Phosphorylation of the small heat-shock protein is regulated by interleukin 1, tumour necrosis factor, growth factors, bradykinin and ATP

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Interleukin 1 (IL1) increased phosphorylation of the small heat-shock protein (hsp 27) in MRC5 fibroblasts. The increase was maintained for at least 30 min, but levels had returned to pre-stimulation values by 2 h. When hsp 27 was metabolically labelled with [³H]leucine, about 15% was phosphorylated in resting confluent cells; this rose to 90% upon stimulation by IL1. Peptide maps of the three differently charged phosphorylated forms were consistent with their arising by phosphorylation of increasing numbers of serine residues. IL1 had the same effect on hsp 27 in pig articular chondrocytes, endothelial cells from human umbilical vein and an epidermoid carcinoma cell line (KB). Certain other agents were found selectively to increase phosphorylation of hsp 27 in MRC5 cells besides IL1 [and tumour necrosis factor (TNF)]. Platelet-derived growth factor had a similar effect to that of IL1; bradykinin, acid fibroblast growth factor and ATP caused an intermediate effect; phorbol myristate acetate (PMA) and 1-oleoyl-2-acetylgllycerol had smaller effects. Dibutyryl cyclic AMP and forskolin had no effects on hsp 27 phosphorylation. When cells had been depleted of protein kinase C (PKC) by prolonged treatment with PMA, stimulation by IL1, TNF or bradykinin still increased hsp 27 phosphorylation. The stimulation by all three agents was also unaffected by the PKC inhibitor staurosporine. IL1, TNF and bradykinin each caused hsp 27 phosphorylation by a pathway independent of PKC. The results are consistent with IL1 activating a serine kinase which remains to be identified.

INTRODUCTION

Interleukin 1 (IL1) is a potent inflammatory cytokine which mediates many local and systemic features of inflammation (Dinarello, 1988). It shares a number of its biological actions with tumour necrosis factor (TNF), but acts through different receptors. Whereas there has long been general agreement over the biological effects of IL1 and their importance, the biochemical basis of its action on cells has remained controversial (Mizel, 1990; O'Neill *et al.*, 1990). Some investigators have found that IL1 stimulates cellular accumulation of cyclic AMP (Chedid *et al.*, 1989) or diacylglycerol (Rosoff *et al.*, 1988), but others have found no evidence for this (Carroll, 1986; Didier *et al.*, 1988). It has been argued that IL1 may work by activating protein kinase A (Mizel, 1990), but it has also been suggested that it could activate protein kinase C (PKC) (Rosoff *et al.*, 1988; Ostrowski *et al.*, 1988). Similar uncertainty exists regarding the intracellular signalling mechanisms involved in the action of TNF. It too may activate PKC (Meichle *et al.*, 1990).

Despite the continuing uncertainty over the importance of recognized second messengers in responses of cells to IL1 and TNF, there is clear evidence that these two cytokines cause rapid increases in protein phosphorylation in stimulated cells. It is therefore likely that they work by regulating protein kinases. The receptor for epidermal growth factor (EGF) was the first protein whose state of phosphorylation was shown to be increased upon stimulation of cells by IL1 or TNF (Bird & Saklatvala, 1989, 1990). Recently, we reported that the small heat-shock protein (hsp 27) was another polypeptide whose state of phosphorylation was increased in fibroblasts stimulated by either of these two cytokines (Kaur *et al.*, 1989). We have now characterized this response, and show that it is caused by IL1 in a variety of cell

types. Other agents, including certain growth factors, bradykinin and ATP, also cause changes in phosphorylation of hsp 27. These findings have implications for the signalling mechanism used by the cytokines.

MATERIALS AND METHODS

Materials

IL1 α and TNF α were human recombinant proteins made in *Escherichia coli* as described elsewhere (Bird & Saklatvala, 1990). Human recombinant platelet-derived growth factor (PDGF) (c-sis) was from Amersham International, Aylesbury, Bucks., U.K. Acidic fibroblast growth factor (FGF) (bovine brain) was from Biogenesis, Bournemouth, U.K. Receptor-grade EGF, bombesin, bradykinin, ATP, calcium ionophore A23187, phorbol myristate acetate (PMA), 1-oleoyl-2-acetylgllycerol (OAG), Tos-Phe-CH₂Cl-treated trypsin, proteinase and phosphatase inhibitors, Protein A-agarose and t.l.c. plates (Sigmacell type 100) were all from Sigma, Poole, Dorset, U.K. Ampholines were from Pharmacia LKB, Milton Keynes, U.K. Chemicals for electrophoresis were all of the best available grade from BDH, Poole, Dorset, U.K., or Fisons, Loughborough, Leics., U.K. Culture media were from Flow Laboratories, Rickmansworth, Herts., U.K.; foetal calf serum (FCS) was from Gibco, Uxbridge, Middx., U.K.

MRC5 (human embryonic lung) fibroblasts and KB cells (human oral epidermoid carcinoma line) were from Flow Laboratories. Rabbit articular chondrocytes were prepared by sequential digestion (hyaluronidase, 1 mg/ml, 40 min; bacterial collagenase, 1.5 mg/ml, 18 h) of cartilage dissected from knee joints. Vascular endothelial cells were prepared from human

Abbreviations used: IL1, interleukin 1; TNF, tumour necrosis factor; PKC, protein kinase C; EGF, epidermal growth factor; hsp 27, small heat-shock protein; PDGF, platelet-derived growth factor; FGF, fibroblast growth factor; PMA, phorbol 12-myristate 13-acetate; OAG, 1-oleoyl-2-acetylgllycerol; FCS, foetal calf serum; DMEM, Dulbecco's modified Eagle's medium; PBS, phosphate-buffered saline; NP40, Nonidet P40.

umbilical veins by standard techniques and were generously given by Dr. J. Pearson, M.R.C. Clinical Research Centre, Northwick Park Hospital, London, U.K.

[^{32}P]P_i (8500–9120 Ci/mmol; carrier-free) was from DuPont/NEN, Stevenage, Herts., U.K. Na ^{125}I (> 2000 Ci/mmol), L-[^{35}S]methionine (> 1300 Ci/mmol) and L-[4,5- ^3H]leucine (120–190 Ci/mmol) were from Amersham International.

The rabbit antiserum to hsp 27 was raised to a protein antigen that had been highly purified from HeLa cells; it has been characterized previously (Arrigo & Welch, 1987). This antiserum was generously given by Dr. W. J. Welch, San Francisco General Hospital, San Francisco, CA, U.S.A.

Cell culture

Cells were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% (v/v) heat-inactivated FCS, and kept at 37 °C in a humidified atmosphere of CO₂/air (1:19).

Metabolic labelling with [^{32}P]P_i

Confluent cells were washed for 20 min in phosphate-free DMEM containing 10% FCS (dialysed) and 20 mM-Hepes and adjusted to pH 7.4. Cells were next incubated for 8 h in the same fresh medium (0.5 ml/30 mm dish) containing 100 μCi of [^{32}P]P_i/ml. They were then stimulated by adding the chosen agent for the required time. Stimulation was terminated by rapidly washing the cells twice in ice-cold phosphate-buffered saline (PBS) consisting of 0.15 M-NaCl, 3 mM-NaH₂PO₄ and 18 mM-Na₂HPO₄ at pH 7.4. Unless the sample was to be immunoprecipitated, the proteins were next rapidly precipitated by adding 0.5 ml of 3% trichloroacetic acid to the cell layer. Precipitated material was scraped into 1.5 ml vials and centrifuged in a Microfuge (2 min, 10000 g). The pellets were taken up in 50 μl of 8 M-urea containing 1% (v/v) Nonidet P40 (NP40), then diluted 1:1 in isoelectric focusing sample buffer [10% (v/v) 2-mercaptoethanol, 20% (v/v) glycerol, 8 M-urea, 0.8% (v/v) Ampholines pH 5–7 and 0.2% (v/v) Ampholines pH 3–10].

Heat shock and labelling with [^3H]leucine

Newly confluent cells were washed for 20 min in leucine-free DMEM containing 10% FCS (dialysed). Next they were incubated for 30 h in the same fresh buffer containing 100 μCi of [^3H]leucine/ml. Cells were then heat-shocked at 45 °C for 20 min by immersing the flask in a water bath, and subsequently maintained for a further 8 h at 37 °C under 5% CO₂. Some were then stimulated with IL1 for 10 min, rapidly washed with ice-cold PBS and then processed for immunoprecipitation.

Immunoprecipitation

After stimulation by cytokine, the cells were rapidly washed in ice-cold PBS, scraped into 1.5 ml vials and centrifuged. Pellets were solubilized in ice-cold 10 mM-triethanolamine, pH 8.0, containing 0.5 M-NaCl, 1 mM-EGTA, 50 mM-NaF, 100 μM -sodium orthovanadate, 0.1% (w/v) SDS, 0.2% (w/v) sodium deoxycholate, 1% (v/v) Triton X-100, 10 μg of leupeptin/ml, 10 μg of pepstatin A/ml, 20 μg of aprotinin/ml and 1 mM-phenylmethanesulphonyl fluoride. Lysates were clarified by centrifuging and then mixed (10 min, 4 °C) with an equal volume of solubilizing buffer containing Protein A-agarose beads [50% (v/v) slurry]. After centrifuging, the supernatant was incubated with antiserum to hsp 27 (3 μl /500 μl of lysate) for 3 h at 4 °C. Finally, Protein A-agarose was added and mixed for 1 h at 4 °C. The immunosorbent was centrifuged, washed three times in 10 mM-triethanolamine, pH 8.0, containing 0.5 M-NaCl, 1%

(v/v) NP40 and 0.25% (w/v) sodium deoxycholate, and then washed twice in 50 mM-Tris/HCl, pH 6.8. Absorbed proteins were solubilized by adding the sample buffer for two-dimensional electrophoresis to the agarose.

Electrophoresis

Isoelectric focusing was carried out in gel rods (10 cm $\times$ 2.5 mm) containing 8 M-urea, 3.5% acrylamide (acrylamide/*NN*-methylene bisacrylamide, 19:1), 4% (v/v) Ampholines pH 5–7 and 1% (v/v) Ampholines pH 3–10. Samples were focused overnight (250 V for 15 min, 400 V for 16 h and 1000 V for 1 h). Reservoirs contained 25 M-NaOH (upper) and 20 mM-H₃PO₄ (lower).

After focusing, gels were extruded and equilibrated in SDS sample buffer for 20 min. For measurement of the pH gradient, some gels were sliced after extrusion and the slices were eluted in degassed water. For the second dimension on SDS/PAGE, the buffer system described by Laemmli (1970) was used. The separating gel was 12.5% acrylamide, and the stacking gel was 5%.

After electrophoresis gels were stained in Coomassie Brilliant Blue solution, destained, processed for fluorography if necessary and dried.

Fluorography and autoradiography

For detection of ^3H , stained gels were soaked for 1 h in two changes of dimethyl sulphoxide, then for 30 min in 22% (w/v) 2,5-diphenyloxazole in dimethyl sulphoxide, then washed in water and dried. Gels were exposed for the required time at –80 °C to RX medical X-ray film (Fuji). For detection of ^{32}P , dried gels were exposed with an image intensifying screen.

Autoradiographs were scanned in an LKB Gelscan system. Incorporation of ^{32}P into specific proteins was quantified by means of the LKB 2400 Gelscan XL software package.

Two-dimensional phosphopeptide mapping

Two-dimensional gels were autoradiographed without drying in order to localize proteins. The piece of gel containing the required protein spot was excised, crushed and eluted with 50 mM-NH₄HCO₃ containing 0.1% SDS and 5% 2-mercaptoethanol. Protein was precipitated from the eluate by trichloroacetic acid in the presence of carrier γ -globulin (40 μg /ml), washed, oxidized with performic acid and digested with Tos-Phe-CH₂Cl-treated trypsin, all as described by Beemon & Hunter (1978). Lyophilized phosphopeptides were dissolved in 1% NH₄HCO₃, pH 8.9, applied to 20 μm cellulose t.l.c. plates and electrophoresed in this buffer (1 kV for 25 min). Ascending chromatography was carried out in *n*-butanol/acetic acid/pyridine/water (15:3:12:10, by vol.) for 4 h. Plates were autoradiographed as described for two-dimensional electrophoresis gels.

EGF radiolabelling and binding

EGF was radioiodinated with ^{125}I to a specific radioactivity of about 2200 Ci/mmol by a method employing chloramine T (Carpenter & Cohen, 1976).

EGF binding to MRC5 fibroblasts grown to confluence in 12-well tissue culture clusters was measured. For experiments, cells were treated as described in the legend to Table 2 and then rinsed twice with a warm (37 °C) buffer [Hanks balanced salt solution supplemented with 25 mM-Hepes, pH 7.4, 0.1% (w/v) glucose and 0.1% (w/v) bovine serum albumin]. Triplicate wells were then stimulated, or not, by PMA in the same buffer (1 ml/well) for 15 min, washed with two changes of chilled (4 °C) buffer and then incubated in buffer (0.5 ml/well) containing 0.5 ng of ^{125}I -

EGF/ml, with or without 50 ng of unlabelled EGF/ml. After 3 h at 4 °C, the buffer was removed and cells were washed four times with fresh chilled binding buffer (2 ml/well), lysed with 1 % (v/v) Triton X-100/0.1 M-NaOH (1 ml/well) and bound radioactivity counted.

RESULTS

IL1 induces phosphorylation of most newly synthesized hsp 27

Previous experiments (Kaur *et al.*, 1989) had shown that stimulation of fibroblasts by IL1 causes an early increase in their content of three phosphorylated forms of hsp 27. In order to assess whether this increase involved modification of a significant amount of hsp 27, cells were metabolically labelled with either [³H]leucine (hsp 27 has only a single internal methionine) or [³²P]P_i. Confluent cells were incubated with [³H]leucine for 30 h to allow equilibration with the intracellular pool of leucine. They were then subjected to heat shock (45 °C, 20 min), and incubated for a further 8 h. This procedure was necessary to label hsp 27 because it is synthesized at only a very low rate in unstressed cells. For labelling with phosphate, cells were incubated with the radioisotope for 8 h. The cells labelled in these two ways were stimulated for 10 min with IL1, by which time phosphorylation was optimal. The distribution of the labels in hsp 27 is shown in Fig. 1. Figs. 1(a) and 1(b) show the ³H label and Figs. 1(c) and 1(d) show the ³²P label, in protein from resting (a, c) or stimulated (b, d) cells. In unstimulated cells, 90 % of the [³H]leucine-labelled hsp 27 was unphosphorylated, and was focused at a position corresponding to pH 6.5 (Fig. 1a, spot a). By contrast, the hsp 27 from the cytokine-stimulated cells consisted largely of the two more basic putative phosphorylated forms (Fig. 1b, spots b and

c) and relatively little of the unphosphorylated form (spot a). The putative phosphorylated forms contained 90 % of the label and corresponded exactly in position with the phosphorylated forms identified by labelling with [³²P]P_i (Figs. 1c and 1d, spots b, c and d with respective focusing positions of pH 6.0, 5.7 and 5.0). The changes upon stimulation in the pattern of the ³²P-labelled forms did not exactly parallel those seen with the ³H-labelled protein. The least acidic phosphorylated form (spot b in Figs. 1c and 1d) would be expected to have increased more markedly in intensity when one compares its change in leucine-labelled cells (spot b in Figs. 1a and 1b). Perhaps the two isotopes were not entering the same pools of hsp 27 to an equal extent. Nevertheless, it can be concluded that a significant amount (75 %) of recently synthesized hsp 27 became phosphorylated upon stimulation of the cells by IL1.

IL1-induced phosphorylation is sustained for at least 30 min

It was shown previously (Kaur & Saklatvala, 1988) that the cytokine-induced phosphorylation of hsp 27 is an early event. It became detectable after 3–5 min of stimulation, and was very marked by 10 min. Fig. 2 shows that the phosphorylation induced by IL1 was maintained for at least 30 min, but after 2 h of stimulation the amount of phosphorylated hsp 27 had apparently returned to the level present before stimulation.

Multiple forms of hsp 27 probably arose due to differences in phosphorylation

It was previously shown (Kaur *et al.*, 1989) that the three phosphorylated forms of hsp 27 contained phosphoserine, but not phosphothreonine or phosphotyrosine. The differences in charge between the four forms of hsp 27 are large: on isoelectric focusing, one pH unit separates the unphosphorylated protein (spot a in Fig. 1) from the most acidic form (spot d in Fig. 1). Experiments were carried out to see if these differences could be attributed solely to phosphorylation, or whether some other modification of the protein was occurring.

Attempts to dephosphorylate the [³H]leucine-labelled protein by treating immunoprecipitated protein with alkaline phosphatase were unsuccessful because the protein was lost, probably

Fig. 1. Two-dimensional electrophoresis of immunoprecipitates shows an effect of IL1 on [³H]leucine-labelled hsp 27 in fibroblasts

MRC5 fibroblasts were labelled either with [³H]leucine and heat-shocked (a and b) or with [³²P]P_i (c and d) as described in the Materials and methods section. For stimulation, IL1 (20 ng/ml) was added for 10 min. Cells were then rapidly washed with ice-cold PBS and processed for immunoprecipitation with antiserum to hsp 27, as described in detail in the Materials and methods section. Fluorographs (a and b) and autoradiographs (c and d) of immunoprecipitated hsp 27 that had been run on two-dimensional electrophoresis are shown. (a) and (c) show protein from unstimulated cells, and (b) and (d) show protein from IL1-stimulated cells. The position of unphosphorylated hsp 27 is marked as spot a (pH 6.5); spots b, c and d (pH 6.0, 5.7 and 5.5) mark the three phosphorylated forms; v indicates vimentin, which contaminates the immunoprecipitates. + and - show focusing orientation; the arrow indicates the direction of SDS/PAGE.

Fig. 2. Time course of IL1-induced phosphorylation of hsp 27

MRC5 fibroblasts were metabolically labelled with [³²P]P_i and then stimulated by adding 20 ng of IL1 for increasing periods of time before lysis and immunoprecipitation. Immunoprecipitates were subjected to two-dimensional electrophoresis. Details of autoradiographs show the three phosphorylated forms of hsp 27 (b–d). + and - show focusing orientation. Stimulation times are indicated (min).

as a result of degradation. As a second approach, tryptic phosphopeptide maps were made from each of the three phosphorylated forms eluted from two-dimensional gels (Fig. 3). The least acidic phosphoprotein contained five major phosphopeptides (Fig. 3c). The intermediate-phosphorylated form (Fig. 3b) showed marked intensification in the region of peptide 3; this could have been due to a new peptide adjacent to peptide 3. The most acidic form (Fig. 3a) showed pronounced intensification of peptide 5. The maps suggest the initial phosphorylation of serine residues to generate the protein of spot b (Fig. 1), with further phosphorylations causing the appearance of a new phosphopeptide in each of the two more acidic forms (spots c and d). The number of serine residues phosphorylated in spot b is unknown. Digestion with chymotrypsin, thermolysin or a mixture of thermolysin and trypsin all generated at least three major phosphopeptides (results not shown). This evidence, together with the change in pI of 0.5 unit, suggests that phosphorylation of more than one serine residue is involved in the generation of the first phosphorylated form, spot b. There are 21 serine residues in the sequence of hsp 27 (Hickey *et al.*, 1986; Carper *et al.*, 1990).

Effect of IL1 on hsp 27 in other cell types

Cells considered to represent important physiological targets for IL1 were tested. The cytokine increased phosphorylation of hsp 27 in human vascular endothelial cells (Figs. 4a and 4b) and in chondrocytes from rabbit articular cartilage (Figs. 4c and 4d). The identity of the chondrocyte proteins could not be confirmed by immunoprecipitation because an antiserum to rabbit hsp 27 was lacking. Nevertheless, the highly characteristic appearance of the three phosphoprotein spots makes it probable that they are hsp 27. IL1 also increased hsp 27 phosphorylation in the KB epidermoid cell line (Figs. 4e and 4f).

Effects of other agents upon phosphorylation of hsp 27

The ability of various agents to stimulate phosphorylation of hsp 27 was tested with a view to obtaining some indication of whether or not IL1 might be acting through a known signalling pathway. IL1 shares many biological actions with the tumour promoter PMA, a substance which works by activating PKC. PMA (or OAG) increased hsp 27 phosphorylation, but only weakly in comparison with the effect of IL1 (Fig. 5). In particular, the very large increase in spot d was not seen after stimulating MRC5 fibroblasts with PMA or OAG. Fig. 6 shows the effects of some other agents that were found to markedly increase phosphorylation of hsp 27. Besides TNF (Kaur & Saklatvala, 1988), only PDGF produced a response comparable with that of IL1 (Fig. 6). FGF, ATP and bradykinin all produced smaller

Fig. 4. Two-dimensional electrophoresis shows that IL1 induces phosphorylation of hsp 27 in different cells

(a) and (b) Human umbilical vein endothelial cells were labelled with [32 P]P_i (8 h). Details of autoradiographs show hsp 27 immunoprecipitated from cell extracts and run on two-dimensional electrophoresis: (a) unstimulated cells; (b) cells stimulated by IL1 (20 ng/ml) for 10 min. (c) and (d) Pig articular chondrocytes labelled with [32 P]P_i. Autoradiographs are of two-dimensional gels of extracts: (c) unstimulated cells; (d) cells stimulated by IL1. b–d mark phosphorylated forms of hsp 27; v is vimentin. The arrow indicates the direction of electrophoresis. Molecular mass marker positions are shown by the arrow heads: from top to bottom: 94 kDa, 68 kDa, 45 kDa and 29 kDa. The orientation of the pH gradient is shown at the bottom. (e) and (f) Same protocol as for (c) and (d), but KB cells were used.

increases (Fig. 6). Ca²⁺ ionophore A23187 caused a marked generalized increase in phosphorylation of many proteins, including hsp 27. A variety of other agents, which were tested at a

Fig. 3. Tryptic phosphopeptide maps of the different phosphorylated forms of hsp 27

MRC5 cells labelled with [32 P]P_i were stimulated by IL1 (20 ng/ml) for 10 min. Extracts were run on two-dimensional gels and the three phosphorylated forms of hsp 27 were located by autoradiographing the wet gels. Areas containing the spots (b–d in Fig. 1) were excised. Elution, digestion by trypsin, electrophoresis and chromatography were done as described in the Materials and methods section. Autoradiographs show (a) the most acidic form (spot d in Fig. 1), (b) the intermediate form (spot c in Fig. 1), and (c) the least acidic form (spot b in Fig. 1). The arrow indicates the direction of electrophoresis to the anode. The sample application point is the spot at the bottom left corner of each panel. Numbers 1–5 identify the main phosphopeptides.

Fig. 5. Effects of PMA and OAG on phosphoproteins of MRC5 fibroblasts

Autoradiographs of two-dimensional gels of extracts of cells labelled with [32 P]P, and stimulated for 10 min as described in the Materials and methods section: (a) unstimulated; (b) IL1 (20 ng/ml); (c) PMA (100 ng/ml); (d) OAG (50 μ g/ml). Spots b, c and d are the pI 6.0, 5.7 and 5.5 forms of hsp 27; v is vimentin (pI 5.5). The arrow indicates the direction of electrophoresis. Molecular mass markers are shown by arrowheads: from top to bottom: 94 kDa, 68 kDa, 45 kDa and 29 kDa.

range of concentrations and stimulation times, had no discernible effect on the phosphorylation of hsp 27 (results not shown). These were: dibutyl cyclic AMP (up to 1 μ M), dibutyl cyclic GMP (1 μ M), forskolin (to 1 μ M), arachidonic acid (10 μ M), bombesin (6 nM), EGF (50 ng/ml) and insulin (50 ng/ml).

The relative amounts of the three phosphorylated forms of hsp 27 from cells treated with various agents were measured by scanning densitometry of the autoradiographs and were expressed as a percentage of the response to IL1 (Table 1). This shows a ranking order with IL1 and PDGF having the largest effect, FGF, bradykinin and ATP having an intermediate action, and OAG and PMA eliciting the smallest response.

Depletion of PKC does not alter the response to IL1, TNF or bradykinin

Because of the biological similarity of IL1 and PMA and the fact that activation of PKC in cells by PMA or OAG can be associated with increased hsp 27 phosphorylation, it seemed possible that IL1 might be acting in part through PKC. Thus MRC5 fibroblasts were incubated with PMA for 24 h to deplete them of regulatable PKC. During the last 8 h, the cells were labelled with [32 P]P, and their responses to IL1, TNF and bradykinin were examined by two-dimensional electrophoresis and compared with the responses of cells not pretreated with PMA (Fig. 7). The phosphorylation of hsp 27 and the increases caused by all three agents were unaffected by the prior treatment of the cells with PMA (Fig. 7), suggesting that neither the cytokines nor bradykinin was causing the phosphorylation via a route involving PKC.

Fig. 6. Two-dimensional electrophoresis showing the effects of various agents on phosphoproteins in MRC5 cells

Autoradiographs showing two-dimensional electrophoresis of extracts of cells labelled with [32 P]P, as described in the Materials and methods section and stimulated for 10 min: (a) unstimulated; (b) IL1 (20 ng/ml); (c) PDGF (20 ng/ml); (d) A23187 (10 μ M); (e) bradykinin (1 μ M); (f) ATP (100 μ M); (g) acidic FGF (100 ng/ml). Spots b–d indicate the phosphorylated forms of hsp 27; v is vimentin. Molecular mass markers are as described in the legend to Fig. 5.

In order to check that PKC had been depleted by the prolonged treatment with PMA, fresh PMA was added, and its ability to decrease the binding of EGF to its receptor was measured. Table 2 shows that, when PMA was added to untreated cells, it caused a 74% decrease in EGF binding; this was due to the expected PKC-induced decrease in affinity of the receptor, or 'transmodulation' (Iwashita & Fox, 1984; Davis & Czech, 1984). When PMA was added to the pretreated cells it caused no change in the amount of EGF that the cells were able to bind, showing an absence of regulatable PKC.

We also looked at the response of cells pretreated for 30 min with an inhibitor of PKC, staurosporine (100 nM). This treatment was sufficient to inhibit PMA-induced phosphorylation of the EGF receptor (Bird & Saklatvala, 1990), but it had no effect on

Table 1. Relative potency of various agents in stimulating phosphorylation of hsp 27

^{32}P -labelled MRC5 cells were stimulated with various agents for 10 min. Extracts were run on two-dimensional electrophoresis. The amounts of each phosphorylated form of hsp 27 (spots b, c and d in Figs. 5 and 6) were estimated by densitometric scanning. The fold increase in each form (relative to its intensity in unstimulated cells) is shown. The data are compiled from three experiments.

Stimulant	Increase (fold)		
	Spot d	Spot c	Spot b
IL1	60	14	1.5
PDGF	50	7	1.0
FGF	15	5	1.5
Bradykinin	15	5	1.5
ATP	10	5	1.4
OAG	3	3	1.5
PMA	2	2	1.3

Table 2. Effect of PMA on the specific binding of ^{125}I -EGF to MRC-5 cells

Untreated cells and cells incubated for 24 h with PMA (100 ng/ml) were either stimulated for 15 min by freshly added PMA, or not stimulated. The binding of ^{125}I -EGF was done as described in the Materials and methods section. Values in parentheses indicate the decrease in EGF binding elicited by PMA.

	Specific ^{125}I -EGF binding (c.p.m./ 10^6 cells)
Untreated cells	
Control	3600 $\pm$ 107
PMA-stimulated	941 $\pm$ 141 (74 %)
PMA-pretreated cells	
Control	3749 $\pm$ 270
PMA-stimulated	3465 $\pm$ 149 (7.5 %)

the hsp 27 phosphorylation induced by IL1, TNF or bradykinin (results not shown).

DISCUSSION

Characteristics of hsp 27 phosphorylation

The experiments carried out on [^3H]leucine-labelled cells showed that a large proportion of hsp 27 was phosphorylated in response to stimulation by IL1. Metabolic labelling of the protein with an amino acid was hampered by its lack of methionine, and by the fact that the cells required to be heat-shocked to induce detectable synthesis of the protein. Therefore the experiment only gave information regarding the phosphorylation state of the hsp 27 synthesized during 8 h following heat-shock, and not of the total amount of hsp 27 in the cell. If this recently synthesized protein was representative of the whole, then IL1 increased the amount phosphorylated from 15% to 90%.

Phosphorylation reached a maximum after about 10 min of stimulation, and this was maintained for at least 30 min. In some experiments the increase lasted for at least 60 min; in others it was declining by this time. In general, the amount of phosphorylated hsp 27 appeared to have returned to the pre-stimulation level after 2 h. The kinetics of the response were similar to those of the phosphorylation of the EGF receptor in fibroblasts stimulated by IL1 or TNF (Bird & Saklatvala, 1989, 1990). Whether the decrease in phosphorylation was due to dephosphorylation, degradation or both is not known. In the case of the EGF receptor, there was dephosphorylation rather than degradation (Bird & Saklatvala, 1990).

The three phosphorylated forms of hsp 27 appeared to arise from increasing phosphorylation, although other covalent modifications could not be entirely excluded. To see such large differences in isoelectric point with no appearance of intermediates is unusual. The protein contains 13 threonine residues in addition to its 21 serines; however, we have detected only phosphoserine on phosphoamino acid analysis, even in protein from cytokine-stimulated cells (Kaur *et al.*, 1989). Three of the serine residues (at positions 15, 78 and 82) are potential phosphorylation sites for PKC (Arg-Xaa-Xaa-Ser). The motifs Ser-Arg and Ser-Xaa-Asp (Glu) both occur five times, but they do not conform to any consensus sequence for known kinases. The diverse agents that increase phosphorylation all cause the same forms to appear, suggesting that there may be one or two rather specific hsp 27 kinases upon which more than one signalling pathway may converge. Such serine kinase activity might be generated in several ways; for instance, there could be

Fig. 7. Effects of depletion of PKC on the cellular response to IL1, TNF and bradykinin

MRC5 fibroblasts were left untreated (a-d) or were pretreated with PMA (100 ng/ml) for 24 h (e-h) as described in the Materials and methods section. During the last 8 h of the pretreatment, all cells were labelled with [^{32}P]P_i as described, then treated for 10 min with vehicle (a, e), IL1 (20 ng/ml) (b, f), TNF (100 ng/ml) (c, g) or bradykinin (1 μM) (d, h). Autoradiographs show two-dimensional gels of cell extracts (c, g, d and h show only details). Spots b-d are hsp 27; v is vimentin. Molecular mass markers and orientation of the pH gradient are given in Fig. 5.

(a) activation of the enzyme, (b) inactivation of an inhibitor, or (c) inactivation of a specific phosphatase.

IL1 causes hsp 27 phosphorylation in a variety of cell types

The effect was not cell-specific. Fibroblasts, chondrocytes, vascular endothelial cells and an epidermoid cell line all displayed the response. Previously we reported it occurring in hepatoma (Hep G2) and leukaemic (U937) cell lines (the latter in response to TNF; Kaur *et al.*, 1989). There have also been independent reports of TNF stimulating phosphorylation of hsp 27 in Hela cells (Arrigo, 1990) and of a small stress protein in endothelial cells (Hepburn *et al.*, 1988; Robaye *et al.*, 1989). We have not so far observed it in any lymphocytic lines. However, the only IL1-responsive T cell line which we have studied (EL4) is murine, and we have been unable to identify any protein corresponding to hsp 27 undergoing phosphorylation in these cells before stimulation. We have also been unable to detect any phosphorylated hsp 27 in murine fibroblasts (3T3). The antiserum to the human protein does not react with hsp 27 of other species, so cannot be used to aid detection in mouse cells. The response was prominent in other species, however. It was seen in pig and rabbit chondrocytes, and has also been observed in rat fibroblasts in response to serum or PMA (Welch, 1985).

Induction of hsp 27 phosphorylation by other agents

PMA and OAG were weakly active in stimulating hsp 27 phosphorylation in MRC5 fibroblasts. Other types of fibroblast may show a stronger response to these agents (Welch, 1985). More marked effects were seen in MRC5 cells stimulated by bradykinin, ATP or FGF, but the only agent that caused a response of similar magnitude to IL1 was PDGF. Bradykinin and ATP have previously been reported to stimulate phosphorylation of a 26 kDa protein in human endothelial cells (Demolle *et al.*, 1988). The phosphorylation followed a time course similar to the one reported here (Demolle *et al.*, 1988), and it is highly likely that this protein was hsp 27.

We were unable to induce any change in the phosphorylation of hsp 27 with agents expected to increase the activity of protein kinase A. Evidence has been presented that IL1 may activate this enzyme by elevating cyclic AMP in cells (Mizel, 1990).

The weak response of MRC5 cells in terms of hsp 27 phosphorylation to activators of PKC, and the failure of PKC depletion or staurosporine to attenuate the response, all suggested that IL1 did not cause this phosphorylation via PKC. Interestingly, the effect of bradykinin was also unaffected by either PKC depletion or staurosporine. The kinin is known to cause hydrolysis of phosphoinositide, and would be expected to increase diacylglycerol production and activate PKC (Derian & Moskowitz, 1986; Lambert *et al.*, 1986). However, it may also cause an increase in intracellular Ca^{2+} , (Lambert *et al.*, 1986) and its effect on hsp 27 might be via activation of a Ca^{2+} /calmodulin-dependent kinase. Interestingly, hsp 27 phosphorylation was enhanced by a Ca^{2+} ionophore (although this was part of a general increase in the phosphorylation of many proteins) and by ATP, which also elevates intracellular Ca^{2+} (Hallam & Pearson, 1986; Piroton *et al.*, 1987). Neither IL1 nor TNF has been found to cause changes in intracellular Ca^{2+} .

PDGF and FGF receptors are tyrosine kinases (Hanks *et al.*, 1988; Ruta *et al.*, 1989), but not enough is known about their signalling pathways for the significance of their ability to cause hsp 27 phosphorylation to be assessed.

The only biological effect that all of these agents appear to have in common is that they stimulate prostaglandin synthesis in cells (Hong & Deykin, 1982; Forsberg *et al.*, 1987; Habenicht *et al.*, 1985; Dinarello, 1988; Liu *et al.*, 1989).

Significance of hsp 27 phosphorylation in intracellular signalling

The function of hsp 27 is largely unknown. Its synthesis is induced by stress, but it is present in significant amounts in unstressed cells. Results of experiments in which a cDNA to hsp 27 was transfected into CHO cells suggested that it may confer upon cells a resistance to thermal injury (Landry *et al.*, 1989). It has been studied less than other stress proteins, perhaps because deletion of a gene coding for a homologue in yeast had no discernible effect on the cells (Petko & Lindquist, 1986). Its striking phosphorylation in cells responding to a number of agents suggests that it may play a role in intracellular signalling. As a cytoplasmic protein it is appropriately placed for such a role. It may enter the nucleus upon heat shock (Arrigo *et al.*, 1988). It forms aggregates (Arrigo & Welch 1987; Arrigo *et al.*, 1988) whose nature might be affected by phosphorylation. It is not yet known whether it interacts specifically with other proteins.

Whatever the function of hsp 27 and the significance of its phosphorylation, it should be possible to use it as a substrate with which to identify protein kinases that are activated in response to several important agents involved in inflammation and cell growth.

We are grateful to the Arthritis and Rheumatism Council and the Medical Research Council of the U.K. for financial support.

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Received 18 December 1990/22 March 1991; accepted 2 April 1991