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Interleukin-1-Induced Intracellular Signaling Pathways Converge in the Activation of Mitogen-Activated Protein Kinase and Mitogen-Activated Protein Kinase-Activated Protein Kinase 2 and the Subsequent Phosphorylation of the 27-Kilodalton Heat Shock Protein in Monocytic Cells

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SUMMARY

Interleukin (IL)-1 plays a central role in human host defense. Binding of IL-1 to its receptor is associated with phosphorylation of various cellular target proteins, most of which are unidentified. The kinases responsible for target protein phosphorylation after IL-1 stimulation are also still not completely understood. We report here that IL-1 induced activation of mitogen-activated protein (MAP) kinase in primary monocytes and in the human monocytic leukemia cell line U-937. Activation of MAP kinase was followed by activation of MAP kinase-activated protein (MAPKAP) kinase 2, a serine/threonine kinase, leading to subsequent phosphorylation of the small heat shock protein [27-kDa heat shock protein (Hsp27)]. Phosphorylation of Hsp27 triggered by IL-1 was both dose and time dependent. IL-1 failed to phosphorylate Hsp27 when cells had been previously deactivated with tyrosine kinase inhibitors such as genistein. In those cells, however, Hsp27 phosphorylation could be reconstituted when activated immunoprecipitated MAP kinase or purified MAPKAP kinase 2 was added. Phosphorylation of Hsp27 could also be inhibited when NaF, a serine/threonine phosphatase inhibitor, was omitted. Taken together, our findings indicate that IL-1-induced intracellular signaling pathways converge in the activation of MAP kinase and MAPKAP kinase 2 and the subsequent phosphorylation of Hsp27.

IL-1, a 17-kDa protein consisting of an α and β species, is a pleiotropic cytokine operating as an important regulator of human host defense (1). It exerts a multitude of biological effects on diverse cell types, including induction of secondary cytokines in fibroblasts, endothelial cells, T lymphocytes, and monocytes (2–6), as well as stimulation of proteinases and release of eicosanoids in connective tissue cells (7). IL-1 also stimulates secretion of acute-phase reactants in liver cells and regulates as a cofactor the proliferation and differentiation of hematopoietic progenitor cells (6, 8, 9). IL-1 binds to type I and II IL-1 receptors (10). Receptor occupation by IL-1 is followed by the intracellular release of H_2O_2 (11) and the activation of G proteins (12). Furthermore, signaling events triggered by IL-1 involve phosphorylation of heat shock proteins (13, 14) and induce activation of the transcription factors NF-κB and NF-IL6 (11, 15). Recent studies have demonstrated that signaling of IL-1 is predominantly, if not exclusively, mediated by the type I IL-1 receptor (16, 17), whereas the type II receptor serves as a negative regulator for IL-1 activity (18). Like other cytokine receptors, the type I IL-1 receptor does not contain an intrinsic kinase domain. Exposure to IL-1 is associated with rapid phosphorylation of several cellular target proteins, most of which are unidentified (19, 20). We show here that exposure of human monocytes and the monocytic leukemia cell line U-937 to IL-1 activates MAP kinase, which in turn activates MAPKAP kinase 2 and thereby leads to serine phosphorylation of Hsp27.

Materials and Methods

Purification and culture of monocytes. Leukocytes were obtained by leukapharesis of consenting healthy volunteer blood donors. Mononuclear cells were separated by density gradient centrifugation

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ABBREVIATIONS: IL, interleukin; MAP, mitogen-activated protein; MAPKAP, mitogen-activated protein kinase-activated protein; MBP, myelin basic protein; Hsp27, 27-kDa heat shock protein; PBS, phosphate-buffered saline; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; PMSF, phenylmethylsulfonyl fluoride; TNF-α, tumor necrosis factor-α; MOPS, 3-(*N*-morpholino)propanesulfonic acid.

on a Ficoll-Hypaque cushion (Seromed Biochrom, Berlin, Germany). Monocytes were purified from the unfractionated mononuclear leukocyte preparation by counter-current centrifugal elutriation (21). The purity of the monocyte preparation was assessed by morphological means and cytochemistry (nonspecific esterase staining) and was always >95%, with <5% contaminating cells with the morphology of basophils and eosinophils. Viability assessed by trypan blue dye exclusion was >98%. Monocytes were cultured at a density of 10^5 to 10^6 cells/ml under nonadherent conditions, either in 96-well round-bottomed polypropylene microtiter plates (Wilks Precision Instruments, Rockville, MD) or in Teflon bottles (Nalge, Baltimore, MD), in RPMI 1640 culture medium (Seromed Biochrom) supplemented with 10% low-endotoxin fetal calf serum (Hazelton, Vienna, VA), 2 mM L-glutamine, 20 mM HEPES, 100 units/ml penicillin, 100 units/ml streptomycin, and 10 μg/ml polymyxin-B (Sigma, Munich, Germany) (standard culture medium). Some of the cultures were treated with recombinant human IL-1β (Boehringer Mannheim, Mannheim, Germany) at 5–500 units/ml for the indicated time periods.

Cell line and cell culture. The human monocytic cell line U-937 was obtained from Dr. H. G. Drexler, German Collection of Microorganism and Cell Cultures (Braunschweig, Germany), and was maintained in standard culture medium. Exponentially growing U-937 cells were washed twice in PBS and incubated for 24 hr in serum-free medium supplemented with 0.5% bovine serum albumin in the absence of growth factors, to deprive cells of factors. Cells were equilibrated at 37° before stimulation with IL-1β. IL-1β was added for up to 2 hr in various concentrations (5–500 units/ml), followed by the MAPKAP kinase 2 assay. In selected experiments cells were treated with genistein (10 μg/ml; Biomol, Hamburg, Germany) for 2 hr before IL-1 stimulation.

Immunoprecipitation and Western blot analysis. Immunoprecipitation and Western blot analysis were performed essentially as described previously (22). Briefly, cells were lysed in RIPA buffer (20 mM Tris, pH 7.5, 150 mM NaCl, 1 mM EDTA, 0.5% deoxycholate, 1% Triton X-100, 0.1% SDS), followed by centrifugation at 20,000 × *g* for 20 min at 4° to remove insoluble material. MAP kinase was immunoprecipitated with monoclonal antibodies directed against the 42-kDa MAP kinase (Upstate Biotechnology, Lake Placid, NY), followed by SDS-PAGE. Proteins were transfered to Immobilon P membranes (Milipore, Eschborn, Germany) and filters were probed with a specific antityrosine monoclonal antibody (Upstate Biotechnology).

MAP kinase assay. The MAP kinase assay was performed as described previously (23). Briefly, after stimulation cells were resuspended in ice-cold PBS and washed twice. The cell pellet was lysed by addition of a buffer containing 20 mM HEPES, pH 7.5, 10 mM EGTA, 2.5 mM Mg_2Cl_2, 1 mM PMSF, 1 mM dithiothreitol, 3 μg/ml leupeptin, 2 mM sodium orthovanadate, and 40 mM β-glycerol-phosphate, and cytosolic fractions were subjected to fractionation on a Mono Q column (Pharmacia) equilibrated in Mono Q buffer (20 mM MOPS pH 7.0, 1 mM EDTA, 5%, v/v, glycerol, 0.01% Brij 35). The column was subjected to a linear 0–0.8 M NaCl gradient using a Pharmacia fast protein liquid chromatography system. Fractions of 1 ml were collected, and protein concentrations were quantified using the Bradford assay (24). Equal amounts of eluted proteins were subjected to the MAP kinase assay, using recombinant MBP as substrate.

MAPKAP kinase 2 assay. The MAPKAP kinase 2 assay was performed essentially as described previously (23, 25). Briefly, after stimulation cells were resuspended in ice-cold PBS and washed twice. The cell pellet was lysed by addition of buffer L [20 mM Tris-acetate, pH 7.0, 0.1 mM EDTA, 1 mM EGTA, 1 mM sodium orthovanadate (Sigma), 10 mM α-glycerol-phosphate (Sigma), 50 mM NaF (Sigma), 5 mM pyrophosphate (Sigma), 1% Triton X-100 (Sigma), 1 mM benzamidine (Sigma), 2 mg/ml leupeptin (Sigma), 0.1% β-mercaptoethanol (Sigma), 0.27 M sucrose (Sigma), 0.2 mM PMSF (Sigma)], vortexed for 30 sec, and cleared by centrifugation. Cell lysates were diluted 1/10 in buffer A [20 mM MOPS, pH 7.0, 1 mM EDTA, 5%, v/v, glycerol, 0.01%, v/v, Brij 35 (Sigma), 0.2 mM PMSF, 1 mM benzamidine, 0.1%, v/v, β-mercaptoethanol] and applied to a Mono S column (Pharmacia) equilibrated in buffer A. The column was subjected to a 30-ml linear salt gradient of 0–500 mM NaCl in buffer A. Fractions of 1 ml were collected and protein concentrations were quantified using the Bradford assay (24). Equal amounts of eluted proteins were subjected to the MAPKAP kinase 2 assay. Fractions were incubated for 15 min at 30° with 10 μg of recombinant Hsp27 purified from *Escherichia coli* (26), in 25-ml reactions containing 50 mM α-glycerol-phosphate, 0.1 mM EDTA, 2.5 mM protein kinase A inhibitor (GIBCO, Glasgow, Scottland), 20 mM H-7 (Biomol), 20 mM HA1007 (Biomol), 4 mM magnesium acetate, 0.1 mM ATP, and 2 mCi of [γ-^{32}P]ATP (Amersham Buchler, Braunschweig, Germany). Reactions were terminated by addition of 8 ml of 4× SDS loading buffer (27), and reaction products were separated by SDS-PAGE. ^{32}P-labeled proteins were detected using a BAS 2000 Bio-Imaging Analyzer (Fuji). In some experiments, NaF was omitted from the MAPKAP kinase 2 assay. In selected experiments, immunoprecipitated MAP kinase or purified MAPKAP kinase 2 (kindly provided by P. Cohen, Department of Biochemistry, University of Dundee, Dundee, Scotland, U.K.) was added to cellular proteins obtained from U-937 cells that had received genistein before exposure to IL-1β.

Results

Tyrosine phosphorylation of the MAP kinase has been demonstrated in human megakaryblastic leukemia cells, neutrophils, and factor-dependent myeloma cells after exposure to cytokines such as IL-3, granulocyte-macrophage colony-stimulating factor, stem cell factor, IL-6, and TNF-α (23, 28–31). To examine whether signaling pathways in human monocytic cells triggered by IL-1 also involve the activation of MAP kinase, the human monocytic cell line U-937 was deprived of serum for 24 hr and then treated with recombinant human IL-1β (100 units/ml) for 30 min. Cell lysates were immunoprecipitated using an anti-MAP kinase monoclonal antibody. Immunoprecipitates were subsequently separated on an SDS gel and immunoblotted with a specific antityrosine monoclonal antibody and an anti-MAP kinase antibody. Exposure of U-937 cells to IL-1β enhanced tyrosine phosphorylation of MAP kinase (Fig. 1). These findings suggest that IL-1 activates MAP kinase in monocytic cells.

To determine whether IL-1 treatment was also associated with activation of MAP kinase, equal amounts of cell lysates obtained from peripheral blood monocytes or U-937 cells that had received IL-1β (100 units/ml) for 30 min were fractionated by ion exchange chromatography on a Mono Q column and subjected to the MAP kinase assay using recombinant MBP as

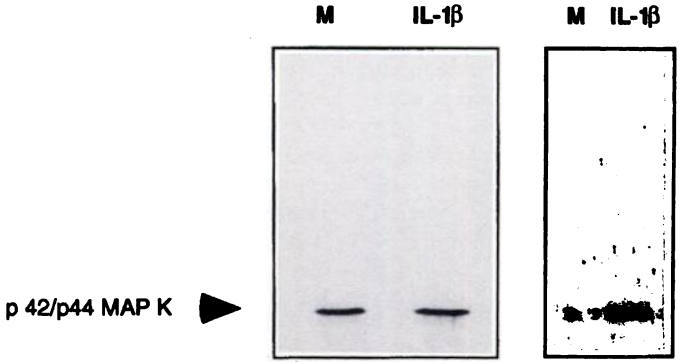

Fig. 1. IL-1β induces tyrosine phosphorylation of p42/p44 MAP kinase in monocytic cells. U-937 cells were deprived of serum for 24 hr and then exposed to serum-free medium without (*M*) or with IL-1β (100 units/ml) for 15 min. Cell lysates were immunoprecipitated with anti-MAP kinase monoclonal antibodies before immunoblotting with anti-MAP kinase monoclonal antibodies (*left*) or antiphosphotyrosine antibodies (*right*). *MAP K*, MAP kinase.

a substrate (28). Resting monocytes do not constitutively display MAP kinase activity. In line with low levels of constitutive tyrosine phosphorylation of MAP kinase seen in serum-deprived U-937 cells, little MAP kinase activity was detectable in fraction 27 from these cells. Upon expsoure to IL-1β, MAP kinase activity was induced in monocytes, whereas U-937 cells responded to IL-1β with an approximately 2-fold enhancement of constitutive MAP kinase activity in fraction 27 (Fig. 2). U-937 cells that had been previously exposed to genistein failed to respond to IL-1β with enhanced MAP kinase activity, indicating that activation of MAP kinase resulted from tyrosine phosphorylation induced by IL-1β.

The capacity of IL-1β to activate MAP kinase was dose and time dependent. U-937 cells were deprived of serum for 24 hr before addition of IL-1β (5–500 units/ml) for 30 min. Cell lysates were fractionated and equal amounts of cellular proteins were subjected to the MAP kinase assay. As shown in Fig. 3, exposure of U-937 cells to 5 units/ml IL-1β was also associated with significant activation of MAP kinase activity. MAP kinase activity further increased when doses of up to 100 units/ml were applied, whereas higher doses did not result in additional increases in MAP kinase activity.

MAP kinase activation upon exposure to IL-1β displayed biphasic activation kinetics. MAP kinase was activated within 5 min upon exposure to IL-1β (100 units/ml), declined after 10–15 min, increased again within 30–60 min after exposure to IL-1β, and declined thereafter (Fig. 4). These findings indicate that IL-1β induces time-dependent activation of MAP kinase in human monocytic cells.

Substrates of MAP kinases include the AP-1 transcription factor, MAPKAP kinase 1 (also referred to as S6 kinase 2), and MAPKAP kinase 2 (23, 25, 31, 32). MAPKAP kinase 2 has been recently identified as the major enzyme phosphorylating the small heat shock protein (Hsp27) on serine residues (26). To further elucidate signaling events inititated by IL-1β "downstream" of MAP kinase, we investigated the potential of cells exposed to IL-1 to phosphorylate Hsp27 *in vitro*. Cells were deprived of serum for 24 hr before exposure to IL-1β for 30 min. Cell lysates were fractionated using a Mono S column and were subjected to the MAPKAP kinase 2 assay with recombinant Hsp27 as substrate (25). Hsp27 contains phosporylation sites at serines 15, 78, and 82, which have been shown to be the target sequences of MAPKAP kinase 2 (25). Assays

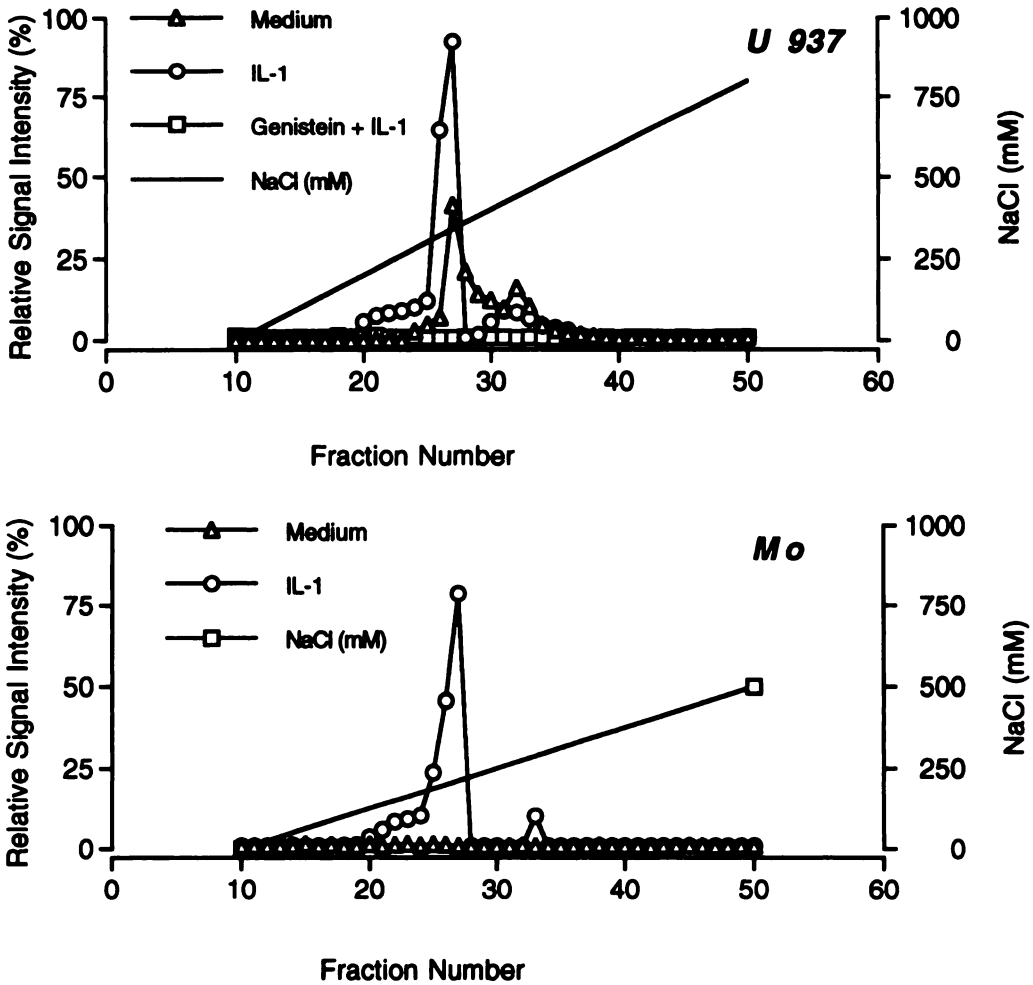

Fig. 2. IL-β induces MAP kinase activity in monocytic cells and in primary monocytes. Human monocytes (*Mo*) or U-937 cells were first deprived of serum for 24 hr and then exposed to serum-free medium in the absence (*Medium*) or the presence of IL-1β (100 units/ml) for 30 min, with or without pretreatment with genistein (10 μg/ml) for 2 hr. Cell lysates were loaded onto a Mono Q column. The column was subjected to a 30-ml linear gradient of 0–800 mM NaCl in Mono Q buffer, at a flow rate of 0.5 ml/min. Column fractions were assayed for MAP kinase activity as described in Materials and Methods, using purified recombinant MBP as substrate. Phosphorylation of MBP was analyzed by quantitation by BAS 2000 phosphoimaging of photostimulated luminescence resulting from ^{32}P-labeled proteins separated by SDS-PAGE and is expressed as signal intensity versus background.

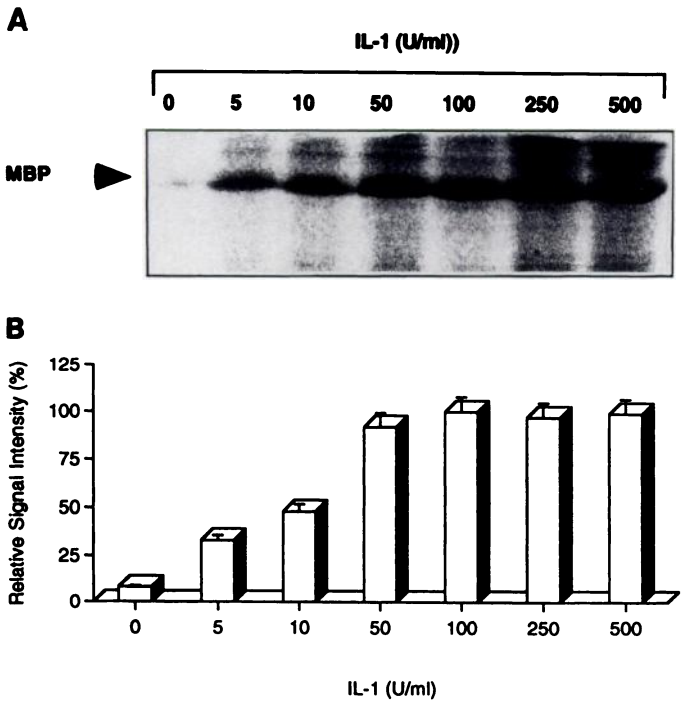

Fig. 3. Dose-response relationship for IL-1 induction of MAP kinase activation in U-937 cells. Cells were deprived of serum for 24 hr and then exposed to increasing concentrations of IL-1β (5–500 units/ml) for 15 min. Cell lysates were assayed for MAP kinase as detailed in Materials and Methods. A, Representative PhosphorImage obtained from U-937 cells. Comparable results were obtained in two additional experiments. B, Phosphorylation of MBP, analyzed by quantitation of photostimulated luminescence as described for Fig. 2 and expressed as relative signal intensity. Maximum signal intensity was arbitrarily set as 100%. Values are expressed as means ± standard deviations of three independent experiments.

were carried out in the presence of protein kinase A inhibitor and the protein kinase inhibitors H-7 and H191077, at concentrations known not to interfere with MAPKAP kinase 2 activity while inhibiting a series of other cellular kinases (24, 25), such as cGMP-dependent protein kinase, smooth muscle myosin light chain kinase, protein kinase C, and insulin-stimulated kinase 1. Little if any MAPKAP kinase 2 activity was detectable in unfractionated lysates of untreated U-937 cells. One peak of MAPKAP kinase 2 activity was observed in the Mono S fractions when cell lysates obtained from IL-1β-treated cells were fractionated (Fig. 5). MAPKAP kinase 2 activity eluted at about 220 mM NaCl in Mono S buffer 20 mM MOPS pH 7.0, 1 mM EDTA, 5% (v/v) glycerol, 0.01% Brij 35, 0.2 mM PMSF, 1 mM benzamidine, 0.1% (v/v) β-mercaptoethanol. In line with these observations, MAPKAP 2 kinase activity was previously identified in rabbit muscle cells (24) and Ehrlich ascites tumors (25), in fractions eluting between 200 and 250 mM NaCl. No MAPKAP kinase 2 activity was measurable in the flow-through fraction or fractions that followed the gradient during washing of the column with 1 M NaCl.

The capacity of IL-1β to activate MAPKAP kinase 2 was both dose and time dependent (Fig. 6). MAPKAP kinase 2 activity was detectable upon exposure of U-937 cells to as little as 5 units/ml IL-1β and further increased when concentrations of up to 100 units/ml were used. Further escalation of the IL-1 dose did not result in additional increases in MAPKAP kinase 2 activity. Although untreated U-937 cells constitutively displayed little, if any, MAPKAP kinase 2 activity, as assessed by phosphorylation of Hsp27, exposure to IL-1β enhanced Hsp27 phosphorylation within 5–10 min. Hsp27 phosphorylation peaked at 30–60 min and declined thereafter.

To prove the notion that phosphorylation of Hsp27 resulted from activation of MAPKAP kinase 2 and required activation of MAP kinase, U-937 cells were pretreated with the tyrosine kinase inhibitor genistein, which has been shown to interfere with the activation of MAP kinase (Fig. 2) (28). Genistein-pretreated U-937 cells failed to respond to IL-1β with enhanced MAPKAP kinase 2 activity in all fractions investigated (Fig. 7A). When activated MAP kinase immunoprecipitated from U-937 cells that had received IL-1β (100/ml, for 30 min) (Fig. 4) was added to genistein-pretreated cellular extracts, phosphorylation of Hsp27 could be reconstituted, whereas addition of

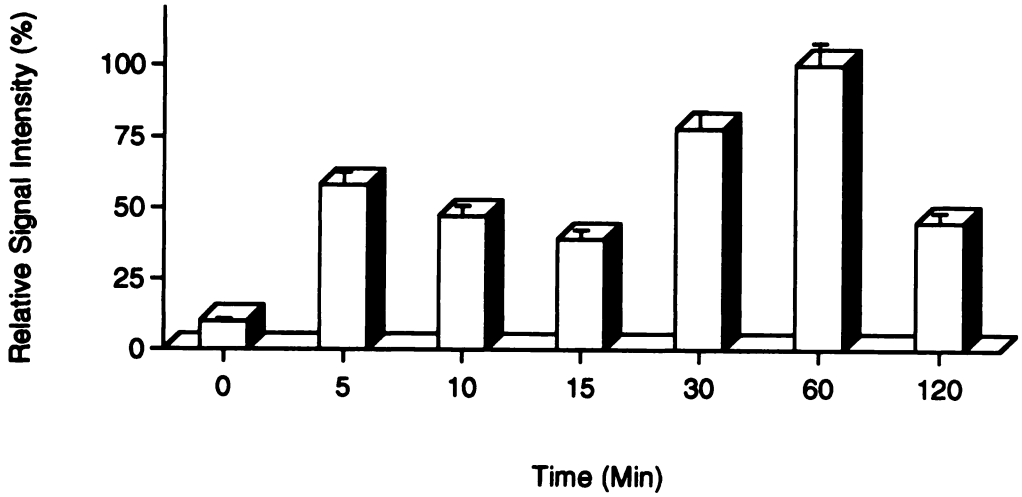

Fig. 4. Time-Kinetics of IL-1β induction of MAP kinase activation in U-937 cells. Cells were deprived of serum for 24 hr and then exposed to IL-1β (100 units/ml) for up to 120 min. Cell lysates were fractionated on a Mono Q column and the column was subjected to a 30-ml linear gradient of 0–800 mM NaCl in Mono Q buffer, at a flow rate of 0.5 ml/min. Column fractions were assayed for MAP kinase as detailed in Materials and Methods. Phosphorylation of MBP was analyzed by quantitation of photostimulated luminescence as described for Fig. 2 and is expressed as relative signal intensity. Maximum signal intensity was arbitrarily set as 100%. Values are expressed as means ± standard deviations of three independent experiments.

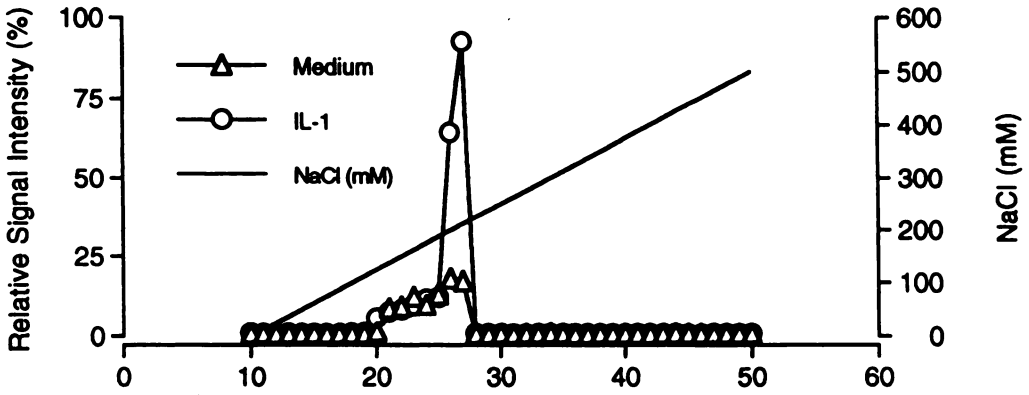

Fig. 5. Identification of MAPKAP kinase 2 activity in U-937 cells. U-937 cells were deprived of serum for 24 hr before IL-1β (10 units/ml) was added. Cell lysates were prepared and separately loaded onto a Mono S column. The column was subjected to a 30-ml linear gradient of 0–500 mM NaCl in buffer A, at a flow rate of 0.5 ml/min. Column fractions were assayed for MAPKAP kinase 2 activity as described in Materials and Methods, using purified recombinant Hsp27 as substrate. Phosphorylation of Hsp27 was analyzed by quantitation of photostimulated luminescence as described for Fig. 2 and is expressed as signal intensity versus background.

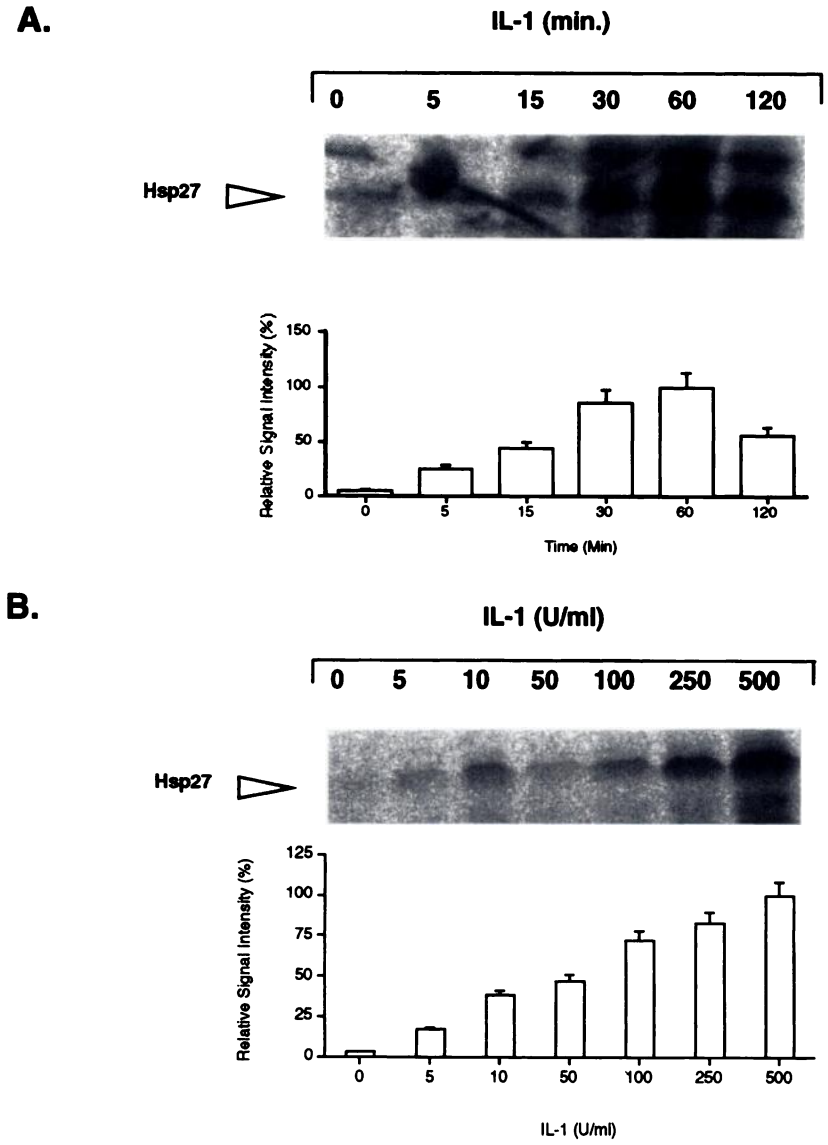

Fig. 6. Dose- and time-dependent phosphorylation of Hsp27 induced by IL-1. A, U-937 cells were deprived of serum for 24 hr before IL-1β (5–500 units/ml) was added for 30 min. Cell lysates were fractionated on a Mono S column. Fractions eluting at 200–250 mM NaCl were pooled, and equal amounts of cellular proteins were subjected to the MAPKAP kinase 2 assay, using purified recombinant Hsp27 as substrate. *Upper*, representative experiment; *lower*, phosphorylation of MBP, analyzed by quantitation of photostimulated luminescence as described for Fig. 2 and expressed as relative signal intensity. Maximum signal intensity was arbitrarily set as 100%. Values are expressed as means ± standard deviations of three independent experiments. B, U-937 cells were deprived of serum for 24 hr before IL-1β (100 units/ml) was added for up to 120 min. Cell lysates were fractionated on a Mono S column, followed by the MAPKAP kinase 2 assay as described. *Upper*, representative experiment; *lower*, phosphorylation of MBP, analyzed by quantitation of photostimulated luminescence as described for Fig. 2 and expressed as relative signal intensity. Maximum signal intensity was arbitrarily set as 100%. Values are expressed as means ± standard deviations of three independent experiments.

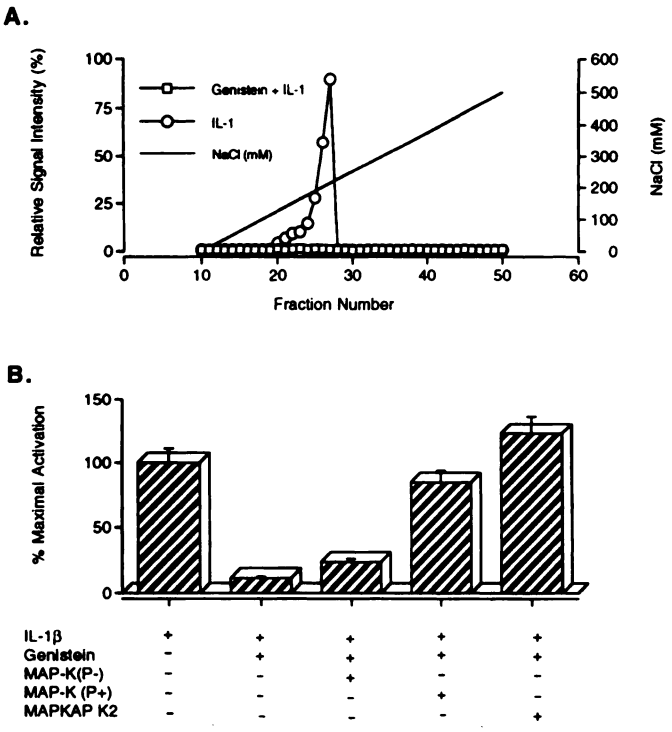

Fig. 7. Prevention by genistein of IL-1β-mediated phosphorylation of Hsp27 and restoration by addition of activated immunoprecipitated MAP kinase or purified MAPKAP kinase 2. A, U-937 cells were deprived of serum and factors for 24 hr and exposed (or not) to genistein (10 μg/ml) for 2 hr before the addition of IL-1β (100 units/ml) for 30 min. Cell lysates were fractionated on a Mono S column. Fractions eluting at 200–250 mM NaCl were pooled, and equal amounts of cellular proteins were subjected to the MAPKAP kinase 2 assay, using purified recombinant Hsp27 as substrate. Shown is a representative experiment. Three additional experiments gave comparable results. B, U-937 cells were deprived of serum for 24 hr and were then exposed to serum-free medium, with or without genistein (10 μg/ml), for 2 hr, followed by exposure to IL-1β (100 units/ml) for 30 min. Cell lysates were prepared and combined with or without activated or inactivated MAP kinase immunoprecipitated from U-937 cells that had received IL-1β after preincubation for 2 hr in the absence [*MAP-K (P+)*] or presence [*MAP-K (P–)*] of genistein, followed by the MAPKAP kinase 2 assay using Hsp27 as substrate. Alternatively, purified MAPKAP kinase 2 (*MAPKAP K2*) (24) was added to cell lysates, followed by assays of Hsp27 phosphorylation as described. Activity obtained from IL-1β-stimulated U-937 cells was arbitrarily set as 100%.

inactive MAP kinase immunoprecipitated from U-937 cells maintained in the absence of IL-1β (Fig. 4) failed to restore Hsp27 phosphorylation (Fig. 7B). In addition, neither activated nor inactivated immunoprecipitated MAP kinase was capable of phosphorylating Hsp27 by itself. Moreover, when purified MAPKAP kinase 2 (kindly provided by P. Cohen) (23) was added to cellular lysates obtained from U-937 cells that had received genistein before IL-1β treatment, Hsp27 phosphorylation could also be restored. Taken together, these findings further substantiate the notion that MAP kinase is responsible for the activation of MAPKAP kinase 2, which then results in phosphorylation of Hsp27.

Previous studies have indicated that MAPKAP kinase 2 phosphorylates Hsp27 on serine residues (26, 33). In accord with these data, we showed that the capacity of IL-1β to induce phosphorylation of Hsp27 was abolished when NaF, a serine/threonine phosphatase inhibitor, was omitted from *in vitro* kinase assays (data not shown).

Discussion

We show here that signaling events triggered by IL-1β involve the activation of MAP kinase and MAPKAP kinase 2, thereby leading to phosphorylation of the small stress protein Hsp27. Hsp27 has been previously identified by others as a target for IL-1 (13, 14). However, the intracellular pathways mediating Hsp27 phosphorylation have remained unclear (35, 36). Our data indicated that IL-1β-mediated activation of MAP kinase and MAPKAP kinase 2 preceded the phosphorylation of Hsp27. Moreover, inactivation of MAP kinase by tyrosine inhibitors interfered with the capacity of IL-1β to induce phosphorylation of Hsp27, which could be reconstituted by addition of activated immunoprecipitated MAP kinase but not by addition of inactivated immunoprecipitated MAP kinase. These findings provide additional support for the notion that activation of MAP kinase is required for IL-1β-mediated phosphorylation of Hsp27. Activation of MAP kinase and phosphorylation of Hsp27 was both dose and time dependent in the human monocytic cell line U-937. Biphasic activation kinetics of MAP kinase activity in U-937 cells upon IL-1β exposure were observed. The second peak of MAP kinase activation at 30–60 min after IL-1β exposure may result from the induction of release of secondary cytokines such as TNF-α, which also is known to induce MAP kinase (31). MAPKAP kinase 2 represents one of the substrates of MAP kinase and has recently been identified as the major enzyme mediating serine phosphorylation of Hsp27 (25). In accord with these data, we showed that addition of purified MAPKAP kinase 2 reconstituted Hsp27 phosphorylation in lysates obtained from U-937 cells that had received genistein before exposure to IL-1β and displayed no Hsp27 phosphorylation. IL-1β-induced phosphorylation of Hsp27 was abolished when NaF, an inhibitor of serine/threonine phosphatase, was omitted from the assay buffer. Taken together, these findings indicate that phosphorylation of Hsp27 by IL-1β is a result of activation of MAP kinase and MAPKAP kinase 2 in human monocytic cells.

Our data provide additional evidence for the notion that IL-1 must be added to the list of cytokines known to activate MAP kinases, i.e., IL-3, IL-6, granulocyte-macrophage colony-stimulating factor, stem cell factor, and TNF-α (22, 18–30). These findings also strengthen the hypothesis that activation of MAP kinase is not linked to a specific receptor or a specific cellular response but may be associated with both induction of proliferation and/or functional activation of the target cell. Activation of G proteins has been previously identified as an important effector mechanism in IL-1-mediated signal transmission (36). In view of the more recent data that linked the activation of G proteins such as $p21^{ras}$ to the activation of c-Raf-1 kinase and to subsequent activation of MAP kinase (37), the identification of MAP kinase as a substrate for IL-1β signaling, although not previously shown, was not surprising.

Expression and phosphorylation of small heat shock proteins are well conserved during evolution (34). The exact function of heat shock proteins is still enigmatic. Recently, Hsp27 has been characterized as a molecular chaperone (38). Chaperones regulate transport and folding of newly synthesized molecules. Most cells constitutively express heat shock proteins even in the absence of stress, favoring the notion that heat shock proteins are of importance in supporting cell viability under unstressed conditions as well. Treatment of human monocytes with IL-1 induces synthesis of secondary cytokines, spurs the oxidative burst, stimulates tumoricidal and bactericidal activites, and enhances the antigen presentation ability of monocytes (1). These processes may also require a molecular chap-

erone such as Hsp27 to ensure appropriate folding and transportation of newly synthesized proteins.

More importantly, heat shock proteins have been implicated in the protection of tumor cells from the cytotoxic effects of cytokines such as TNF-α (40). Pretreatment of tumor cells with low doses of TNF-α or IL-1 induces phosphorylation of Hsp27 and thereby protects these cells from the cytotoxic effects of a subsequent challenge with TNF-α (39, 40). Based on these findings, it is tempting to speculate that the phosphorylation of Hsp27 upon exposure to IL-1, which is released, for example, in the course of the acute-phase reaction, may constitute a mechanism whereby these cells protect themselves from the effects of secondary cytokines elicited by IL-1, such as TNF-α (1).

Note Added in Proof:

Freshney et al. (*Cell* **70**:1039–1049 (1994)) have recently identified a new MAP kinase cascade in KB cells, which, in parallel to the mechanism demonstrated in this paper, could activate MAPKAP kinase 2 and increase Hsp 27 phosphorylation.

References

1. Dinarello, C. A. Interleukin-1 and interleukin-1 antagonism. *Blood* **77**:1627–1632 (1991).
2. Oster, W., N. A. Cicco, H. Klein, T. Hirano, T. Kishimoto, A. Lindemann, R. H. Mertelsmann, and F. Herrmann. Participation of the cytokines interleukin 6, tumor necrosis factor-*alpha*, and interleukin 1-*beta* secreted by acute myelogenous leukemia blasts in autocrine and paracrine leukemia growth control. *J. Clin. Invest.* **84**:451–457 (1989).
3. Bagby, G. C., C. A. Dinarello, P. Wallace, C. Wagner, S. Hefeneider, and E. McCall. Interleukin-1 stimulates synthesis of granulocyte-macrophage colony-stimulating factor by vascular endothelial cells. *J. Clin. Invest.* **81**:92–97 (1986).
4. Didier, M., C. Aussel, C. Pelassy, and M. Fehlmann. IL-1 signalling for IL-2 production in T cells involves a rise in phosphatidylserine synthesis. *J. Immunol.* **141**:3078–3083 (1988).
5. Brach, M. A., B. Löwenberg, L. Mantovani, U. Schwulera, R. Mertelsmann, and F. Herrmann. Interleukin-6 (IL-6) is an intermediate in IL-1-induced proliferation of leukemic human megakaryoblasts. *Blood* **76**:1972–1979 (1990).
6. Herrmann, F., W. Oster, S. C. Meuer, A. Lindemann, and R. H. Mertelsmann. Interleukin 1 stimulates T-lymphocytes to produce granulocyte-monocyte colony-stimulating factor. *J. Clin. Invest.* **81**:1415–1418 (1988).
7. Mizel, S. B., J. M. Dayer, S. M. Krane, and S. E. Mergenhagen. Stimulation of rheumatoid synovial cell collagenase and prostaglandin production by partially purified lymphocyte activating factor (interleukin-1). *Proc. Natl. Acad. Sci. USA* **78**:2474–2477 (1981).
8. Mortenson, R. F., J. Shapior, B. F. Lin, S. Douches, and R. Neta. Interaction of recombinant IL-1 and recombinant tumor necrosis factor in the induction of mouse acute phase proteins. *J. Immunol.* **140**:2260–2266 (1988).
9. Mochizuki, D. Y., J. A. Eisenmann, P. J. Conclon, A. D. Larsen, and R. J. Tushinski. Interleukin-1 regulates hematopoietic activity, a role previously ascribed to hematopoietin-1. *Proc. Natl. Acad. Sci. USA* **84**:5267–5271 (1987).
10. Kroggel, R., M. Martin, V. Pingould, J. M. Dayer, and K. Resch. Two-chain structure of the interleukin 1 receptor. *FEBS Lett.* **229**:59–62 (1988).
11. Schreck, R., P. Rieber, and P. A. Bäuerle. Reactive oxygen intermediates as apparently widely used messengers in the activtion of the NF-κB transcription factor and HIV-I. *EMBO J.* **10**:2247–2252 (1991).
12. O'Neil, L. A. J., T. A. Bird, A. J. H. Gearing, and J. Saklatvala. Interleukin-1 signal transduction. *J. Biol. Chem.* **265**:3146–3152 (1990).
13. Kaur, P., W. J. Welch, and J. Saklatvala. Interleukin-1 and tumor necrosis factor increase phosphorylation of the small heat shock protein. *FEBS Lett.* **258**:269–273 (1989).
14. Saklatvala, J., P. Kaur, and F. Guesdon. Phosphorylation of the small heat-shock protein is regulated by interleukin 1, tumor necrosis factor, growth factors, bradykinin and ATP. *Biochem. J.* **277**:635–639 (1991).
15. Isshiki, H., S. Akira, O. Tanabe, T. Nakajuma, T. Shimamoto, T. Hirano, and T. Kishimoto. Constitutive and IL-1 inducible factors interact with the IL-1 responsive element in the IL-6 gene. *Mol. Cell. Biol.* **10**:2757–2763 (1990).
16. Sims, J. E., M. A. Gayle, J. L. Slack, M. R. Alderson, T. A. Bird, J. G. Giri, F. Colotta, F. Re, A. Mantovani, K. Shanebeck, K. H. Grabstein, and S. K. Dower. Interleukin 1 signaling occurs exclusively via the type I receptor. *Proc. Natl. Acad. Sci. USA* **90**:6155–6159 (1993).
17. Stylianou, E., L. A. O'Neil, L. Rawlinson, M. R. Edbrooke, P. Woo, and J. Saklatvala. Interleukin-1 induces NF-κB through its type I but not its type II receptor in lymphocytes. *J. Biol. Chem.* **267**:15836–15842 (1992).
18. Colotta, F., F. Re, M. Muzio, R. Bertini, N. Polentarutti, M. Sironi, J. G. Giri, S. K. Dower, J. E. Sims, and A. Mantovani. Interleukin-1 type II receptor: a decoy target for IL-1 that is regulated by IL-4. *Science (Washington D. C.)* **261**:472–475 (1993).
19. Guy, G. R., S. P. Chua, N. S. Wong, S. B. Ng, and Y. H. Tan. Interleukin-1 and tumor necrosis factor activate common multiple protein kinases in human fibroblasts. *J. Biol. Chem.* **266**:14343–14348 (1991).
20. Kaur, P., and J. Saklatvala. Interleukin-1 and tumor necrosis factor increase phosphorylation of fibroblast proteins. *FEBS Lett.* **241**:6–10 (1988).
21. Brach, M. A., C. Arnold, M. Kiehntopf, H. J. Gruss, and F. Herrmann. Transcriptional activation of the M-CSF gene by IL-2 is associated with secretion of bioactive M-CSF protein by monocytes and involves activation of the transcription factor NF-κB. *J. Immunol.* **150**:5535–5543 (1993).
22. Brach, M. A., H. J. Gruss, C. Sott, and F. Herrmann. The mitogenic response to tumor necrosis factor-α requires c-Jun/AP-1. *Mol. Cell. Biol.* **13**:4824–4830 (1993).
23. Stokoe, D., D. G. Campbell, S. Nakielny, H. Hidaka, S. Leevers, C. Marshall, and P. Cohen. MAPKAP kinase 2: a novel protein kinase activated by mitogen-activated protein kinase. *EMBO J.* **10**:3985–3991 (1992).
24. Bradford, M. M. A rapid and sensitive method for the quantification of protein-dye binding. *Anal. Biochem.* **72**:234–244 (1976).
25. Stokoe, D., K. Engel, D. G. Campbell, P. Cohen, and M. Gaestel. Identification of MAPKAP kinase 2 as a major enzyme responsible for the phosphorylation of the mammalian small heat shock proteins. *FEBS Lett.* **313**:307–312 (1992).
26. Gaestel, M., W. Schroder, R. Benndorf, C. Lippmann, K. Buchner, F. Hucho, V. A. Erdmann, and H. Bielka. Identification of the phosphorylation sites of the murine small heat shock protein hsp25. *J. Biol. Chem.* **266**:14721–14724 (1991).
27. Laemmli, U. K. Cleavage of structural proteins during assembly of the head of bacteriophage T4. *Nature (Lond.)* **227**:680–682 (1970).
28. Okuda, K., J. S. Sanghera, S. L. Pelech, Y. Kanakura, M. Hallek, J. D. Griffin, and B. J. Druker. Granulocyte-macrophage colony-stimulating factor, interleukin-3, and stem cell factor induce rapid phosphorylation of p42 and p44 MAP kinase. *Blood* **79**:2880–2885 (1992).
29. Raines, M. A., D. W. Golde, M. Daeipour, and A. E. Nel. Granulocyte-macrophage colony-stimulating factor activates microtubule-associated protein 2 kinase in neutrophils via a tyrosine kinase-dependent pathway. *Blood* **79**:3350–3354 (1992).
30. Deiapour, M., G. Kumar, M. C. Amaral, and A. E. Nel. Recombinant IL-6 activates p42 and p44 mitogen-activated protein kinase in the IL-6-responsive B cell line, AF-10. *J. Immunol.* **150**:4743–4738 (1993).
31. Engel, K., A. Ahlers, M. A. Brach, F. Herrmann, and M. Gaestel. MAPKAP kinase 2 is activated by heat shock and TNF-α phosphorylation of small heat shock protein results from stimulated MAP kinase cascade. *J. Cell Biochem.*, in press.
32. Pulverer, B. J., J. M. Kyriakis, J. Avruch, E. Nikolakaki, and J. R. Woodgett. Phosphorylation of c-Jun mediated by MAP kinases. *Nature (Lond.)* **353**:670–672 (1991).
33. Landry, J., H. Lambert, M. Zhou, J. N. Lavoie, E. Hickey, L. A. Weber, and C. W. Anderson. Human HSP27 is phosphorylated at serines 78 and 82 by heat shock and mitogen-activated kinases that recognize the same amino acid motif as S6 kinase II. *J. Biol. Chem.* **267**:794–803 (1992).
34. Gething, M. J., and J. Sambrook. Protein folding in the cell. *Nature (Lond.)* **355**:38–46 (1992).
35. Guesdon, F., and J. Saklatvala. Identification of a cytoplasmic protein kinase regulated by IL-1 that phosphorylates the small heat shock protein, hsp27. *J. Immunol.* **147**:3402–3407 (1991).
36. Ray, K., N. Thompson, N. Kennard, P. Rollins, S. Grenfell, S. Witham, N. Smithers, and R. Solari. Investiagtion of guanine-nucleotide-binding protein involvement and regulation of cyclic AMP metabolism in interleukin 1 signal transduction. *Biochem. J.* **282**:59–65 (1992).
37. Moodie, S. A., B. M. Wilumsen, M. J. Weber, and A. Wolfman. Complexes of Ras-GTP with Raf-1 and Mitogen-activated protein kinase kinase. *Science (Washington D. C.)* **260**:1659–1661 (1993).
38. Jacob, U., M. Gaestel, K. Engel, and J. Buchner. Small heat shock proteins are molecular chaperones. *J. Biol. Chem.* **268**:1517–1521 (1993).
39. Konig, M., D. Wallach, K. Resch, and H. Holtmann. Induction of hyporesponsiveness to an early post-binding effect of tumor necrosis factor by tumor necrosis factor itself and interleukin-1. *Eur. J. Immunol.* **21**:1741–1745 (1991).
40. Jaattela, M., D. Wissing, P. A. Bauer, and S. C. Li. Major heat shock protein hsp 70 protects tumor cells from tumor necrosis factor cytotoxicity. *EMBO J.* **11**:3507–3512 (1992).

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