min after which the cells were washed and prepared for two-dimensional gel electrophoresis, as described above.

**Separation of Phosphatase Catalytic Subunits from MRC-5 Cytosolic Extracts and Location by Western Blotting**—Six large flasks of MRC-5 cells were solubilized in sample loading buffer (see above), applied to a heparin-Sepharose column in 20 mM Tris-HCl, pH 7.4, 0.1 mM EDTA, and 0.1% Nonidet P-40, and then later eluted with 0.5 M NaCl. The resulting fractions were divided equally, and each fraction was concentrated by lyophilization. One fraction was boiled with Laemmli buffer and run on three identical 10% SDS-PAGE minigels. The gels were then electrophoretically transferred to Immobilon-P, and each was immuno-blotted with polyclonal antibodies specific for PP1, PP2A, or PP2B. The immunoreactivity of the primary antibody was visualized after incubation with a secondary anti-rabbit antibody conjugated with horseradish peroxidase and subsequent development with 3,3-diaminobenzidine tetrahydrochloride. The other fraction was assayed for its activity on immunoprecipitated recombinant Hsp27.

**Assay for Hsp27 Phosphatase Using Immunoprecipitated Hsp27**—MRC-5 cells were labeled for 3 h with 33P as described above. To increase the phosphorylation of the more acidic isoforms of Hsp27 the cells were treated with 100 units/ml IL-1β for 15 min prior to cell lysis. The cells were lysed, Hsp27 was immunoprecipitated, and the immunoprecipitates were washed extensively prior to be used as the substrate for the putative Hsp27 phosphatase. The phosphatase assay took place for 10–20 min at 30 °C with the Hsp27 still attached to the immunobeads. The beads were divided into aliquots containing approximately 50,000 cpm, and the assay was performed as described for the recombinant protein. After the assay was terminated the Hsp27 protein was removed from the immunobeads by sonication in two-dimensional sample loading buffer, and each sample was subjected to twodimensional electrophoresis, autoradiography, and image analysis. Comparative experiments were performed by sequentially comparing the immunoprecipitated pattern of Hsp27 phosphatase with that from cytosolic extracts to show that the phosphorylation pattern was not altered in any way during the immunoprecipitation and subsequent elution processes.

**Tyrosine Phosphorylation of PP2A and Subsequent Assay of Activity**—Purified PP2A catalytic subunit was phosphorylated by src kinase for 1 h essentially as described by Chen et al. (47) using either [γ-32P]ATP or [γ-35S]thio-ATP. Hsp27 immunoprecipitated from 32P-labeled, IL-1-treated MRC-5 cells was then added to the reaction mixture, which contained thio-phosphate-labeled PP2A, for a further 30 min at 30 °C. The samples were then subjected to the same procedure as described above.

**Sequence Analysis of Substrate Proteins**—Hsp27 that was phosphorylated in vitro was separated from the kinase and SDS-PAGE gels. Identical Coomassie blue-stained bands from dried gels were excised with a scalpel and subjected to in gel proteolytic digestion using trypsin. Excised spots were resuspended in 200 μM amionborbic acid buffer, pH 8.9, containing 50 mM acetaldehyde. A 2-μl aliquot of trypsin (modified trypsin, Promega) at a concentration of 0.01 μg/μl was added to each gel piece and allowed to soak into the matrix. This was repeated a second time, after which similar volumes of 200 μM amionborbic acid buffer, pH 8.9, were added until each gel piece had resuspended to its original size. Gel pieces were then placed into an Eppendorf tube, covered with the ammonium bicarbonate buffer (~200 μl), and incubated at 37 °C for 4–6 h. After incubation the buffer was transferred to a clean tube and pooled with two successive 30-min washes of 0.1% trifluoroacetic acid containing 60% acetonitrile. These washes were then lyophilized. The residue was resuspended in 0.1% trifluoroacetic acid and injected onto a C18 narrow bore (2.1 × 250 mm) reversed-phase HPLC column (Vydac, Hesperia, CA). Elution and subsequent separation of the peptides were performed by subjecting the column to a gradient of increasing acetonitrile concentration in 0.1% trifluoroacetic acid. Eluting peptides were monitored at 214 nm and collected into tubes manually.

**Sequence Analysis of the purified peptides was carried out using a Milligen 6600 ProSequencer (Millipore). Reverse phase HPLC was used to separate the tryptic peptides using a Waters 600 pump, M990 diode array detector, and incorporating the following parameters. Column size was 2.1 × 250 mm C18 (Vydac). Buffer A was 0.1% trifluoroacetic acid in Milli Q water. Buffer B was 0.08% trifluoroacetic acid in acetonitrile (far UV grade). The elution gradient was linear from 2% B to 60% B over 100 min. The flow rate was 200 μl/min, and the detection was above 214 nm. The fractions were collected manually and analyzed for protein using a Lowry assay (100 μl in 0.1% acetic acid B in 0.1% trifluoroacetic acid).

**Sequence Analysis**—Sequence analysis of labeled peptides was done using a model 6600 ProSequencer (Millipore). Trifluoroacetic acid was used as the AT2-amino acid extraction solvent on this instrument, which is advantageous during the extraction of the polar phosphoamino acids. The program used (50–60) splits the reconstituted phenylhydantoin derivative so that 50% can be collected for counting while the remaining 50% is injected into the on-line HPLC system for amino acid determination.

**Results**

**Effect of Cell-permeant Phosphatase Inhibitors on the Phosphorylation of Hsp27 in MRC-5 Fibroblasts**—High resolution two-dimensional gel electrophoretic analysis of early protein phosphorylation in MRC-5 cells treated with TNF/IL-1 or OA indicated that the Hsp27 complex was significantly hyperphosphorylated (29). In this study, MRC-5 cells were preincubated with two other phosphatase inhibitors unrelated to OA and derived from different sources. Calyculin A inhibits PP2A and PP1, and cantharidin binds the A and C subunits of PP2A (45). The results show that these inhibitors induce a pattern of phosphorylation of cellular proteins in MRC-5 fibroblasts similar to that which is typically induced by OA (Fig. 1). Calyculin A and cantharidin, like OA, induce the hyperphosphorylation of the Hsp27 complex. Treatment of MRC-5 cells with OA, cantharidin, or calyculin A (Fig. 1, a, b, d, and f) produces the phosphorylation of at least four acidic Hsp27 isoforms (Fig. 1, which exist as a single species in control cells (Fig. 1, a, c, and e).

Experiments were performed to show which protein phosphatase is the most likely target of these inhibitors or TNF/IL-1 as reported in the next sections.

**Dephosphorylation of Recombinant Hsp27 Phosphorylated by Protein Kinase A in Vitro by the Catalytic Units of Purified PP1, PP2A, and PP2B**—A number of protein kinases (MAP-2 kinase, p34cdc2, casein kinase 2, and the catalytic unit of cAMP kinase) were tested to see which of the kinases was most effective in phosphorylating Hsp27 in vitro. The catalytic subunit of CAMP kinase was the most effective, hence it was used to produce [32P]Hsp27 for use in determining which protein phosphatase is the most effective in dephosphorylating Hsp27 in vitro. None of the other kinases catalyzed a significant phosphorylation of Hsp27 in vitro. When CAMP kinase was used in the in vitro phosphorylation of Hsp27 three major isoforms were separated on two-dimensional gels. These were compared with “native” Hsp27 either by comparing the Hsp27 from immunoprecipitations or by or runnning the CAMP kinase-catalyzed product with cytosolic extracts of 3P-labeled fibroblasts. The major isoforms ran at the same pl on two-dimensional gels, and microsequencing analysis showed that serines 15, 78, and 82 were the major phosphorylated sites (data not shown) as has been demonstrated for other agonists (27, 31).

In comparison with PP1 and PP2B, PP2A was most effective in the dephosphorylation of Hsp27 (Fig. 2 A). OA and DOA (both inhibitors of PP2A) blocked or partially blocked the dephosphorylation of Hsp27 by PP2A, whereas the PP1 inhibitor did not block either PP2A or PP2B (data not shown). Cyclosporin could not be used as an in vitro inhibitor as it forms a complex with cyclophilins in vivo to achieve inhibition of PP2B (42). The dephosphorylation of [32P]Hsp27 in vitro was also analyzed by two-dimensional electrophoresis (Fig. 2 B). In the controls, [32P]Hsp27 is shown to consist of at least four isoforms, similar to those observed in vivo (Fig. 1). PP2A preferentially dephosphorylates these phosphorylated isoforms of Hsp27 and is sensitive to OA. PP1 did not appear to cause any significant

**Hsp27 Is an in Vivo Substrate of PP2A**
dephosphorylation (less than 5% of control), suggesting that PP1 is not involved in Hsp27 dephosphorylation. Calcineurin (PP2B), in the presence of Ca\(^{2+}\) and calmodulin, dephosphorylated Hsp27 by 23%, albeit to a lesser extent than PP2A but suggesting that PP2B can also dephosphorylate Hsp27.

**Dephosphorylation of \(^{32}\)P-Hsp27 Immunoprecipitated from MRC-5 Cells by the Catalytic Subunits of Purified PP1, PP2A, and PP2B in Vitro**—Attempts using more native phosphorylated Hsp27 substrates were made. MRC-5 cells were phosphorylated in vivo, and \(^{32}\)P-Hsp27 was immunoprecipitated from extracts of MRC-5 cells treated with IL-1β to stimulate the phosphorylation of Hsp27 (29). The immunoprecipitated Hsp27 was subjected to dephosphorylation by purified phosphatases while immobilized on Sepharose beads. The radioactive phosphate released was collected and counted, and the results were consistent with those shown in Fig. 2A (data not shown). The substrate was then eluted from the beads and analyzed by two-dimensional gel electrophoresis. The results of this analysis show that PP2A (Fig. 3b) was most active (apparent removal of 90% of \(^{32}\)P from Hsp27), PP2B (Fig. 3d) had minimal activity (15%), and PP1 (Fig. 3c) was the least active (less than 6%) of the three phosphatases in the dephosphorylation of the immunoprecipitated \(^{32}\)P-Hsp27 complex.

**Immunoprecipitation of Hsp27 Phosphatase in Cell Lysates after Treatment of Cells with Phosphatase Inhibitors**—Having shown PP2A and PP2B to dephosphorylate Hsp27 in vitro (Figs. 2 and 3) the activity of the three protein phosphatases was compared in the extracts of MRC-5 cells, using \(^{32}\)P-Hsp27 as substrate. Living MRC-5 cells were treated with phosphatase inhibitors. 500 nM OA inhibited Hsp27 phosphatase activity by 38% (Fig. 4). Since this concentration of OA is known to inhibit 90–95% of PP2A activity, other OA-insensitive phosphatases such as calcineurin (PP2B) may be involved in the dephosphorylation of Hsp27 as well. The experiment was repeated with cyclosporin A, an inhibitor of PP2B (42). When cells were treated with cyclosporin, the cell extracts show a 34% inhibition of Hsp27 phosphatase activity. When cyclosporin A-treated cell lysates were additionally treated with 500 nM OA, the dephosphorylation of Hsp27 was inhibited by 75%. Various doses of both OA and cyclosporin were tested, and those used above were found to be optimal. Calyculin A at 0.1 μM gave effects similar to those of OA. Cyclosporin cannot be used in vitro as an inhibitor of PP2B unless it is complexed to a low molecular weight cyclophilin protein.

This additive effect suggests that calcineurin and PP2A may both dephosphorylate Hsp27 in vivo.

**Effect of Cyclosporin A on the Phosphorylation of Hsp27 Complex in Vivo**—MRC-5 fibroblasts were treated with 100 nM cyclosporin for 30 or 60 min, the cells were lysed, and the lysates were analyzed by two-dimensional gel electrophoresis (Fig. 5). No change in the phosphorylation of the Hsp27 complex was observed as a consequence of cyclosporin A treatment, although changes in the phosphorylation of at least six other phosphoproteins were observed. This is in contrast to the results obtained in MRC-5 cells treated with OA, which causes a signifi-
9180

**Hsp27 Is an in Vivo Substrate of PP2A**

To investigate the possibility of PP2B involvement in the phosphorylation status of Hsp27 in *vivo* we adopted a pulse-chase method to determine whether the inhibition of PP2B would prevent the dephosphorylation of Hsp27 (43). Cells were prelabeled with $^{32}$P and activated with TNF-α for 15 min to induce the phosphorylation of the Hsp27 complex *in vivo*. The treated cells were washed rapidly and chased with excess unlabeled phosphate. Various treatments were then applied to the TNF-treated cells subsequently divided into different groups; 100 nM cyclosporin was added to one group, 1 μM OA to another, and nothing to the control group. After a 1-h chase the cells were extracted, $^{32}$P-Hsp27 was immunoprecipitated and analyzed by two-dimensional PAGE, and the gels were subjected to autoradiography. OA but not cyclosporin inhibited the dephosphorylation of Hsp27 during the cold phosphate chase. There was no difference between the resultant phosphorylation of the Hsp27 complex in control and cyclosporin-treated cells, suggesting that the inhibitor of PP2B has no effect in preventing the dephosphorylation of Hsp27 (not shown) and thus suggesting that PP2B is not primarily responsible for dephosphorylating Hsp27 in *vivo* at least in IL-1- or TNF-treated cells.

It is unlikely that the results in Fig. 5A are due to cyclosporin not inhibiting PP2B in the MRC-5 cells because we tested the ability of cyclosporin to inhibit the activity of PP2B in MRC-5 cells using the synthetic 19-amino acid peptide substrate. Cyclosporin but not OA inhibited PP2B activity of MRC-5 cells (Fig. 5B), and PP2B can be completely inhibited by treatment of MRC-5 cells with 100 nM cyclosporin A for 30 min.

The above results that demonstrate that the inhibition of PP2B by cyclosporin *in vivo* seem to disagree with the results from the previous section, where PP2B extracted in lysates from MRC-5 cells could dephosphorylate $^{32}$P-labeled Hsp27 in *in vitro* phosphatase assays, and this could be inhibited by phosphorylation of phosphorylated recombinant Hsp27 by the catalytic subunit of cAMP kinase. The protein was attached to beads to facilitate handling, and an aliquot of labeled substrate was incubated with standardized, purified PP1, PP2A, or PP2B, with or without phosphatase inhibitors. The released, labeled phosphate was collected and counted, following which the remaining substrate was released from the beads and run on two-dimensional gels. The resultant autoradiographs were analyzed for changes in the distribution of radioactivity in the Hsp27 isoforms. Panel A shows the radiolabeled phosphate released when labeled Hsp27 was treated with PP1 (protein phosphatase catalytic subunit), PP1 plus OA (okadaic acid at 400 nM), PP2A (the catalytic subunit of PP2A), PP2A plus OA (400 nM), PP2A plus OA + OA (10 μM), PP2B (phosphatase 2B (calcineurin)) and PP2B plus OA. CON is the control, which consisted of the substrate with reaction buffer only. Panel B shows autoradiographs that resulted from running the recombinant, $^{32}$P-labeled Hsp27, on two-dimensional gels at the end of the experiment shown in panel A.

![Fig. 2](image-url)

**Fig. 2. Dephosphorylation of phosphorylated recombinant Hsp27 *in vitro* by the catalytic subunits of purified PP1, PP2A, and PP2B.** Recombinant Hsp27 was phosphorylated by the catalytic subunit of cAMP kinase. The protein was then attached to beads to facilitate handling, and an aliquot of labeled substrate was incubated with standardized, purified PP1, PP2A, or PP2B, with or without phosphatase inhibitors. The released, labeled phosphate was collected and counted, following which the remaining substrate was released from the beads and run on two-dimensional gels. The resultant autoradiographs were analyzed for changes in the distribution of radioactivity in the Hsp27 isoforms. Panel A shows the radiolabeled phosphate released when labeled Hsp27 was treated with PP1 (protein phosphatase catalytic subunit), PP1 plus OA (okadaic acid at 400 nM), PP2A (the catalytic subunit of PP2A), PP2A plus OA (400 nM), PP2A plus OA (10 μM), PP2B (phosphatase 2B (calcineurin)) and PP2B plus OA. CON is the control, which consisted of the substrate with reaction buffer only. Panel B shows autoradiographs that resulted from running the recombinant, $^{32}$P-labeled Hsp27, on two-dimensional gels at the end of the experiment shown in panel A.

![Fig. 3](image-url)

**Fig. 3. Activity of purified phosphatase subunits on immunoprecipitated, $^{32}$P-labeled Hsp27 from IL-1β-stimulated primary human fibroblast cells.** Confluent MRC-5 cells were prelabeled with $^{32}$P for 3 h, stimulated with IL-1β for 15 min, before the Hsp27 was immunoprecipitated and used as a substrate for the various catalytic units of the major serine/threonine phosphatases. At the end of the experimental time the protein was detached from the immunobeads and subjected to two-dimensional electrophoresis and autoradiography. Panel a, control experiment with no added phosphatase. Panel b, PP2A was added to the incubation mixture as outlined under "Experimental Procedures." Panel c, addition of PP1; and panel d, addition of PP2B and calcimodulin.

![Fig. 4](image-url)

**Fig. 4. Dephosphorylation of phosphorylated recombinant Hsp27 *in vitro* by lysates from primary fibroblast cells previously treated with phosphatase inhibitors.** Recombinant Hsp27 was phosphorylated by the catalytic subunit of cAMP kinase, and the assay was performed as outlined under "Experimental Procedures." The released, labeled phosphate was collected and counted. The CONTROL contained no lysate in the incubation mixture; LYS. is the lysate from nontreated cells; LYS + OA is the lysate from cells treated with 500 nM okadaic acid for 30 min prelysis. CSA is the lysate from cells treated with cyclosporin (10 μM) for 30 min prelysis; CSA + OA is the lysate from cyclosporin-treated cells, with 500 nM OA added in after lysis. The experiment was performed in duplicate and was repeated twice.
Dephosphorylation of primary human lung fibroblast cells for activity to Hsp27 phosphatase. Cytosolic extracts from MRC-5 human fibroblast cells were fractionated by a heparin-Sepharose column as described under “Experimental Procedures.” The fractions were concentrated and assayed for Hsp27 phosphatase activity using immunoprecipitated Hsp27 from MRC-5 cells prelabeled with 32P and prestimulated with IL-1p for 15 min prior to cell lysis. The assay for Hsp27 phosphatase was performed as described under “Experimental Procedures.” The 32P-labeled Hsp27 was separated on two-dimensional gels, and the dried gels were quantitated for distribution of Hsp27 phosphorylation. Panel B, Western blotting of separated Hsp27 phosphatase with antibodies against PP1, PP2A, and PP2B. The Hsp27 phosphatase fractions eluted from the heparin-Sepharose column were concentrated and further subjected to SDS-PAGE separation. The proteins on SDS-PAGE gels were transferred to Immobilon-P membranes and blotted with polyclonal antibodies against the catalytic subunits for PP1, PP2A, or PP2B. The locations of the phosphatase catalytic subunits were indicated by incubating the blots with a secondary antibody conjugated to horseradish peroxidase and further incubation with 3,3'-diaminobenzidine tetrahydrochloride.

phosphatase activity using immunoprecipitated [32P]Hsp27 as substrate. The treated enzyme as well as the dephosphorylated substrate were examined on two-dimensional electrophoresis gels. Autoradiographs of the PP2A incubated with pp60src show that the catalytic subunit of PP2A is phosphorylated (Fig. 7A). Autoradiographs of the digested [32P]Hsp27 substrates show the tyrosine-labeled PP2A (labeled with γ-[32P]thio-ATP to minimize autodephosphorylation) dephosphorylated by Hsp27 by only 14% in comparison with the untreated control enzyme (Fig. 7B).

**DISCUSSION**

A redox-sensitive Hsp27 phosphatase was shown to be inactivated in TNF/IL-1 signal transduction (43). This phosphatase was inactivated in vitro by okadaic acid (an inhibitor of PP1 and PP2A) which mimicked the effects of TNF/IL-1 on early protein phosphorylation, suggesting that either PP1 and/or PP2A inactivation is an early event of TNF/IL-1 signaling (29, 43, 44, 46). A recent report indicated that PP2B is the enzyme responsible for the dephosphorylation of Hsp25 (the murine...
The apparent anomaly between the ability of PP2B to dephosphorylate Hsp27 in cell lysates but not in whole cells could be explained by the enzyme and its substrate being in different cellular compartments. Although we have no direct proof of this the data presented are consistent with this hypothesis.

It should also be noted that we have used the catalytic subunits of various phosphatases in this work. There is emerging evidence that the A and B subunits of phosphatase 2A can effect the substrate specificity in different ways (34). Furthermore, the specificity of phosphatases to certain substrates is conferred via adaptor proteins (50), and these would not be present in vitro assays using recombinant protein substrates. With such limitations in phosphatase assays a role for PP1 in the in vivo dephosphorylation of Hsp27 in such circumstances cannot be totally excluded.

At least two protein kinases are implicated in the phosphorylation of cellular Hsp27 (37–39). One of them, MAPKAP kinase 2, which recognizes the sequence LXRXS (38), has a restricted number of substrate proteins including Hsp27, C/EBPβ, glycogen synthase, and laminin (according to analysis of current, protein sequence data bases). Another kinase specific for Hsp27 and stimulated by TNF and IL-1 was recently described in MRC-5 cells (37), and it is possible that it is the same as MAPKAP kinase 2. Considering that the phosphorylation of cellular proteins is maintained in homeostasis by the protein phosphatases actively opposing kinases (40), we suggest that both MAPKAP 2 and MAP kinases and PP2A are closely linked in the phosphorylation of Hsp27 according to the scheme in Fig. 8. Activation of the kinases or inactivation of the
phosphatase together or separately can lead to the enhanced phosphorylation of Hsp27. Furthermore, MAPKAP kinase 2 and MAP kinase are regulated by PPA2 since the dephosphorylation of MAP kinase is increased leading to an increase in the phosphorylation and activity of its substrate MAPKAP kinase 2, which in turn leads to the increased phosphorylation of Hsp27.

Acknowledgments—We thank Dr. C. J. Vallen for helpful discussions with the above work and for reading the manuscript and Melinda Ong for technical assistance.

REFERENCES

1. Hickey, E., Brandon, S. E., Potter, R., Stein, G., Stein, J., and Weber, L. A. (1986) Nucleic Acids Res. 14, 4127-4146
2. Ingolia, T. D., and Craig, E. A. (1982) Proc. Natl. Acad. Sci. U.S.A. 79, 2360-2364
3. DeNagel, D. C., and Piers, S. K. (1989) Immunol. Rev. 106, 66-78
4. De Jong, W. W., Leunissen, J. A., Leenen, P. J., Zwaar, A., and Versteeg, M. (1985) J. Biol. Chem. 260, 5141-5149
5. Lingquist, S., and Craig, E. A. (1988) Annu. Rev. Genet. 22, 631-677
6. Vitek, M., and Berger, E. M. (1984) J. Biol. Chem. 259, 173-189
7. Fuqua, S. A., Blum-Salingaros, M., and McGuire, W. L. (1989) Cancer Res. 49, 4126-4129
8. Ciocca, D. R., Adams, D. J., Edwards, D. P., Bjereke, R. J., and McGuire, W. L. (1983) Cancer Res. 43, 1294-1300
9. Thor, A., Benz, C., Moore, D., Goldman, E., Edgerton, S., Landry, J., Schwartz, L., Mayall, B., Hickey, E., and Weber, L. A. (1991) J. Natl. Cancer Inst. 83, 170-178
10. Tetu, B., Lacasse, B., Bouchard, H., Lagace, R., Huet, J., and Landry, J. (1992) Cancer Res. 52, 2325-2328
11. Landry, J., Chretien, P., Lambert, H., Hickey, E., and Weber, L. A. (1989) J. Cell Biol. 109, 7-15
12. Landry, J., Chretien, P., Laszlo, A., and Lambert, H. (1991) J. Cell. Physiol. 147, 93-101
13. Arigo, A. P., and Welch, W. J. (1987) J. Biol. Chem. 262, 15359-15369
14. Welch, W. J. (1985) J. Biol. Chem. 260, 3058-3065
15. Regazzi, R., Ripponer, U., and Fabbin, D. (1988) Biochem. Biophys. Res. Commun. 158, 62-68
16. Welsh, M. J., and Ireland, M. E. (1992) Biochem. Biophys. Res. Commun. 184, 217-224
17. Bitar, K. N., Kaminski, M. S., Hailait, N., Cease, K. B., and Strahler, J. R. (1991) Biochem. Biophys. Res. Commun. 181, 1192-1200
18. Chambard, J.-C., and Pouyssegur, J. (1983) Biochem. Biophys. Res. Commun. 111, 1034-1044
19. Deonille, D., Lecomte, M., and Boeysesans, J.-M. (1988) J. Biol. Chem. 263, 18459-18465
20. Shiharuma, U., Kurup, T., and Koshi, K. (1991) Cell Growth & Differ. 2, 583-591
21. Michishita, M., Sato, M., Yamaguchi, M., Hirayoshi, K., Okuma, M., and Nagata, K. (1991) Biochem. Biophys. Res. Commun. 176, 979-984
22. Darbon, J.-M., Bournier, J.-F., Tauber, J.-P., and Bayard, F. (1986) J. Biol. Chem. 261, 8005-8008
23. Arigo, A. P. (1980) Mol. Cell. Biol. 10, 1276-1290
24. Kaur, P., Welch, W. J., and Saklatvala, J. (1989) FEBS Lett. 258, 289-297
25. Guy, G. R., Chau, S. P., Wong, N. S., Ng, S. B., and Tan, Y. H. (1991) J. Biol. Chem. 266, 14343-14352
26. Robey, P., Hepsturn, A., Lecorg, B., Fiers, W., Boeysesans, J.-M., and Dumont, J. E. (1989) Biochim. Biophys. Acta 193, 301-308
27. Gaestel, M., Schroder, W., Benndorf, R., Lippmann, C., Buchner, K., Hucn, F., Erdmann, V. A., and Bielka, H. (1991) J. Biol. Chem. 266, 14721-14724
28. Guesdon, F., and Saklatvala, J. (1991) J. Immunol. 147, 3402-3407
29. Guy, G. R., Cao, X., Chua, S. P., and Tan, Y. H. (1992) J. Biol. Chem. 267, 18458-18462
30. Huet, J., Boy, G., Lambert, H., Chretien, P., and Landry, J. (1991) Cancer Res. 51, 5245-5252
31. Landry, J., Lambert, H., Zhou, M., Lavoie, J. N., Hickey, E., Weber, L. A., and Anderson, C. W. (1992) J. Biol. Chem. 267, 794-803
32. Crete, P., and Landry, J. (1990) Radiat. Res. 121, 320-327
33. Strahler, J. R., Kruck, R., and Hanash, S. M. (1991) Biochem. Biophys. Res. Commun. 178, 134-142
34. Ferrigno, P., Lanagan, T. A., and Cohen, P. (1993) Mol. Cell. Biol. 4, 669-677
35. Kennerly, P. J., and Krebs, E. G. (1991) J. Biol. Chem. 266, 15555-15558
36. Zhou, M., Lambert, H., and Landry, J. (1991) J. Biol. Chem. 266, 36-43
37. Guesdon, F., Fresehney, N., Waller, R. J., Rawlinson, L., and Saklatvala, J. (1991) J. Biol. Chem. 266, 4236-4243
38. Stokoe, D., Campbell, D. G., Nocis, S., Hida, K., Levers, S. J., Marshall, C., and Cohen, P. (1992) EMBO J. 11, 3945-3954
39. Stokoe, D., Engel, K., Campbell, D. G., Cohen, P., and Gaestel, M. (1993) FEBS Lett. 333, 307-313
40. Tan, Y. H. (1993) Science 260, 376-377
41. Gaestel, M., Benndorf, R., Hayes, K., Priemer, E., and Engel, K. (1992) J. Biol. Chem. 267, 21067-2101
42. Schreiber, S. L., and Crabbe, G. R. (1992) Immuno. Today 13, 136-142
43. Guy, G. R., Cairns, J., Ng, S. B., and Tan, Y. H. (1993) J. Biol. Chem. 268, 15241-15248
44. Cao, X., Mademden, R., Gi, R., and Tan, Y. H. (1993) J. Biol. Chem. 267, 12991-12997
45. Li, Y.-M., and Casida, J. E. (1992) Proc. Natl. Acad. Sci. U.S.A. 89, 11867-11870
46. Menon, S. D., Qin, S., Guy, G. R., and Tan, Y. H. (1993) J. Biol. Chem. 268, 26805-26812
47. Chen, J., Martin, B. L., and Brautigan, D. L. (1992) Science 257, 1261-1264
48. Guo, H., and Damuni, Z. (1993) Proc. Natl. Acad. Sci. U.S.A. 90, 2500-2504
49. Guo, H., Reddy, S. A. G., and Damuni, Z. (1993) J. Biol. Chem. 268, 11193-11198
50. Hubbard, M. J., and Cohen, P. (1993) Trends Biochem. Sci. 18, 172-177