Volume 258, number 2, 269–273 FEB 07879 December 1989

Interleukin 1 and tumour necrosis factor increase phosphorylation of the small heat shock protein

Effects in fibroblasts, Hep G2 and U937 cells

Paramjit Kaur, William J. Welch* and Jeremy Saklatvala

Cytokine Biochemistry Group, Strangeways Research Laboratory, Worts' Causeway, Cambridge CB1 4RN, England and **Lung Biology Center, San Francisco General Hospital, San Francisco, CA 94110, USA*

Received 6 October 1989

Interleukin 1α and tumour necrosis factor-α stimulated phosphorylation of three 27 kDa phosphoproteins in MRC-5 fibroblasts which was sustained for up to 2 h after adding the cytokines All three phosphoproteins were immunoprecipitated by a specific antiserum to the small mammalian heat shock protein, hsp 27 The three phosphoproteins from stimulated or control cells contained phosphoserine but not phosphothreonine or phosphotyrosine Similar increases in phosphorylation of immunoprecipitable 27 kDa proteins were seen in U937 cells stimulated by TNFα and Hep G2 cells stimulated by IL1α

Heat shock protein 27, Interleukin 1, Tumour necrosis factor, Protein phosphorylation

1. INTRODUCTION

Interleukin 1 (IL1) and tumour necrosis factor (TNF) are major pro-inflammatory cytokines with similar biological activities. They cause fever, endothelial cell activation, leucocyte accumulation, tissue resorption and the acute phase response [1,2]. To investigate their signalling mechanism, we set out to identify early changes in protein phosphorylation in stimulated cells. We found that they induced phosphorylation of a triad of cytosolic 27 kDa proteins in dermal fibroblasts [3]. By use of a specific antiserum we show here that these are phosphorylated forms of the small mammalian heat shock protein, hsp 27.

Hsp 27 (or 28) is an ubiquitous protein whose synthesis is induced by heat shock. It is homologous with lens α-crystallin and may protect cells from thermal killing [4–7]. On the basis of immunological cross reactivity, a minor portion of hsp 27 was shown to exist in phosphorylated forms markedly more acidic than the unmodified protein [5]. Since human hsp 27 expressed in transfected rodent cells is converted to the three expected phosphorylated derivatives [7], the native hsp 27 forms are probably post-translational modifications of the same protein.

Correspondence address J Saklatvala, Strangeways Research Laboratory, Worts' Causeway, Cambridge CB1 4RN, England

2. MATERIALS AND METHODS

2 1 *Cell cultures*

Cells were cultured in Dulbecco's modified medium (DMEM) containing 10% foetal calf serum (FCS) and maintained at 37°C in a humidified atmosphere of air enriched with 5% CO_2 MRC5 fibroblasts were from Flow Laboratories, U937 cells from the American Tissue Culture Collection, and Hep G2 cells from Dr J Gauldie, McMaster University, Canada.

2 2. *Reagents*

Human recombinant IL1α (~2 × 10^7 LAF units/mg) and TNFα (10^7 U/mg) were purified from extracts of *Escherichia coli* expressing the proteins [^{32}P]Orthophosphoric acid (8500–9120 Ci/mmol) was from Dupont/NEN. L-[4,5-^{3}H]leucine (120–190 Ci/mol) was from Amersham plc, England The rabbit antiserum to hsp 27 was raised against protein purified to homogeneity from HeLa cells and has been characterized elsewhere [5]. Protein A-agarose was from Sigma Ampholines were from Pharmacia/LKB

2 3 *Radiolabelling and stimulation of cells*

The radiolabelling buffer was phosphate-free DMEM containing 20 mM Hepes, 10% FCS (dialysed against 0 9% NaCl) and adjusted to pH 7 4 Cells were labelled (8 h) in buffer containing [^{32}P]orthophosphate (100 μCi/ml unless stated) then stimulated and prepared for electrophoresis as described previously [3]

2 4. *Immunoprecipitation*

Labelled, stimulated cells were rapidly washed in phosphate-buffered saline (PBS), scraped, centrifuged and then solubilised in 10 mM triethanolamine pH 8 containing 0 5 M NaCl, 1 mM EGTA, 50 mM NaF, 100 μM Na orthovanadate, 0 1% SDS, 0.2% Na deoxycholate, 1% Triton X-100, 10 μg/ml leupeptin, 10 μg/ml pepstatin A, 20 μg/ml aprotinin, 1 mM phenylmethylsulphonyl fluoride. The lysate was clarified by centrifugation, then mixed (10 min, 4°C) with

Published by Elsevier Science Publishers B.V (Biomedical Division)
00145793/89/$3.50

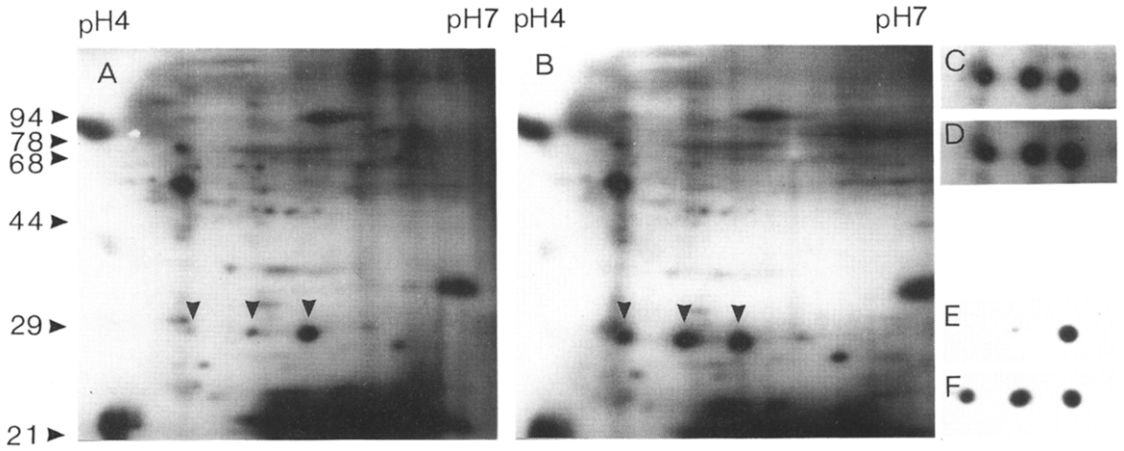

Fig.1. Autoradiographs of 2D electrophoresis of phosphoproteins of MRC5 fibroblasts metabolically labelled with [^{32}P]orthophosphate. (A) Control, (B) 20 ng/ml IL1α for 10 min, (C) and (D), 20 ng/ml IL1α added for 30 and 120 min, respectively to cells in labelling buffer, (E) control for (F) 100 ng/ml TNFα for 10 min. (A,B) Whole gels, 27 kDa proteins (arrowed). (C–F) Details showing 27 kDa proteins.

an equal volume of solubilising buffer containing protein A-agarose beads (50% slurry v/v). After centrifuging, the supernatant was incubated with antiserum (3 μl/500 μl lysate) for 3 h at 4°C. Protein A-agarose was added and mixed for 1 h at 4°C. The immunosorbent was centrifuged, then washed firstly 3 × in 10 mM triethanolamine pH 8 containing 0.5 M NaCl, 1% Nonidet P40, 0.2% Na deoxycholate and then secondly 2 × in 50 mM Tris-HCl pH 6.8. Finally the absorbed proteins were solubilised in sample buffer for electrophoresis.

2.5. *Electrophoresis*

Two-dimensional (2D) electrophoresis was carried out according to O'Farrell [8] under conditions described previously [3]. One-dimensional SDS-PAGE was carried out according to Laemmli [9]. Stained gels were dried and autoradiographed for 24–48 h with RX film (Fuji) with an image intensifying screen. Marker proteins were muscle phosphorylase (94 kDa), transferrin (78 kDa), albumin (68 kDa), IgG (50 kDa), ovalbumin (44 kDa), carbonic anhydrase (29 kDa) and soybean trypsin inhibitor (21 kDa).

2.6. *Phosphoamino acid analysis*

The selected areas of 2D gels were excised and eluted. Proteins were precipitated by TCA with albumin as carrier and then partially hydrolysed and analysed by 2D thin layer electrophoresis [10].

3. RESULTS

Fig.1 shows the effect of IL1 on the phosphorylation of proteins in MRC5 fibroblasts that had been metabolically labelled to equilibrium with [^{32}P]orthophosphoric acid and then stimulated with the cytokine for 10 min. As in human foreskin fibroblasts [3], there was a striking increase in the phosphorylation of a triad of 27 kDa proteins. When cells were extracted after 30 (C) and 120 (D) min continuous stimulation, the increased amounts of phosphorylated 27 kDa proteins were still seen. TNF (100 ng/ml) produced the same effect (E and F).

Because of similarities in size and charge, we wondered whether the cytokine-induced phosphoproteins were the phosphorylated forms of the small mammalian heat shock protein, hsp 27 [4,5]. Fig.2 shows the effect of heat shock on the incorporation of [^{3}H]leucine into proteins newly synthesised by MRC5 fibroblasts. There are several changes, including strong induction

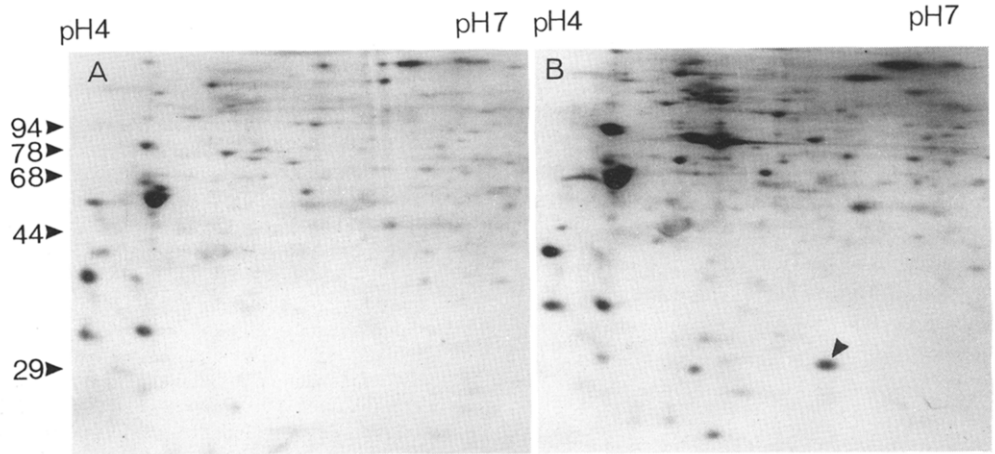

Fig.2. Effect of heat shock on MRC5 fibroblasts. Non-stressed cells (A) or cells subjected to heat shock for 90 min at 42.5°C (B) were labelled with [^{3}H]leucine (25 μCi/ml) for 3 h in leucine-free DMEM, then harvested and analysed by 2D electrophoresis. Arrowhead indicates hsp 27.

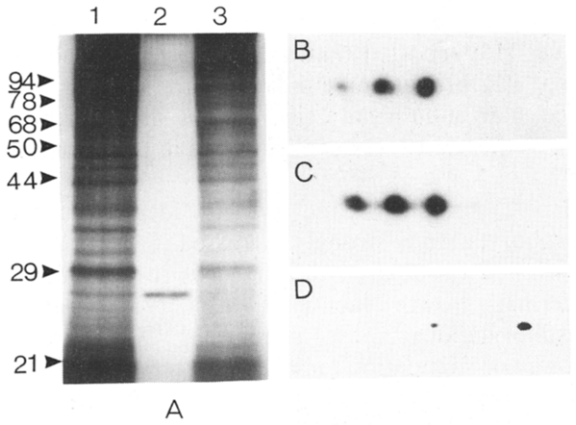

Fig.3. Immunoprecipitation of hsp 27. (A) Autoradiographs of 1D SDS-PAGE of phosphoproteins of MRC5 fibroblasts metabolically labelled with [^{32}P]orthophosphate. Cell extract (lane 1); protein precipitated by antiserum to hsp 27 (lane 2); supernatant of extract after immunoprecipitation (lane 3). (B–D) Autoradiographs of 2D electrophoretic analysis of fibroblast proteins precipitated by antiserum to hsp 27. (B) Cells labelled with [^{32}P]orthophosphate. (C) Cells labelled as for (B), then stimulated by IL1 (20 ng/ml, 10 min). (D) Cells labelled with [^{3}H]leucine (100 μCi/ml in leucine-free DMEM, 10% FCS) for a total of 30 h and heat shocked (20 min, 44°C) 8 h before harvesting.

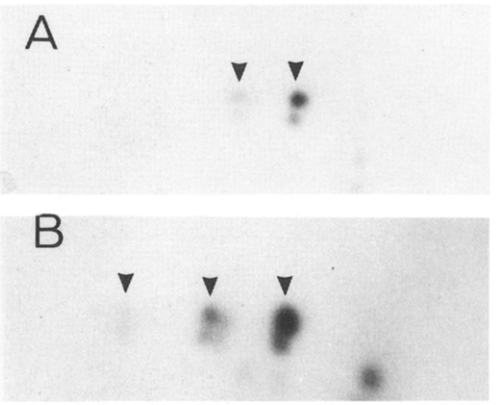

Fig.5. Immunoprecipitation of phosphoproteins from U937 cells (10^6/ml) labelled as described but [^{32}P]orthophosphate was 250 μCi/ml. (A) Control, (B) 100 ng/ml TNFα for 10 min, hsp 27 forms shown by arrowheads.

of synthesis of a protein of 27 kDa and p*I* ~6.5: the small heat shock protein (arrowhead). We next used a specific antibody to hsp 27 to immunoprecipitate cell extracts of MRC5 fibroblasts metabolically labelled with [^{32}P]orthophosphate and stimulated by IL1. Fig.3A shows that the serum precipitated a single 27 kDa phosphoprotein (lane 2) from the extract (lane 1): removal of this component was complete (lane 3). The band was not precipitated by a non-immune serum. Immunoprecipitates of extracts of IL1-stimulated and unstimulated cells labelled with [^{32}P]orthophosphate were subjected to 2D electrophoresis and autoradiography: three phosphoprotein species were seen and their state of phosphorylation was markedly increased in the stimulated extract (fig.3B,C). A separate batch of cells was heat-shocked and metabolically labelled with [^{3}H]leucine, then lysed and immunoprecipitated with the antiserum. Autoradiography after 2D electrophoresis of the precipitate showed a major [^{3}H]leucine-labelled protein of 27 kDa of higher p*I* than the phosphoproteins (fig.3D): this is the unphosphorylated form of hsp 27 and corresponds to the [^{3}H]leucine-labelled heat shock protein identified in whole cell extracts (fig.2). There is also a [^{3}H]leucine-labelled component corresponding to the most basic phosphoprotein seen in the parallel gels (fig.3, lanes 1,2). These results showing that most of hsp 27 is un-

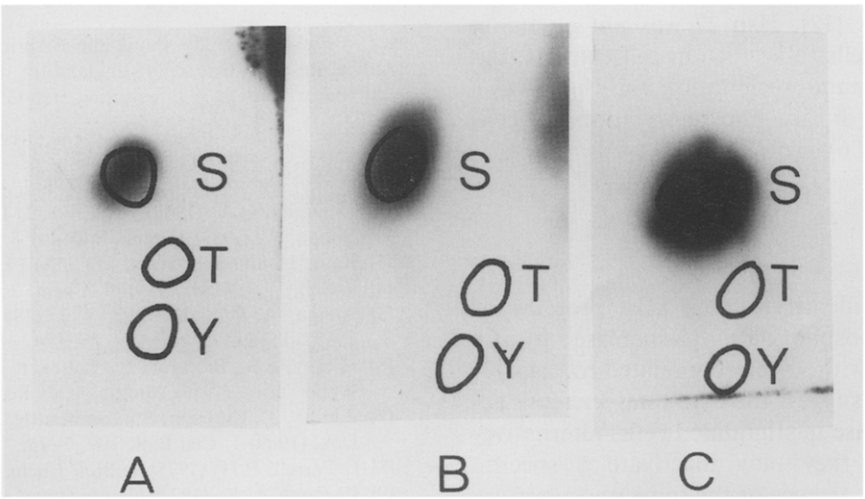

Fig.4. Phosphoamino acid analysis of the three phosphorylated forms of hsp 27 from IL1-stimulated MRC5 fibroblasts metabolically labelled with [^{32}P]orthophosphate. Circles indicate the position of the standard phosphoamino acids. (A) Most acidic, (B) middle, (C) most basic phosphoprotein.

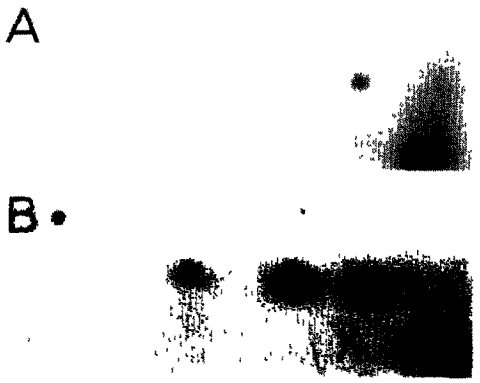

Fig.6 Immunoprecipitation of phosphoproteins from Hep G2 cells. (A) Control, (B) 20 ng/ml IL1α for 10 min. Cells radiolabelled as described for fibroblasts

phosphorylated are in close agreement with those reported by Arrigo and Welch [5].

The phosphorylated forms of hsp 27 contain phosphoserine [5]. We carried out phosphoamino acid analysis of the three phosphoproteins excised from a 2D gel. Fig.4 shows that the proteins from IL1-stimulated cells contained phosphoserine, but not phosphothreonine or phosphotyrosine. The proteins from unstimulated cells also contained only phosphoserine (data not shown).

Both IL1 and TNF act on a variety of cell types. Recently TNF was reported to increase phosphorylation of a 27 kDa protein in the histiocytoid line U937 [11]. The antiserum precipitated the characteristic 27 kDa phosphoproteins from extracts of labelled U937 cells, and their phosphorylation was strongly increased in cells incubated with TNF (fig.5, there is some degradation of the proteins; the most acidic one is faintly seen in TNF-treated cells). IL1 stimulates hepatocytes or hepatoma cells such as Hep G2 to make acute phase proteins [12]. Hsp 27 was not abundant enough in Hep G2 cells to be seen in cell extracts, but was visible after immunoprecipitation and there was a very large increase in its phosphorylation in cells treated with IL1 (fig.6).

4. DISCUSSION

The experiments identify the 27 kDa proteins of fibroblasts whose phosphorylation is increased by IL1 or TNF as identical with, or closely related to, hsp 27. The results strongly suggest the cytokines increase activity of a serine kinase in stimulated cells; alternatively, but less likely, they may inactivate a specific phosphatase. The occurrence of the same phosphorylation in histiocytic (U937) and hepatoma (Hep G2) cell lines suggests that the cytokines may act on different cell types via a similar signalling mechanism.

While this work was in progress, Robaye and colleagues [13] reported that TNF induced phosphorylation of a 27 kDa arsenate-inducible stress shock protein in vascular endothelial cells. This is likely to be the same as the hsp 27 we have identified in fibroblasts and U937.

Hsp 27 is the second protein identified whose phosphorylation is strongly increased in IL1- or TNF-stimulasted fibroblasts; the first was the receptor for epidermal growth factor (EGFR) [14]. The two phosphorylations show similarities. Both require 3–5 min of stimulation to become detectable [3,14] and involve serine. The phosphorylation of EGFR was thought to be due to a serine kinase distinct from protein kinase C [14]: it is possible that phosphorylation of hsp 27 is due to the same enzyme. IL1 may also activate a serine kinase in glucocorticoid-treated mononuclear leucocytes which phosphorylates a 65 kDa cytosolic protein [15].

The signal transduction mechanisms of these cytokines are still unclear. IL1 has been reported to increase diacylglycerol [16] and cAMP [17] which might activate protein kinases A or C, respectively. While our results, and those of others [3,11–16], now provide strong evidence that both IL1 and TNF cause early changes in protein phosphorylation, they do not implicate any particular signalling system. The phosphorylation of hsp 27 is induced by a range of agents including serum, Ca ionophore, phorbol myristate acetate [4], bradykinin and ATP [17], and it is likely to be a substrate for a number of protein kinases.

The significance of hsp 27 phosphorylation and its role in the cellular response to the cytokines is unknown. Perhaps the cytokines evoke some features of the stress response or perhaps the protein is involved in cellular signalling.

Acknowledgements. We thank the Arthritis and Rheumatism and Medical Research Council of England for financial support, and Dr Carl Richards for help in culturing Hep G2 cells

REFERENCES

[1] Dinarello, C A. (1988) FASEB J 2, 108–115.
[2] Billiau, A. (1987) Immunol. Today 8, 84–87
[3] Kaur, P. and Saklatvala, J. (1988) FEBS Lett. 241, 6–10.
[4] Welch, W J. (1985) J Biol Chem. 260, 3058–3062
[5] Arrigo, A -P and Welch, W J (1987) J Biol Chem. 262, 15359–15369
[6] Hickey, E.S., Brandon, E., Potter, R , Stein, G., Stein, J. and Weber, L.A (1986) Nucleic Acids Res 14, 4127–4145
[7] Landry, J , Chrétien, P , Lambert, H , Hickey, E. and Weber, L.A (1989) J. Cell Biol 109, 7–15
[8] O'Farrell, P.H (1975) J. Biol. Chem 260, 3058–3062
[9] Laemmli, U K (1970) Nature (Lond) 227, 680–685
[10] Cooper, J A , Safton, B.M and Hunter, T. (1983) Methods Enzymol. 99, 387–402.
[11] Schutze, S , Scheurich, P , Pfizenmaier, K and Kronke, M (1989) J Biol. Chem 264, 3562–3567

[12] Gouldie, J , Richards, C., Harnish, D., Lansdorp, P. and Baumann, H (1987) Proc. Natl. Acad. Sci. USA 84, 7251–7255.
[13] Robaye, B , Hepburn, A., Lecocq, R., Fiers, W., Boeynaems, J M. and Dumont, J E (1989) Biochem. Biophys Res. Commun. 163, 301–308
[14] Bird, T.A. and Saklatvala, J (1989) J. Immunol 142, 126–133
[15] Matsushima, K , Shiroo, M., Kung, H.-F and Copeland, T D. (1988) Biochemistry 27, 3765–3770.
[16] Rosoff, P M., Savage, N and Dinarello, C A. (1988) Cell 54, 73–81
[17] Chedid, M., Shirakawa, F., Naylor, P and Mizel, S.B. (1989) J. Immunol. 142, 4301–4306
[18] Demolle, D., Lecomte, M. and Boeynaems, J M. (1988) J. Biol Chem 263, 18459–18465