Identification of a protein transiently phosphorylated by activators of endothelial cell function as the heat-shock protein HSP27

A possible role for protein kinase C

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Several agonists of endothelial cell function (thrombin, histamine, dioctanoylglycerol, phorbol 12-myristate 13-acetate, interleukin-1) have previously been shown to enhance the level of phosphorylation of an undefined 29000- M_r protein (P29). Comparison of this protein with other phosphoproteins suggested that it may be related to the mammalian heat-shock protein HSP27. Immunoprecipitation and immunoblot analysis with antibodies specific for human HSP27 demonstrated that P29 was immunochemically identical with HSP27. Further characterization of agonist-induced phosphorylation of HSP27 indicated that phosphorylation occurred exclusively on serine residues, and phosphopeptide analysis of tryptic- and chymotryptic-cleavage products demonstrated that the phosphopeptides generated were identical for each agonist and okadaic acid. Down-regulation of protein kinase C- α by prolonged treatment with phorbol esters eliminated the ability of phorbol 12-myristate 13-acetate, dioctanoylglycerol, thrombin and histamine to phosphorylate HSP27 above background levels and decreased interleukin-1-stimulated HSP27 phosphorylation by 60%. These data suggest that the various agonists employed stimulate HSP27 phosphorylation through similar mechanisms and that protein kinase C is probably involved.

INTRODUCTION

We have previously reported that the treatment of human umbilical-vein endothelial cells with various activators of endothelial cell function leads to the phosphorylation of a protein of approximate M_r 29000 [1,2]. The phosphorylation of this protein (P29) after thrombin, histamine, interleukin-1 (IL-1), and dioctanoylglycerol (DiC₈) treatment was transient, lasting no more than 1 h, whereas phorbol 12-myristate 13-acetate (PMA) treatment increased phosphorylation for prolonged periods of time (> 4 h). Thrombin, histamine, DiC₈ and PMA stimulated phosphorylation by 2.5–4-fold, and IL-1 increased the level up to 18 times that of control. The transient phosphorylation induced by IL-1, thrombin or histamine was followed by the onset of a refractory period during which time readdition of the homologous agonist did not stimulate secondary phosphorylation. However, P29 phosphorylation could be enhanced a second time by treatment with any of the heterologous agonists. If the primary agonist was removed after desensitization had occurred and the cells incubated in its absence, rephosphorylation could once again be stimulated by the homologous compound. Thus phosphorylation of P29 appeared to be controlled by receptor occupancy-dependent homologous down-regulation. Treatment of cells with okadaic acid also promoted the phosphorylation of this protein, although more slowly than the other agonists listed (approx. 1 h compared with 30 s), and inhibited the decline in the level of phosphorylation when added simultaneously with IL-1 [2]. Therefore the level of phosphorylation of this protein may depend on an equilibrium between a protein kinase(s) and a protein phosphatase(s).

Although the identity of P29 was not readily apparent during these previous studies, comparison of other phosphoproteins

with P29 indicated that a protein with similar characteristics was heat-shock protein 27 (HSP27) [3–7]. Among these similarities were the agonists which enhance the phosphorylation of the two proteins [6,8–10] and the number and relative migration on two-dimensional gels of the phosphoproteins which comprise HSP27 and P29 [3,11]. Thus it became apparent that a likely candidate for P29 was HSP27. We report here that P29 is indeed HSP27 and further characterize and compare agonist-induced phosphorylation of this stress protein. We also suggest that protein kinase C (PKC) is probably involved in the phosphorylation of this protein.

EXPERIMENTAL

Materials

All tissue culture reagents were purchased from sources previously described [12]. PMA and DiC₈ were from Calbiochem. Trypsin and chymotrypsin were from Worthington Biochemicals. Phosphoamino acid standards, Sigma cell type 100 cellulose t.l.c. plates, and the components of the t.l.c. marker dye were from Sigma Chemical Co. ³²PO₄ (specific radioactivity 8500–9120 Ci/mmol) was purchased from DuPont/NEN, and ampholines were obtained from Pharmacia LKB. Okadaic acid was purchased from Gibco/BRL, thrombin was provided by Dr. J. W. Fenton II (New York Department of Health, Albany, NY, U.S.A.) and had a specific activity of 4014 units/mg of protein. Recombinant human IL-1 was provided by Dr. Martin Lotz (Department of Molecular and Experimental Medicine, Scripps Clinic) and had a specific activity of 2 × 10⁷ units/mg of protein in thymocyte co-stimulation assays. The rabbit polyclonal antibody to human HSP27 was a gift from Dr. J. Landry, University of Laval, Quebec, Canada. The monoclonal antibody to PKC- α

Abbreviations used: DiC₈, dioctanoylglycerol; PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate; IL-1, interleukin-1; HSP27, heat-shock protein 27; Tos-Phe-CH₂Cl, *N*-tosyl-L-phenylalanylchloromethane ('TPCK'); DNP, dinitrophenyl.

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Endothelial cell culture

Endothelial cells were isolated from human umbilical cord veins as previously described [13] and were cultured into 75 cm² tissue culture flasks coated with 20 mg of calf skin gelatin/ml. Cells were grown to confluence in RPMI 1640 containing 10% fetal calf serum, 200 units of penicillin/ml, 200 µg of streptomycin/ml, 10 µg of endothelial cell growth factor/ml and 90 µg of heparin/ml. Passaged cells were subcultured into 12-well dishes and allowed to grow to confluence under the same conditions as primary cultures except that 50 µg of endothelial cell growth factor/ml was used. All experiments employed once-passaged cultures. Average cell densities at confluence were 6×10^4 cells/cm².

Phosphate labelling of endothelial cells

Confluent monolayers in 12-well tissue culture dishes were washed twice with phosphate-free RPMI 1640 and incubated in phosphate-free medium containing 100 (for one-dimensional gels) or 200 (for two-dimensional gels) µCi of ³²PO₄/ml for 1 h, and the test compounds were added directly to the medium for the specified times. For phosphoamino acid and phosphopeptide analysis, the ³²PO₄ concentration was increased to 250 µCi/ml and the cells were incubated for 2 h. Stock solutions of PMA and okadaic acid were made in dimethyl sulphoxide. In no case was the final concentration of dimethyl sulphoxide added to cells greater than 0.01%. The presence of endothelial cell growth factor, heparin or dimethyl sulphoxide had no effect on the phosphorylation or dephosphorylation of the proteins studied in the presence or absence of any of the compounds used. To determine the effect of PKC down-regulation on HSP27 phosphorylation, cultures were treated with 100 nM-PMA or vehicle for 24 h in 10% fetal calf serum, the medium was removed, and the cells were washed twice with phosphate-free RPMI 1640. Phosphate-free medium containing 50 µCi of ³²PO₄/ml was added and the cells were labelled for 1 h. The agonists were added directly to the medium at the prescribed concentration for 10 min (1 h for okadaic acid), and then the cells were extracted and prepared for analysis. Experiments to be analysed by one-dimensional electrophoresis were stopped by removing the medium and adding 500 µl of 10% trichloroacetic acid, the wells washed once with 1 ml of water, and the protein was solubilized with 200 µl of 62.5 mM-Tris/HCl (pH 6.8)/1% SDS/well. For two-dimensional electrophoresis, the cells were lysed (no trichloroacetic acid treatment) in 100 µl of 9.5 M-urea/2% Nonidet P-40/2% Ampholine, pH 9–11. All lysis buffers were supplemented with proteinase inhibitors: 1 mM-phenylmethanesulphonyl fluoride, 100 µg of leupeptin/ml, 100 units of aprotinin/ml, 10 mM-N-ethylmaleimide. The insoluble material was removed by centrifugation at 13000 g for 10 min before electrophoresis.

Electrophoresis

One-dimensional SDS/PAGE was performed according to the procedure of Laemmli [14] using a 12.5% separating gel and a 4% stacking gel unless otherwise specified. A final protein content of 20 µg/sample was reduced with 5% 2-mercaptoethanol and boiled for 3 min before application to the gel. All gels were stained with Coomassie Brilliant Blue and dried before autoradiography. In some cases, the gels were exposed to film for various lengths of time to attain optimum visualization of the various bands of interest. When the relative intensities of HSP27 were determined by laser densitometry, autoradiographs were scanned perpendicular to the plane of electrophoresis so that no

other bands were detected and the signal could return to baseline between each lane. The intensities of the various bands were normalized to a phosphoprotein the level of phosphorylation of which did not change in response to any of the agonists used (*M_r* 37000).

Two-dimensional gel electrophoresis was performed with 17.5 µg of cell extract reduced with 5% 2-mercaptoethanol using Ampholines, pH range 3–10 according to the method of O'Farrell [15]. Proteins were separated by isoelectric focusing on cylindrical gels in the first dimension (pH range 5–8) in the presence of 2% Nonidet P-40, and the gels were transferred directly to SDS/PAGE gels consisting of 12.5% polyacrylamide and 0.1% SDS. *M_r* markers and a sample of cell extract were applied to the second-dimension gel and run simultaneously with the samples from the first-dimension gel.

Phosphoamino acid and phosphopeptide analysis

For phosphoamino acid analysis, the portion of the gel containing the HSP27 bands was excised, allowed to rehydrate in 50 mM-NH₄HCO₃, pH 7.5, and the phosphoprotein eluted overnight at room temperature by diffusion in the presence of 0.1% SDS and 5% 2-mercaptoethanol. A second elution was performed in the same buffer for 45 min at 37 °C and the eluates were combined. The eluted proteins in a total volume of 1.2 ml were transferred to a screw-capped tube and precipitated for 4 h at 4 °C with 20% trichloroacetic acid using 25 µg of human α-globulin as carrier. The precipitated proteins were washed with ethanol/ether (1:1) followed by ethanol, and the samples dried with N₂. The precipitates were suspended in 100 µl of 5.7 M-HCl, boiled for 5 min, and hydrolysed at 110 °C for 1 h. The hydrolysate was dried by evaporation and the residue was dissolved in 5 µl of pH 1.9 buffer (88% formic acid/acetic acid/water, 59:156:1794, by vol.) containing 67 µg each of standard phosphoserine, phosphotyrosine and phosphothreonine. Each sample was applied to one corner of a 10 cm × 10 cm cellulose t.l.c. plate and subjected to electrophoresis at 30 mA for 20 min at 18 °C. Second-dimension electrophoresis was performed in pH 3.5 buffer (acetic acid/pyridine/water, 10:1:200, by vol.) at 35 mA for 15 min at 18 °C [16]. The phosphoamino acid standards were visualized by ninhydrin staining and the thin-layer plates subjected to autoradiography at –70 °C.

For phosphopeptide mapping, the eluted protein in 1.2 ml was first precipitated with 20% trichloroacetic acid in the presence of 20 µg of RNAase A as carrier. The precipitate was collected by centrifugation after incubation at 4 °C for 60 min, washed with ice-cold ethanol, and the pellet dried under nitrogen. The pellet was dissolved in 100 µl of performic acid and the sample allowed to oxidize at 4 °C for 60 min. The reaction was stopped by adding 400 µl of ice-cold deionized water and the sample immediately frozen on dry ice before freeze-drying. The oxidized protein pellet was resuspended in 50 µl of 50 mM-NH₄HCO₃, pH 8.0, 10 µg of tosylphenylalanylchloromethane (Tos-Phe-CH₂Cl; 'TPCK')-treated trypsin or chymotrypsin added, and the sample incubated overnight at 37 °C. Another 10 µl of the proteinase was then added and the mixture incubated once more for 3 h at 37 °C. After proteolytic digestion, the sample was diluted in 400 µl of deionized water, frozen and freeze-dried to 10 µl. A second portion of water was added and the sample freeze-dried to dryness. The peptide mixture was resuspended in 300 µl of pH 1.9 buffer (2.2% formic acid/7.8% acetic acid), freeze-dried once and redissolved in 5 µl of pH 1.9 buffer. The peptides were separated by thin-layer electrophoresis and t.l.c. Electrophoresis was performed as described above except the marker dye consisted of 5 mg of *ε*-DNP-lysine/ml plus 1 mg of xylene/ml/cyanol FF in 30% pH 4.7 buffer (butan-1-ol/

pyridine/acetic acid/water, 2:1:1:40, by vol.). Electrophoresis was for 25 min at 21 mA at 18 °C. Second-dimension chromatography was performed in butan-1-ol/pyridine/acetic acid/water 15:10:3:12, by vol. After completion of the chromatography, the plates were air-dried and the ^{32}P -containing peptides detected by autoradiography.

Immunoprecipitation and Western blotting

For immunoblot analysis of PKC, cells were scraped into 10 ml of cold PBS and centrifuged for 5 min at 800 rev./min at 4 °C. Cell pellets were resuspended in lysis buffer (0.025 M-Tris/HCl, pH 7.5, 2.5 mM-MgCl₂, 2 mM-EDTA, 2 mM-EGTA, 5 mM-dithiothreitol, 100 μM -phenylmethanesulphonyl fluoride and 100 μg of leupeptin/ml) and sonicated for two 10 s bursts using a Sonifier cell disrupter microprobe at setting number 3. The lysate was centrifuged at 100 000 *g* for 1 h at 4 °C followed by extraction of the membrane pellet in lysis buffer containing 1% Nonidet P-40. Remaining particulate matter was removed by centrifugation at 13 000 *g* for 10 min. The entire cytosol and membrane-derived fractions from each flask were subjected to electrophoresis on 10% SDS/polyacrylamide gels followed by transfer to nitrocellulose for 3 h at 250 mA at 4 °C in 25 mM-Tris/glycine, pH 8.3, and 20% (v/v) methanol. After transfer the membrane was exposed to monoclonal antibodies specific for PKC- α as described [17]. The presence of antibody to PKC- α was detected by the addition of goat anti-mouse IgG conjugated to horseradish peroxidase (Vectastain). Immunoblot analysis of HSP27 was performed with a 1:500 dilution of rabbit anti-(human HSP27) antibody under the same conditions as described for PKC. Detection of anti-HSP27 was achieved with goat anti-rabbit antibody-horseradish peroxidase conjugate (Vectastain).

Immunoprecipitation studies were performed as follows: cells labelled with $^{32}\text{PO}_4$ and treated with the various agonists were extracted into 10 mM-Tris/HCl (pH 8.0)/0.15 M-NaCl/0.1% Triton X-100/1 mM-phenylmethanesulphonyl fluoride/10 mM-EDTA/100 μg of leupeptin/ml/10 mM-N-ethylmaleimide/100 U of aprotinin/ml/100 nM-okadaic acid/1% BSA. Rabbit anti-(human HSP27) (0.1 mg/ml; 1:500 dilution) was added and the mixture incubated at 4 °C for 24 h. Immunocomplexes were precipitated with 50 μl of protein A-Sepharose (0.1 mg/ml), followed by centrifugation at 13 000 *g*. The beads were washed twice with 50 mM-Tris/HCl (pH 8.7)/0.1% Nonidet P40/1 M-NaCl, twice with 50 mM-Tris/HCl (pH 6.8)/1% Nonidet P40/0.3% SDS and boiled for 3 min. The supernatants were analysed by SDS/PAGE.

RESULTS

The identity between P29 and HSP27 was examined by immunoprecipitation and Western blot analysis using a polyclonal antibody specific for human HSP27 [5]. Immunoprecipitation studies were performed by incubating the HSP27 antibody with extracts from $^{32}\text{PO}_4$ -labelled cells treated with the various agonists which induce phosphorylation of P29 (Fig. 1). SDS/PAGE of the immunoprecipitates revealed a single specific band which migrated to a position identical with that of P29 (IL-1- and DiC₈-treated samples are shown). Non-immune rabbit IgG showed no band at the corresponding position. Western blot analysis was performed with extracts fractionated by two-dimensional gel electrophoresis (Fig. 2). Cultures containing samples from IL-1-treated gels probed with anti-HSP27 antibodies were compared with the corresponding autoradiograph of ^{32}P -labelled proteins. The spots in Fig. 2(a), previously referred to as P29, migrated to positions identical with those of four of the five spots detected by anti-HSP27 antibodies (Fig. 2b). A single unphosphorylated spot was also detected. Analysis of

extracts from histamine-treated cells showed identical results (results not shown). Immunoblot analysis was also performed on single-dimension gels containing cell extracts prepared before

Fig. 1. Immunoprecipitation of HSP27

Endothelial cells were labelled with 100 μCi of ^{32}P /ml for 1 h and treated with either 10 ng of IL-1/ml or 150 μM -DiC₈ for 10 min. Cells were extracted with 0.5% Triton X-100, the extracts treated with antibody to HSP27, and immunoprecipitates fractionated by PAGE. The left lane contains whole cell extracts prepared by SDS treatment. NI, non-immune rabbit IgG.

Fig. 2. Immunoblot analysis of HSP27 fractionated by two-dimensional gel electrophoresis

$^{32}\text{PO}_4$ -labelled endothelial cells were treated with 10 ng of IL-1/ml for 10 min and the cell extracts prepared and analysed by two-dimensional gel electrophoresis as described in the Experimental section. The proteins were transferred on to nitrocellulose, immunoblot analysis was performed with anti-(human HSP27), and then the nitrocellulose placed on to autoradiographic film. (a) Autoradiographic analysis of the gel showing the multiple species of HSP27. (b) Immunoblot analysis of the same sample. The pH range of the isoelectric focusing gel was 4.7(+) and 7.2(-) and the M_r range ($\times 10^3$) of the second dimension is shown on the left.

Fig. 3. Immunoblot analysis of HSP27 before and after histamine treatment

Cells were labelled with 100 μ Ci of 32 P $_4$ /ml for 1 h and treated with vehicle or 10 μ g of histamine/ml for 10 min. Cells were extracted and fractionated on one-dimensional gels, the proteins transferred to nitrocellulose, and immunoblot analysis performed with anti-(human HSP27) as described in the Experimental section. The nitrocellulose sheet was then placed on to autoradiographic film. (a) Immunoblot analysis. (b) Autoradiographic analysis. The time of exposure to histamine is shown at the bottom of the figure. The arrow shows the position of the band formerly referred to as P29.

Fig. 4. Phosphoamino acid analysis of HSP27

Bands were excised from SDS/polyacrylamide gels, and the protein was eluted, precipitated and hydrolysed in acid for 1 h. Amino acids were separated by cellulose t.l.c. and subjected to electrophoresis in two dimensions. The sample radioactive spot migrated to the same position as the phosphoserine standard. The phosphothreonine (PT) and phosphotyrosine (PY) standards are marked by the circles. Th, thrombin; HIS, histamine; DiC₈/PMA, samples from cultures treated with PMA or DiC₈; the results were identical.

and after treatment with histamine for 10 min (Fig. 3). Single bands of HSP27-reactive material with similar intensities were observed in both cases. These bands migrated to the same position as the radioactive band appearing in the lane representing extracts of histamine-treated cultures. Thus the set of proteins we have previously referred to as P29 are immunologically identical with HSP27 and are constitutively expressed in endothelial cells before enhanced phosphorylation.

Comparative characterization of HSP27 phosphorylated by the various agonists was performed by phosphoamino acid and phosphopeptide analysis. As previously reported for IL-1 [2],

Fig. 5. Tryptic phosphopeptide analysis of HSP27

The 32 P-labelled HSP27 band was excised from SDS/polyacrylamide gels, digested with trypsin and the peptides were fractionated in the first dimension by electrophoresis at pH 1.9 (horizontal direction) and in the second dimension by chromatography (vertical direction) as described in the Experimental section. The origin is indicated by the x. Th, thrombin; HIS, histamine; PMA/DiC₈, samples from cultures treated with PMA or DiC₈; the results were identical.

Fig. 6. Down-regulation of PKC- α by prolonged phorbol ester treatment

Cells were treated with 100 nM-PMA for 10 min or 24 h and cell extracts prepared by sonication. Membrane and cytosol fractions were separated, fractionated by SDS/PAGE, and immunoblot analysis was performed with anti-PKC- α monoclonal antibodies. The duration of PMA treatment is shown at the top of the Figure. M, membrane. C, cytosol.

serine was the only phosphoamino acid detected after thrombin, histamine, DiC₈ and PMA treatment; neither phosphotyrosine nor phosphothreonine were observed (Fig. 4). Phosphopeptide mapping of HSP27 tryptic-cleavage products showed the same pattern regardless of the agonist used to enhance HSP27 phosphorylation; three spots representing phosphorylated peptides were observed at identical positions in each case (Fig. 5). Chymotryptic digests were also prepared after enhanced phosphorylation of HSP27. The number of phosphopeptides observed after treatment with chymotrypsin increased to seven. Just as we found with trypsin, the relative migration of these peptides was the same regardless of the agonist used to enhance HSP27 phosphorylation (results not shown). Thus it appears that each of the agonists employed induced the phosphorylation of HSP27 at similar if not identical positions along the polypeptide chain.

The identification of the protein kinase(s) responsible for HSP27 phosphorylation has previously been examined using various protein kinase inhibitors [1,2]. We had found that kinase

Fig. 7. Effect of PMA pretreatment on HSP27 phosphorylation

Cells were treated with 100 nM-PMA (open bars) or vehicle (hatched bars) for 24 h, washed twice with phosphate-free RPMI 1640, and then labelled with 100 μ Ci of 32 PO₄/ml in phosphate-free medium for 1 h. Treatment with the various agonists indicated was for 10 min except for okadaic acid which was added for 1 h. Cell extracts were prepared, fractionated on SDS/polyacrylamide gels, and the HSP27 band visualized by autoradiography. The intensities of the HSP27 bands were determined by laser densitometric scanning and the values normalized to a phosphoprotein band not affected by any of the compounds used. The level of phosphorylation is presented relative to control samples not pretreated with PMA or treated with any agonist. Data provided are the means $\pm$ S.D. for at least three separate experiments. Con, control; HIS, histamine; Th, thrombin; OA, okadaic acid.

inhibitors, such as staurosporine, H7 and W7, and calcium chelators had no apparent effect on the phosphorylation of HSP27. However, the failure of the PKC inhibitors to affect HSP27 phosphorylation contradicted the obvious interpretation of the phorbol ester and DiC₈ results, that is, activation of PKC was involved in HSP27 phosphorylation. We have therefore approached the question of a role for PKC in HSP27 phosphorylation by an alternative method. During prolonged incubation of cultured cells with phorbol esters, there is a loss in the antigen and activity of several subtypes of protein kinase C (down-regulation). One of these forms, PKC- α , is a prominent form of PKC in human endothelial cells (E. G. Levin and N. S. Bartfeld, unpublished work). Fig. 6 demonstrates that PKC- α is found predominantly in the cytosol under resting conditions and responds to PMA treatment by translocating from the cytosol to the membrane within 10 min, a characteristic that represents the activation of this kinase within the cell [18]. After 24 h a complete absence of antigen from either compartment is observed. To determine whether the ability of the various agonists that induce phosphorylation of HSP27 is compromised by the down-regulation of PKC- α , endothelial cells were treated with 100 nM-PMA for 24 h, loaded with 32 PO₄ for 1 h in phosphate-free medium, and then treated with the various agonists for 10 min. The autoradiograph was scanned and the bands representing HSP27 were compared with those in samples treated with the respective agonists without PMA pretreatment (Fig. 7). Cultures not treated with any agonist after PMA treatment but extracted immediately after the labelling period still showed a two-fold enhancement of HSP27 phosphorylation, indicating that the cells retained a residual capacity to phosphorylate this protein in the presence of PMA. However, this was still only 50% of the level of HSP27 phosphorylation observed after 10 min incubation of PMA with no pretreatment. Readdition of fresh PMA had no further effect on the concentration of phosphorylated HSP27, nor was HSP27 further phosphorylated when DiC₈, another activator of PKC, thrombin or histamine were added. As we

have shown before, the concentration of IL-1-generated phosphorylated HSP27 was considerably greater than that occurring after DiC₈, PMA, histamine or thrombin treatment [2]. Although PMA pretreatment also decreased the ability of IL-1 to stimulate maximum phosphorylation of HSP27, the final level was higher than after secondary treatment with the other agonists. In contrast, okadaic acid-dependent increases in HSP27 phosphorylation were not depressed by PMA pretreatment.

DISCUSSION

We have provided evidence that a protein previously described as a 29000- M_r phosphoprotein is immunochemically identical with the low- M_r heat-shock protein of mammalian cells, HSP27. The identity of this phosphoprotein was established by immunoprecipitation and immunoblotting studies using antibodies specific for the human HSP27. Immunoblot analyses of extracts from activated endothelial cells fractionated in two dimensions showed positive staining of five spots, four of which corresponded to 32 P-labelled proteins. The appearance of an unphosphorylated positive-staining spot is characteristic of HSP27 [3]. Thus we conclude that the set of proteins we have described which become more highly phosphorylated in response to PMA, histamine, thrombin, IL-1, and DiC₈ is HSP27.

Two questions concerning HSP27 that have yet to be fully answered are: (1) what is the function of this protein during agonist-induced cell activation and (2) which protein kinases are involved in the phosphorylation of this protein? The former question has been addressed with respect to heat shock; various studies including the isolation of stable heat-resistant variants and transfection of HSP27 into CHO cells demonstrate an association between elevated concentrations of HSP27 antigen and thermotolerance [5,19]. The role of HSP27 in response to agonist treatment is probably different since an increase in protein synthesis of HSP27, aggregate formation and nuclear translocation, all of which occur during heat shock [3,7,9,10], do not occur after treatment of cells with tumour necrosis factor [9]. It has been suggested that HSP27 phosphorylation after treatment with tumour necrosis factor may be responsible for establishing resistance to the cytotoxic effect of monokines [20]. However, neither thermotolerance nor cytotoxicity are issues with thrombin- and histamine-activated endothelial cells. Because of the pleiotropic effects of each of the agonists that have been used to induce HSP27 phosphorylation, it is difficult to speculate a specific role for this protein.

The other factor that has been a focus of speculation is the protein kinase that is responsible for phosphorylating HSP27. Several articles, including our own, have suggested, mainly on the basis of the ability of phorbol esters to induce increased phosphorylation [6,11,21], that PKC is involved. Both thrombin and histamine have the capacity to activate PKC [22], and thrombin has been shown to induce translocation of this kinase [23]. Previously, however, we have failed to inhibit HSP27 phosphorylation with a variety of protein kinase inhibitors, including two that are known to inhibit PKC-mediated events intracellularly [1,2]. These contradictory results are difficult to reconcile, and our use of PKC down-regulation was another attempt to address the issue of a role for PKC in HSP27 phosphorylation. Treatment with thrombin, histamine, DiC₈ or PMA did not stimulate HSP27 phosphorylation above the level observed in control samples after down-regulation. Even IL-1, which stimulates HSP27 phosphorylation to levels four to eight times greater than the other agonists, was severely compromised by PMA pretreatment. Thus loss of PKC- α is associated with a decrease in HSP27 phosphorylation which occurs with agonist treatment. However, the increase in the level of HSP27

phosphorylation during the labelling period suggests that 16 h of PMA pretreatment does not eliminate all of the protein kinase activity responsible for HSP27 phosphorylation. This activity may represent another kinase that is activated by PKC-dependent phosphorylation and remains active for prolonged periods of time. Examples of protein kinases that are modified in such a manner are cyclic AMP-dependent protein kinase and Ca^{2+} /calmodulin-dependent protein kinase [24,25]. Alternatively, this residual kinase activity may involve PKC- ϵ , a PKC subtype which is not affected by PMA-dependent down-regulation and which is Ca^{2+} independent [26]. In contrast, the effect of okadaic acid was not affected by preincubation with PMA. If we are correct in suggesting that the concentration of phosphorylated HSP27 is determined by a phosphorylation/dephosphorylation cycle, then the okadaic acid results would suggest that the kinase responsible for constitutive HSP27 phosphorylation is not a down-regulatable PKC. From these results, we propose that several kinases are involved in HSP27 phosphorylation including PKC. Whether PKC directly phosphorylates the heat-shock protein or whether it phosphorylates and activates a secondary kinase is unknown. These results are in contrast with previous studies showing that down-regulation of PKC has no effect on tumour necrosis factor-induced HSP27 phosphorylation [9].

In summary, we have demonstrated that a protein that is transiently phosphorylated and susceptible to homologous down-regulation by various receptor-dependent agonists is HSP27. The phosphopeptide pattern on two-dimensional t.l.c. is identical for each agonist employed, and a role for PKC has been suggested by the decrease in the capacity of each agonist to phosphorylate HSP27 after down-regulation of PKC.

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