Interleukin 1 and Tumor Necrosis Factor Stimulate Two Novel Protein Kinases That Phosphorylate the Heat Shock Protein hsp27 and $\beta$-Casein*

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We have partially purified and characterized two protein kinases that were strongly activated by interleukin-1 (IL-1) or tumor necrosis factor (TNF) in MRC-5 fibroblasts. The kinases were separated by anion exchange chromatography of cytosolic fractions. They phosphorylated in vitro the small heat shock protein (hsp27) or $\beta$-casein and were stimulated 3- and 4.5-fold, respectively, in cells that had been exposed to IL-1 or TNF for 10 min. They were distinct from the mitogen-activated protein kinases, whose activation by IL-1 or TNF has been reported recently. The hsp27 kinase phosphorylated its substrate on serine residues. Its molecular mass was estimated to be 45-kDa by gel filtration. It is probably involved in the increase in hsp27 phosphorylation seen in intact cells. The $\beta$-casein kinase behaved as a 65-kDa protein. It phosphorylated its substrate on serine and threonine residues and had little activity on $\alpha$-casein.

The hsp27 and $\beta$-casein kinases were not activated after stimulation of the cells with phorbol myristate acetate (PMA). In contrast, the MAP kinases were activated to a similar extent (2–3-fold) by the cytokines and by PMA. The hsp27- and $\beta$-casein kinases probably correspond to novel enzymes whose mechanisms of activation may be independent of protein kinase C or MAP kinases.

Interleukin 1 (IL-1)$^1$ and tumor necrosis factor (TNF) are two cytokines that play a central role in inflammatory processes. They cause endothelial cell activation, leucocyte accumulation, tissue resorption, fever, and the acute phase reaction (1, 2). These responses require the synthesis of specific proteins, the nature of which depends on the responding cell type. Although their biological effects are similar, IL-1 and TNF are structurally unrelated and bind to distinct receptors on their target cells (1, 3, 4).

The intracellular signaling pathways linking the activation of the receptors to the cellular responses are still unclear (5).

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3. The abbreviations used are: IL-1, interleukin-1; E64, trans-epoxysuccinyl-l-leucylamido(4-guanidino)butane; hsp27, small heat shock protein; MAP kinase, mitogen-activated protein kinase; MBP, myelin basic protein; PMA, phorbol 12-myristate 13-acetate; TNF, tumor necrosis factor; TPCK, l-1-tosylamide-2-phenylethyl chloromethyl ketone.

In several cell types, the cytokines increase the phosphorylation of certain proteins within minutes of stimulation (6–15). This suggests that they work by modulating the activities of protein kinases. The ability of phorbol myristate acetate (PMA) to mimic certain effects of IL-1 and TNF has led to the suggestion that protein kinase C might be involved in some of the responses to the cytokines (16–18).

Recent evidence indicates that the 42- and 44-kDa mitogen-activated protein kinases (MAP kinases) may be activated by IL-1 and TNF in fibroblasts and other cells (15, 19). The MAP kinases were originally described as enzymes that phosphorylated microtubule-associated protein 2 or myelin basic protein (MBP) and were stimulated in cells challenged with PMA or growth factors such as nerve growth factor, epidermal growth factor, and insulin (reviewed in Ref. 20). The MAP kinases form a family of enzymes encoded by structurally related genes. The 42- and 44-kDa MAP kinases are coded by the erk2 and erk1 genes, respectively.

One protein that undergoes a prominent increase in phosphorylation in cells stimulated by IL-1 or TNF is the small heat shock protein, hsp27. The response has been seen in fibroblasts (6, 14, 15), articular chondrocytes (14), vascular endothelial cells (7, 14), and several cell lines (6, 13, 14). hsp27 is a 27-kDa protein structurally homologous to the lens $\alpha$-crystallins that is expressed in many cell types (21, 22). Overexpression of hsp27 has been reported to protect cells against heat shock and other stresses (23), but its function is unknown. Recent evidence obtained in vitro suggests that it may participate in the regulation of actin polymerization in cells (24).

Using purified unphosphorylated human hsp27 as a substrate, it has been possible to detect in cytosolic fractions of MRC-5 fibroblasts a cytokine-activated hsp27 kinase (25). We have now used hsp27, and other substrates, to look for cytokine-stimulated protein kinases in chromatography fractions of MRC-5 cytosol preparations. We report here the detection of three cytokine-activated protein kinases. One of them corresponds to p42 MAP kinase, but the two other enzymes, hsp27 and $\beta$-casein kinases, seem to be novel. Unlike the MAP kinase, the two new kinases were not activated in fibroblasts stimulated with PMA. Our evidence suggests that their activation by the cytokines occurs independently of activation of protein kinase C or MAP kinase.

MATERIALS AND METHODS

Reagents—Human recombinant IL-1a and TNF$\alpha$ were purified from extracts of Escherichia coli expressing the proteins (9). The rabbit anti-hsp27 antiserum had been raised against antigen purified from HeLa cells (22). It was kindly given by Dr. W. J. Welch, San Francisco General Hospital, San Francisco, CA. The rabbit antiserum raised against recombinant human p42-MAP kinase was a gift of Pr. C. J. Marshall, Chester Beatty Laboratory, London, United Kingdom.
Cytokine-activated hsp27- and β-casein Kinases

(P. PMA, β-glycerophosphate, protease inhibitors, ATP, GTP, heparin, MBP, dephosphorylated α- and β-caseins, α- and β-crystallins, histone H-1, TPCK-treated trypsin, molecular mass standard proteins and phosphoacids were from Sigma. Staur

(423) 

of 25 mM Tris-Cl, pH 7.4, 154 mM NaCl, 1% non-

beads were detected by the use of 27i-labeled donkey anti-rabbit IgG at 0.2 μCi/ml. 

purification of hsp27 from ME-180 Cells—The purification of hsp27 was monitored by immunoblotting using the rabbit antisem specific for hsp27. Preliminary screening of various cell lines showed that ME-180 cells constitutively express much higher levels of hsp27 than other cell lines (data not shown). 

Lysis of ME-180 Cells—Confluent ME-180 cells (2 x 10^6 cells) were resuspended by treatment with trypsin and EDTA (GIBCO-Bethesda Research Laboratories). After inactivation of the trypsin by addition of culture medium, the suspension was spun at 40 x g for 10 min. The cell-free supernatant (by trypsin) was incubated in ice-cold 50 mM Tris-HCl, pH 8.0, 0.5 mM EDTA, 0.5 mM EGTA, 0.1% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, 1 mM orthophenyldiamine, and 10 μM trans-epoxysuccinyl-α-leucylamido(4-guanidino)butane (E64). The cells were disrupted by passing through 23 gl (twice) and 26 1/8 (10 times) injection needles. 

Ammonium Sulfate Precipitation—The ME-180 lysate, containing about 100 μg of protein, was centrifuged at 100,000 x g for 40 min (4 °C). The ammonium sulfate was then eluted at the supernatant (0.194 g/ml) to a final concentration of 35% of these volumes. The suspens

Anion Exchange Chromatography of the Ammonium Sulfate Pre-

Fractionation of MRC-5 Fibroblasts—Cells grown in 175-cm² flasks were harvested 1-4 days after reaching confluence. They were disrupted by adding IL-1 (20 ng/ml), TNF (100 ng/ml), or PMA (100 ng/ml) to the culture medium and incubating for 10 min at 37 °C. Stimulated and unstimulated cells (six flasks each) were washed twice in ice-cold PBS supplemented with 0.1 mM EGTA (1 ml per wash), then diluted buffer (20 mM β-glycerophosphate, 10 mM NaF, 2 mM dithiothreitol, 0.5 mM EDTA, 0.5 mM EGTA, 0.02% NaN3). After addition of protease inhibitors (10 μM E64, 1 μM pepstatin, 1 mM phenanthroline, and 1 mM phenylmethylsulfonyl fluoride), the suspensions were homoge-

Anion Exchange Chromatography of the Cytosolic Fractions—Chro

mination was done on 5 x 0.5-cm Mono Q fast-protein liquid chromatography column equilibrated in buffer M. Elution was by a linear gradient of NaCl in buffer M (0.5-20 mM over 30 ml). 0.1 ml fractions were collected and stored at 4 °C. 

Gel Filtration Chromatography—Mono Q fractions of interest were pooled and concentrated 4-5-fold by ultrafiltration on a PM10 mem-

Protein Kinase Assays—In vitro phosphorylations were carried out in the presence of 50 mM Tris-HCl, pH 7.2, 10 mM MgCl2, 20 μM ATP, 0.2-0.4 μCi/μl [γ-32P]ATP, and, for casein phosphorylation, 120 mM KCl. hsp27 was used at a final concentration of 70 μg/ml, MBP at 1.7 mg/ml, and bovine α- and β-caseins at 250 μg/ml each. Reaction volumes were 24 μl for hsp27- and casein-kinase assays and 15 μl for MBP-kinase assays, the samples under test constituting one-third of these volumes. Reaction mixtures were started by adding 2 μCi of [γ-32P]ATP and lasted 20 min at room temperature. hsp27- and casein-kinase assays were stopped as described (25). MBP kinase assays were stopped by adding 3 μl of 90% formic acid to the reaction mixtures. Aliquots of the reaction mixtures (8 μl) were then spotted on 8 x 10 cm squares of P81 phosphocellulose paper (Whatman) (27). The paper was washed in four changes of a 0.5% phosphoric acid solution (5 min per change) and left to dry. The squares were then cut out, and the 32P incorporated on the paper was counted. The background due to the phosphorylation of endogenous proteins in each sample kinase assay was carried out in the absence of MBP and subtracted from the counts. 

Phosphoamino Acid Analysis and Phosphopeptide Maps—In vitro phosphorylation of hsp27 or β-casein were carried out as for kinase assays except that hsp27, β-casein, ATP, and [γ-32P]ATP were used at concentrations of 200 μg/ml, 1 mg/ml, 5 μM, and 1 μCi/μl, respectively. After one- dimensional electrophoresis and drying on nitrocellulose paper, the precipitates were extracted from the gel, and precipitated with trichloroacetic acid as described previously (14) except that no carrier protein was used. For phosphoamino acid analysis, the precipitates were hydrolyzed at 110 °C for 2 h in the presence of 6 N HCl (28). The dried hydrolysates were then separated by two-dimensional thin-layer electrophoresis on cellulose plates (28) and detected by staining with ninhydrin (28). For tryptic phosphopeptide mapping, the precipitates (15-40 μg of protein) were oxidized with performic acid (29), then freeze-dried and taken into 40 μl of 0.2 M NH4HCO3. The samples were digested by addition of 10 μl of TPCK-treated trypsin at 0.7 mg/ml in 50 mM in NH4HCO3. After 24 h at 37 °C, a further 50 μg of trypsin

Separation of α and β Forms of hsp27 by Chromatofocusing—The hsp27-containing fractions were pooled (total volume 2.4 ml) and dialyzed against 200 ml of buffer A (25 mM Bis-Tris/HisCl, pH 7.1, 2 mM dithiothreitol, 7.5 μM urea, and 0.1% Brij 35) at 4 °C. The dialyzed fractions were filtered (0.45 μm) and loaded at room temperature on Mono Q anion exchange chromatography column (Pharmacia) equili-

Separation of the a and B Forms of hsp27 by Chromatofocusing—

The hsp27-containing fractions were pooled (total volume 2.4 ml) and dialyzed against 200 ml of buffer A (25 mM Bis-Tris/HisCl, pH 7.1, 2 mM dithiothreitol, 7.5 μM urea, and 0.1% Brij 35) at 4 °C. The dialyzed fractions were filtered (0.45 μm) and loaded at room temperature on Mono Q anion chromatofocusing column (Pharmacia) equili-

The α-caseins were determined by subtracting from the counts. 

Phosphoamino Acid Analysis and Phosphopeptide Maps—In vitro phosphorylation of hsp27 or β-casein were carried out as for kinase assays except that hsp27, β-casein, ATP, and [γ-32P]ATP were used at concentrations of 200 μg/ml, 1 mg/ml, 5 μM, and 1 μCi/μl, respectively. After one-

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were added and the incubation was extended for another 24 h. Separation of phosphopeptides on cellulose plates by electrophoresis at pH 8.9 (first dimension), and ascending chromatography (second dimension) was done as described previously (14).

Miscellaneous—All protein concentrations other than that of the pure hsp27 were determined by the method of Bradford (30), with bovine serum albumin as a standard. Metabolic labeling of MRC-5 fibroblasts with [32P]orthophosphoric acid and immunoprecipitation of hsp27 were carried out as described previously (14). One- and two-dimensional electrophoresis were carried out as before (25). Molecular mass standards for electrophoresis were phosphorylase a (94 kDa), bovine serum albumin (67 kDa), ovalbumin (45 kDa), carbonic anhydrase (29 kDa), and soybean trypsin inhibitor (22 kDa). After electrophoresis, the slab gels were stained with Coomassie Brilliant Blue and dried. Detection of radioisotopes by autoradiography and quantitation of labeled antibodies, phosphoproteins, phosphopeptides, or phosphoamino acids were done as before (14, 25). Counts were corrected for background.

RESULTS

Detection of the Cytokine-activated hsp27 Kinase in Mono Q Chromatography Fractions—The cytokine-activated hsp27 kinase had previously been found to be associated with the cytosolic fraction of MRC-5 fibroblasts (25). In order to characterize it further, cytosolic preparations of MRC-5 cells were chromatographed on the anion exchanger Mono Q. The column was eluted by a gradient of increasing NaCl concentration, and the fractions collected were assayed for phosphorylating activity using pure unphosphorylated hsp27 (hsp27-a) as a substrate. Fig. 2 shows that an hsp27 kinase eluted from the Mono Q column in fractions containing between 50 and 100 mM NaCl (fractions 10 and 11 in Fig. 2). Comparison between fractions of resting cells and of cells that had been stimulated by IL-1 for 10 min showed that there was more activity in fractions of stimulated cells (Fig. 2). The fractions eluting after the main peak contained low levels of hsp27-phosphorylating activity. In some experiments, this activity was resolved into one or two broad peaks that were not regulated by the cytokines (data not shown). All the hsp27 kinase bound to the column and no further active material was eluted when it was washed with 1 M NaCl after the gradient had been run (data not shown).

Quantitation of the 32P incorporated into hsp27 showed that stimulation of the kinase by IL-1 was about 3-fold (Fig. 3). When the effect of TNF was compared with that of IL-1, it was found that each cytokine stimulated the kinase to the same extent (Fig. 3A). By contrast, stimulation of the fibroblasts with PMA induced no significant activation of hsp27 kinase (Fig. 3B).

The specificity of the hsp27 kinase was investigated by testing the ability of the Mono Q fractions containing it to phosphorylate a variety of substrates. When the least acidic phospho form of hsp27, hsp27-b, was used, a kinase activity that coeluted with hsp27 kinase was detected (data not shown). The hsp27-b-phosphorylating enzyme was activated to the same extent as hsp27 kinase in fractions of IL-1-stimulated cells (data not shown). These results suggested that hsp27 kinase was able to phosphorylate both hsp27-a and hsp27-b. However, it had no detectable activity on histone H1 or α-crystallins (data not shown).

Analysis of the in Vitro Phosphorylated hsp27—In metabolically labeled human cells, the phosphorylation of hsp27 on several serine residues generates three increasingly phosphorylated phospho forms that produce a characteristic pattern on two-dimensional gels (6, 13–15). To check whether or not the physiologically relevant phospho forms were produced by hsp27 kinase, we phosphorylated hsp27-a and hsp27-b and then subjected them to phosphoamino acid analysis. Both

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**Fig. 1.** Two-dimensional electrophoresis of the purified fractions containing hsp27-a and hsp27-b. After the final dialysis step the fractions containing hsp27-a (A) and hsp27-b (B) were subjected to two-dimensional electrophoresis and the slab gels were stained with Coomassie Brilliant Blue as described. The pH range of the isoelectric focusing and the positions of molecular mass markers with their masses in kDa are indicated.

**Fig. 2.** Phosphorylation of hsp27 by Mono Q chromatography fractions of MRC-5 cytosols. Cytosolic preparations of unstimulated (A) and IL-1-stimulated (B) fibroblasts were prepared and chromatographed on a Mono Q column as described under "Materials and Methods." The column fractions were then assayed for hsp27 phosphorylating activity, and the reaction mixtures were resolved by electrophoresis as described. Autoradiographs of the gels are shown. The positions of molecular mass standards and of hsp27 are indicated on the sides of the gels, the chromatography fraction numbers on top. The NaCl gradient applied to the column is shown in C.
substrates were found to be phosphorylated exclusively on serine residues (Fig. 4). We also compared by two-dimensional electrophoresis the phospho forms produced in vitro with those found in metabolically labeled fibroblasts (Fig. 5). The initial products of the in vitro phosphorylation of hsp27-a by hsp27 kinase were found to consist of two major phospho forms (spots I and 2 on Fig. 5A). A minor, more acidic phospho form was also detected (spot 3, Fig 5A). Electrophoresis of a mixture of the in vitro-phosphorylated hsp27 and the phospho forms immunoprecipitated from metabolically labeled cells showed that spots 2 and 3 had electrophoretic mobilities identical to those of the b and c forms of hsp27 (Fig. 5C). These results indicate that the IL-1-activated hsp27 kinase is able to generate three hsp27 phospho forms, two of which are identical to those found in cells.

**Gel Filtration Chromatography of hsp27 Kinase**—The IL-1-stimulated hsp27 kinase was further characterized by gel filtration on a Sephacryl S-200 column. The main peak of active material eluted from the column at a position corresponding to a molecular mass of about 45 kDa for the enzyme (Fig. 6). In some experiments, a shoulder of this peak suggested the presence of a second minor component of 70 kDa (Fig. 6). The enzyme was also chromatographed on another gel filtration medium, TSK 3000 (Pharmacia) and eluted sharply at a position corresponding to 45 kDa (data not shown).

**Inhibition of hsp27 Kinase**—The sensitivity of Mono Q-

**FIG. 3.** Activity of hsp27 kinase in Mono Q chromatography fractions of cells stimulated by IL-1, TNF or PMA. A, cytosolic fractions of resting (O), IL-1- (●), and TNF-stimulated (▲) fibroblasts were chromatographed as in Fig. 2. After assay of the fractions for hsp27-kinase activity and electrophoresis, the [32P]orthophosphoric acid and stimulated with IL-1 for 10 min. The cells were then lysed, and hsp27 was immunoprecipitated with the specific antiserum. The in vitro phosphorylation reaction mixture (A), the immunoprecipitate (B), and a mixture of both (C) were then subjected to two-dimensional electrophoresis. Autoradiographs of portions of the gels containing the hsp27 forms are shown. The – and + signs show the focusing orientation. The position of the Coomassie Blue-stained hsp27-a (red) is indicated. The phospho forms of hsp27 produced in vitro are designated 1, 2, and 3, and those immunoprecipitated from metabolically labeled cells are indicated as b, c, and d in order of increasing acidity.

**FIG. 4.** Phosphoamino acid analysis of in vitro-phosphorylated hsp27. Cytosolic fractions of IL-1-stimulated cells were chromatographed on Mono Q and the fractions were assayed as in Fig. 2. The chromatography fraction containing the IL-1-activated kinase was then used to phosphorylate hsp27-a (A) and hsp27-b (B) for phosphoamino acid analysis as described under “Materials and Methods.” Autoradiographs of the cellulose plates are shown. The directions of migration for the first (I) and second (2) dimensions and the positions of standard phosphoserine (S), phosphothreonine (T), and phosphotyrosine (Y) are indicated.

**FIG. 5.** Two-dimensional electrophoresis of hsp27 phospho forms. A cytosolic fraction of IL-1-stimulated MRC-5 cells was prepared and chromatographed on Mono Q as in Fig. 2. hsp27 kinase was then used to phosphorylate hsp27-a. A separate culture of fibroblasts was metabolically labeled with [32P]orthophosphoric acid and stimulated with IL-1 for 10 min. The cells were then lysed, and hsp27 was immunoprecipitated with the specific antiserum. The in vitro phosphorylation reaction mixture (A), the immunoprecipitate (B), and a mixture of both (C) were then subjected to two-dimensional electrophoresis. Autoradiographs of portions of the gels containing the hsp27 forms are shown. The – and + signs show the focusing orientation. The position of the Coomassie Blue-stained hsp27-a (red) is indicated. The phospho forms of hsp27 produced in vitro are designated 1, 2, and 3, and those immunoprecipitated from metabolically labeled cells are indicated as b, c, and d in order of increasing acidity.

purified hsp27 kinase to putative activators or inhibitors was investigated. The kinase was not affected by addition of 200 μM CaCl2 to the reaction mixture (data not shown). Heparin and NaF, which have been reported to be stimulatory and inhibitory, respectively, on MAP kinase (19), had no significant effect on hsp27 kinase (Table I). Of the ATP analogues tested, GTP was the most interesting, reducing the labeling of the substrate by 50% at a concentration (40 μM) that was twice that of the ATP (Table I). This result suggests that hsp27 kinase may be able to use GTP as a phosphate donor. Staurosporine, the protein kinase C inhibitor, had little effect when used at 1 μM (Table I). Protein kinase C is inhibited by nanomolar amounts of the drug (31). 2-Aminopurine (1 mM) inhibited hsp27 kinase only partially, while 6-thioguanine (0.5 mM) and genistein (10 μg/ml) had no effect (data not shown).

**Detection of p42 MAP Kinase in the Mono Q Fractions**—IL-1 and TNF have been reported to stimulate the p42 and p44 mitogen-activated protein kinases (19). We assayed the Mono Q fractions of MRC-5 cytosols for MBP-phosphorylating activity to assess whether or not the cytokine-activated
**FIG. 6. Gel filtration of hsp27 kinase.** The IL-1-activated hsp27 kinase prepared as in Fig. 2 was concentrated by ultrafiltration and chromatographed on Sephacryl S-200. The gel filtration fractions were assayed for hsp27 kinase activity as described except that ATP was 5 μM. The 32P incorporated into the substrate was quantitated by counting. The result of a calibration of the column by the molecular mass standards Blue dextran (Vo), transferrin (78 kDa), ovalbumin (45 kDa), carbonic anhydrase (29 kDa), and cytochrome c (12.5 kDa) is shown.

**TABLE I**

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>hsp27 kinase</th>
<th>β-Casein kinase</th>
<th>Casein kinase II</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heparin 1 μg/ml</td>
<td>112</td>
<td>110</td>
<td>79</td>
</tr>
<tr>
<td>Heparin 10 μg/ml</td>
<td>118</td>
<td>86</td>
<td>38</td>
</tr>
<tr>
<td>GTP 40 μM</td>
<td>50</td>
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<td>94</td>
<td>22</td>
</tr>
<tr>
<td>Staurosporine 100 nM</td>
<td>86</td>
<td>51</td>
<td>109</td>
</tr>
<tr>
<td>Staurosporine 1 μM</td>
<td>70</td>
<td>21</td>
<td>81</td>
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hsp27 kinase corresponded to any of the MAP kinases. A single peak of MBP kinase was detected in Mono Q fractions prepared from cytosol of unstimulated cells (Fig. 3, A and B). This activity was increased 2–3-fold in cells that had been treated by IL-1, PMA, or TNF for 10 min (Fig. 7). The distribution of the MBP kinase in the Mono Q fractions coincided with two antigens of 42 and 44 kDa detected by immunoblotting with an anti-MAP kinase antiserum (Fig. 7). The 42-kDa antigen was also detected by a monoclonal antibody specific for p42 MAP kinase (data not shown). The 44-kDa band may correspond to a cross-reaction of the polyclonal antibody with p44 MAP kinase. These results show that the MBP kinase activity is clearly distinct chromatographically from hsp27 kinase and corresponds to p42 and, probably, p44 MAP kinases.

**Detection of Cytokine-stimulated Casein Kinases in Mono Q Fractions**—Casein kinases I and II are ubiquitous serine/threonine-specific protein kinases that were originally detected by their ability to phosphorylate caseins or phosvitin in vitro (reviewed in Ref. 32). Several recent reports suggest that casein kinase II may be activated within minutes in cells stimulated by insulin or epidermal growth factor (33, 34), which are known activators of MAP kinase (19, 20). We decided to use α- and β-caseins as substrates in phosphorylation assays of the Mono Q fractions to check whether or not an activation of casein kinase II accompanied that of p42 MAP kinase in IL-1- or TNF-stimulated cells.

Three regions of the chromatogram of cytosols from resting cells contained enzymes able to phosphorylate α- and β-caseins (Fig. 8). Casein kinase II is inhibited by low concentrations of heparin (35) and is able to use GTP as a phosphate donor (32). These properties were used to identify it in the fractions. The first and second casein kinases to elute were not inhibited by heparin at up to 10 μg/ml (data not shown). They were designated casein kinases A and B. The third
casein kinase, a relatively abundant enzyme eluting at about 400 mM NaCl, was inhibited by heparin and by GTP (Table I). These results clearly identify it as casein kinase II.

Assays of casein kinase activities in Mono Q fractions of IL-1-stimulated cells showed no significant change in the activity of casein kinase II, relative to fractions of unstimulated cells (Fig. 8). However, the cytokine dramatically activated a kinase that phosphorylated β-casein, but had little activity on α-casein (Fig. 8). This IL-1-regulated enzyme, named β-casein kinase, eluted between casein kinase B and casein kinase II (fractions 21-27) and was therefore chromatographically distinct from hsp27 kinase and MAP kinases. β-Casein kinase was also activated in fractions prepared from TNF-stimulated cells (data not shown). Quantitation of the 32P incorporated into β-casein indicated that the kinase was activated between 3- and 6-fold in fractions of cells stimulated with IL-1 or TNF for 10 min (data not shown). The cytokines also induced a small but reproducible increase in the activity of casein kinase B, but did not activate casein kinase A (Fig. 8). Casein kinase B was also activated in fractions of cells that had been stimulated with PMA, but casein kinase A, β-casein kinase, and casein kinase II were not (Fig. 8).

Characterization of in Vitro Phosphorylated β-Casein—Casein kinase II and β-casein kinase produced phospho forms of β-casein whose electrophoretic mobilities differed from each other and from the unphosphorylated protein, detected by Coomassie Blue staining of gels (Fig. 8). By contrast, β-casein was not electrophoretically retarded after phosphorylation by casein kinase A or B. These results suggested that the casein kinases separated by chromatography on Mono Q phosphorylated β-casein on different residues. We therefore investigated the in vitro phosphorylation sites of β-casein by phosphoamino acid analysis and tryptic peptide mapping.

Phosphoamino acid analysis (Fig. 9) showed that the cytokine-activated β-casein kinase phosphorylated β-casein predominantly on serine residues (89%) and to a smaller extent, on threonine residues (11%). Phosphorylation by casein kinase A also produced mainly phosphoserine, whereas the major targets for casein kinases B and II were threonine residues.

Tryptic phosphopeptide maps confirmed the differences in specificity between the four enzymes (Fig. 10). β-Casein phosphorylated by casein kinases A (A) and B (B), the β-casein kinase (C), and casein kinase II (D) and the protein was subjected to phosphoamino acid analysis. Orientation of the plates is as in Fig. 4. The positions of standard phosphoserine, phosphothreonine, and phosphotyrosine are indicated as in Fig 4.

Cytokine-activated hsp27- and β-Casein Kinases

Fig. 8. Detection of casein kinases in the Mono Q fractions. Cytosolic preparations of unstimulated cells (A) and cells stimulated by IL-1 (B) and PMA (C) were chromatographed as before, and the fractions were assayed for casein kinase activities. Portions of the autoradiographs are shown. The fraction numbers, the positions of the Coomassie Blue-stained α- and β-caseins, and the regions of the chromatograms containing casein kinases A (CKA), B (CKB), and II (CKI) are indicated.

Fig. 9. Phosphoamino acid analysis of β-casein. β-Casein was phosphorylated by casein kinases A (A) and B (B), the β-casein kinase (C), and casein kinase II (D), and the protein was subjected to phosphoamino acid analysis. Orientation of the plates is as in Fig. 4. The positions of standard phosphoserine, phosphothreonine, and phosphotyrosine are indicated as in Fig 4.

Fig. 10. Tryptic phosphopeptide maps of β-casein. β-Casein was phosphorylated by casein kinases A (A) and B (B), the β-casein kinase (C), and casein kinase II (D) and subjected to digestion by trypsin as described under “Material and Methods.” The positions of the origins are indicated (O). The − and + signs show the orientation of electrophoresis, the arrow shows the direction of chromatography.

Fig. 11. Gel Filtration Chromatography of β-Casein Kinase—Gel filtration chromatography of concentrated Mono Q fractions containing the TNF-activated β-casein kinase resulted in the separation of two active components of molecular masses near 45 and 65 kDa (Fig. 11). Similar results were obtained with fractions of IL-1-treated cells (data not shown). However, chromatography of fractions prepared from resting cells
Cytokine-activated hsp27- and β-Caseine Kinases

The kinase generated in vitro two phospho forms of hsp27 that comigrated on two-dimensional gels with the b and c phospho forms immunoprecipitated from metabolically labeled cells. These results are consistent with the IL-1-activated kinase under study being involved in the phosphorylation of hsp27 in cells. A third phospho form that had no counterpart in metabolically labeled cells was also produced in vitro. It may correspond to the phosphorylation of the substrate on a nonphysiological site in artificial conditions.

hsp27 kinase appeared to have a molecular mass of 45 kDa and not to be regulated by Ca²⁺, F⁻, or heparin. None of the kinase inhibitors tested had any strong effect on it. GTP inhibited the labeling of hsp27, which suggests that hsp27 kinase may be able to use it as a phosphate donor. hsp27 kinase had no detectable activity on histone H1 or MBP, a finding that clearly distinguished it from protein kinase C and MAP kinase.

Heat shock and mitogens, which increase hsp27 phosphorylation in the Chinese hamster cell line CCL 39, have been found to stimulate protein kinases that could be detected in crude extracts when purified hsp27 or the ribosomal protein S6 were used as substrates (36). The -Arg-X-X-Ser- motif is present in two of the phosphorylation sites of protein S6, which suggests that S6 kinases could be involved in hsp27 phosphorylation. However, the molecular mass of the cytokine-activated hsp27 kinase is not consistent with it being either of the S6 kinases, whose molecular masses are close to 90 and 70 kDa, respectively (38). The inability of hsp27 kinase to phosphorylate α-crystallins, which are structurally related to hsp27 and possess -Arg-X-X-Ser- motifs (36, 39), suggests that structural features exclusive to hsp27 are involved in its recognition by the kinase. Such a narrow specificity is consistent with our previous observation that hsp27 kinase can phosphorylate the endogenous hsp27 in crude cell extracts where both are at very low concentrations (25). Its specificity for hsp27 and its unusual sensitivity to GTP both strongly suggest that hsp27 kinase is a novel enzyme.

The possibility that MAP kinase might be involved in the signal transduction mechanisms triggered by IL-1 and TNF was first suggested by the results of kinase assays carried out in crude extracts of fibroblasts with microtubule-associated protein 2 as a substrate (15). Subsequently, a protein kinase that was activated by the cytokines and phosphorylated a synthetic substrate of MAP kinases was detected in extracts of a wide range of cell types. This activity was purified from KB carcinoma cells and shown to correspond to p42 and p44 MAP kinases (19). The evidence presented here clearly identifies the major enzyme responsible for the MBP phosphorylation in the Mono Q fractions of MRC-5 fibroblasts as p42 MAP kinase. Only small amounts of p44 MAP kinase were detected immunologically. The MAP kinases had been reported to be activated about 10-fold by IL-1 and TNF in MRC-5 cells (19), but we have found the enzyme to be only activated 2–3-fold. The reason for this discrepancy is unclear. We have found that resting cells contained a significant amount of activity, suggesting that the enzyme was already partially activated in unstimulated cells. However, attempts to reduce this basal activity by leaving the cells in a medium containing 0.5% newborn calf serum for 4 days before the experiments were unsuccessful (data not shown).

We have used caseins as substrates to investigate whether or not casein kinase II, or another casein phosphorylating enzyme, was activated by IL-1 or TNF. Three casein kinases were detected in the column fractions of resting cells, and the major one was identified as casein kinase II on the basis of its sensitivity to heparin and GTP. Whether or not one of the two other casein kinases correspond to casein kinase I is unclear. There are no specific inhibitors of casein kinase I,

**FIG. 11.** Gel filtration of β-casein kinase. Mono Q fractions containing the TNF-activated β-casein kinase (B) and similar fractions prepared from resting cells (A) were concentrated and chromatographed on Sephacryl S-300. The gel filtration fractions were assayed for casein-kinase activity as described except that ATP was 5 μM. Portions of the autoradiograms containing the caseins are shown. The fraction numbers and the positions of elution of transferrin (78 kDa), ovalbumin (45 kDa), and carbonic anhydrase (29 kDa) are shown on top. The positions of α- and β-caseins are indicated.

The kinase activity corresponding to one of the phosphorylation sites of the murine heat shock protein have been recently identified, serine 78 and serine 82 (36). A third phosphorylation site exists that has not yet been identified. It may be serine 15, which is homologous to one of the phosphorylation sites of the murine small heat shock protein (36, 37). All these sites are located in sequence motifs of the form -Arg-X-X-Ser-. Although we have not yet checked whether these sites are phosphorylated in vitro by hsp27 kinase, we have shown that the enzyme phosphorylates exclusively serine residues on the substrate.

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and the name is often given to any casein kinase activity that is not inhibited by heparin or GTP (92). Hybridization experiments have suggested that casein kinase I may correspond to a family of related enzymes rather than to a single one (40). Casein kinase I from rabbit reticulocytes phosphorylates bovine $\beta$-casein on serine 22 (41). The results of the phospho-
amino acid analysis of $\beta$-casein are consistent with casein kinase A having a similar specificity. Because of its preference for threonine residues of $\beta$-casein, casein kinase B seems less likely to correspond to casein kinase I.

We have found no evidence to suggest that casein kinase II and casein kinase A are regulated by the cytokines or by PMA. Casein kinase B appeared to be stimulated to a small extent (1.5-2-fold) by these agents. However, this kinase coloated from the Mono Q column with the MAP kinases, and we cannot rule out the possibility that they were responsible for the increased phosphorylation of casein. Further work will be needed to establish whether or not casein kinase B is another cytokine-regulated enzyme.

The most dramatic effect of IL-1 and TNF was the 4.5-fold stimulation of a $\beta$-casein kinase that had been unnoticed in the fractions of resting cells. The lack of activity of this enzyme on $\alpha$-casein and the characteristics of the in vitro-phosphorylated $\beta$-casein all indicated that its substrate spec-
diety differed from that of casein kinases A, B, and II. The sequences of $\alpha$- and $\beta$-caseins show little homology outside the short acidic regions that bear the phosphorylation sites of casein kinases I and II (42), and $\beta$-casein kinase is probably specific for sites that are absent from $\alpha$-casein. With the exception of stauroporine, none of the protein kinase inhibitors tested had any significant effect on it at the doses used.

Gel filtration chromatography showed the cytokine-activated $\beta$-casein kinase to have a molecular mass of 65 kDa. The occasional detection of stimulated $\beta$-casein kinase activity in fractions eluting near the void volume of the S-300 column suggested that the mass of the native enzyme might conceivably be bigger. In any case, the characteristics of $\beta$-casein kinase suggest that it corresponds to a new activity.

The maintenance of hsp27 and $\beta$-casein kinases in their activated state after partial purification strongly suggests that, like MAP kinases, they are not dependent on second messengers. Activation of the MAP kinases has been found to occur via their phosphorylation on threonine and tyrosine residues by a dual specificity protein kinase termed MAP kinase kinase, or MAP kinase activator (43-45). Whether or not hsp27 and $\beta$-casein kinases are activated by a similar mechanism remains to be investigated.

Stimulation of cells with PMA is known to mimic some of the effects of the cytokines, such as synthesis of prostaglandin E$_2$ (16), the activation of MAP kinase (19), and of the trans-
scription factors NF-$\kappa$B and AP-1 (17, 18). We have found that, unlike MAP kinase, hsp27 and $\beta$-casein kinases were not activated in PMA-treated cells. These results are consist-
ent with previous work on metabolically labeled MRC-5 cells which showed that PMA did not strongly increase hsp27 phosphorylation (14). Furthermore, cytokine-induced phos-
phorylation of hsp27 (13, 14) and of the epidermal growth factor receptor (8, 9) have been shown to be independent of protein kinase C since they occurred in cells depleted of protein kinase C or were not inhibited by stauroporine. The inability of PMA to stimulate the two novel enzymes indicated that, in MRC-5 cells, they are not under the control of either protein kinase C or MAP kinases. Investigation of their regulation will be crucial to the progress of our understanding of the signal transduction mechanisms used by IL-1 and TNF.