

Phosphorylation of the 90 kDa heat shock protein in heat shocked HeLa cell lysates

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The 90 kDa heat shock protein (hsp 90) is a major phosphorylated protein under normal growth conditions. However, it does not incorporate detectable levels of phosphate by incubation of control HeLa cell lysates with [γ -³²P]ATP in vitro. In this paper we show that strong phosphorylation of hsp 90 occurs in lysates prepared from heat shocked HeLa cells. Possible involvement of the eukaryotic initiation factor 2 kinase of the heme-controlled repressor of translation is discussed.

Heat shock; Heat shock protein; Protein phosphorylation; Eukaryotic initiation factor; Protein kinase; (HeLa cell)

1. INTRODUCTION

Heat shock provokes numerous alterations in the metabolism of cells. (i) The synthesis of the heat shock proteins is induced through the transcription of the heat shock genes and the selective translation of corresponding mRNAs [1,2]. The 90 kDa heat shock protein (hsp 90) is a major heat shock protein [3,4] which is strongly phosphorylated in vivo [3]. It has been shown to interact and form stable complexes with various proteins such as actin [5] and untransformed steroid receptors [6]. Several protein kinases also associate with hsp 90, such as retroviral tyrosine kinases [7,8], type II casein kinase [9] and the eukaryotic initiation factor 2 (eIF-2 α) kinase of the heme-controlled repressor of translation (HCR kinase) [10]. (ii) Protein synthesis is inhibited in part through alteration of the phosphorylation state of proteins belonging to the translational apparatus: the initiation factor 2 (eIF-2 α) becomes phosphorylated [11] whereas the S6 ribosomal subunit

[12,13] and initiation factors 4B and 4F (eIF-4B and eIF-4F) [14,15] are dephosphorylated after heat shock.

In vitro phosphorylation of an 80–90 kDa protein had been previously observed in heated rabbit reticulocyte lysates [11] and lysates from heat shocked HeLa cells [16]. In this paper we show that the 90 kDa heat shock protein is a major phosphorylatable protein in heat shocked HeLa cell lysates in contrast to control cell lysates.

2. MATERIALS AND METHODS

2.1. Cell culture and heat shock conditions

HeLa cells (MRL2 strain) were grown at 37°C on tissue culture dishes with Dulbecco's modified Eagle medium supplemented with 10% fetal calf serum. For heat shock, 35 mm culture dishes were immersed for 20 or 45 min in a water bath at 46.5°C. The cells (~10⁶ cells) were immediately lysed or allowed to recover for 3 to 24 h at 37°C.

2.2. In vitro phosphorylated protein analysis

Cells were washed three times in PBS and lysed in 200 μ l of 20 mM Tris-HCl, pH 7.5, 6 mM magnesium acetate, 50 mM NaCl, 10% glycerol, 1% Triton X-100 and 0.5% β -mercaptoethanol (lysis buffer). The cell extracts were centrifuged at 10000 $\times$ g for 10 min and the supernatants (clear cell lysate) were used for in vitro phosphorylation experiments: 20 μ l of

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lysate were incubated for 1 h with 2 μ l of [γ - 32 P]ATP (1 mCi/ml; 0.5–3 Ci/mmol) at 30°C. The reaction was stopped by addition of 20 μ l of Tris-HCl 0.125 M, pH 6.8, 5% β -mercaptoethanol, 2% SDS, 10% glycerol (loading buffer). 20 μ l of the resulting sample solutions were electrophoresed on a 10% polyacrylamide gel. For two-dimensional gel analysis, the sample solutions were saturated with urea and loaded onto an isoelectrofocusing gel containing 0.8% pH 4–6 ampholines (LKB) and 1.2% pH 3.5–10 ampholines (LKB). After migration this gel was loaded onto a 10% polyacrylamide gel [4].

2.3. Immunoadsorption

60 μ l of clear cell lysates were phosphorylated and then incubated for 1 h at 4°C after adding 2 μ l of a 30% serum albumin (fraction V; Sigma) solution and then for 20 min with 40 μ l of protein A Sepharose suspension (v/v in lysis buffer). After a 2 min centrifugation at 10000 $\times$ g, supernatant (80 μ l) was incubated overnight at 4°C with 1 μ l of anti-hsp 90 A1 serum (kindly provided by Dr M. Catelli). Anti-hsp 90 A1 is a rabbit antiserum raised against a synthetic peptide corresponding to a defined region (amino acids 260–280 derived from the chicken hsp 90 cDNA sequence (Binart, N. et al., in preparation)). Immunoreactive materials were precipitated by 20 min incubation with 40 μ l of protein A Sepharose suspension. The pellet was washed three times and 50 μ l of loading buffer were added. 20 μ l of the resulting sample were electrophoresed on a 10% polyacrylamide gel.

3. RESULTS AND DISCUSSION

In 10000 $\times$ g supernatants from lysed HeLa cells (strain MRL2), several soluble proteins incorporate phosphate from [γ - 32 P]ATP (fig.1, lane C). In lysates prepared after heating the cells for 45 min at 46.5°C, some phosphorylated polypeptides disappear while new major bands are detectable, corresponding to 38 and 90 kDa proteins (lanes 2).

The phosphorylation of the 90 kDa protein occurs in lysates prepared immediately or 24 h after a 45 min heat shock at 46.5°C (which results in complete extinction of *in vivo* protein synthesis). Furthermore, the induction of the 90 kDa protein phosphorylation also occurs when the cells are preincubated for 1 h in the presence of 20 μ g/ml of cycloheximide and heat shocked in the same medium (not shown). Thus the phosphorylation of the 90 kDa protein appears to be independent of the synthetic ability of the cells. When milder heat shock conditions (20 min at 46.5°C) are used, the phosphorylation of the 90 kDa protein becomes strongly apparent only when cells are lysed 24 h after the shock (fig.1, lanes 1).

It is well established that heat shock promotes the phosphorylation of eIF-2 α by the HCR kinase

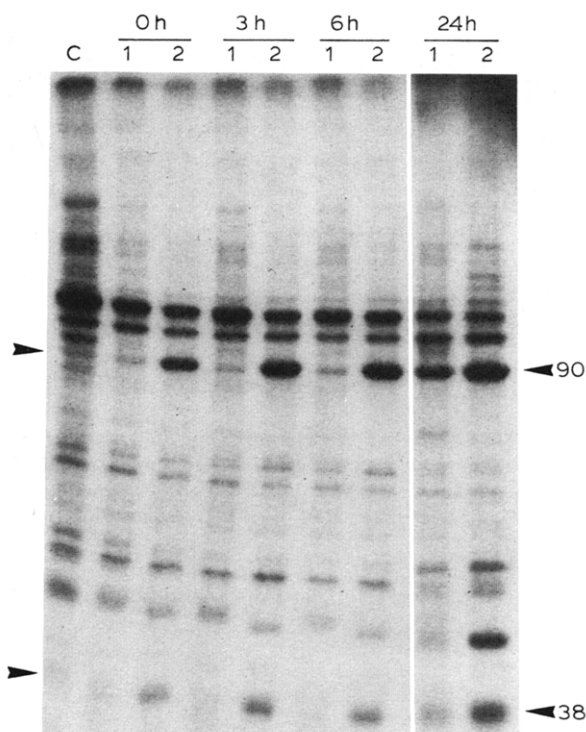


Fig.1. Activation of a 90 kDa polypeptide phosphorylation in heat shocked cell extracts. After incubation with [γ - 32 P]ATP, the extracts are analyzed by electrophoresis on a 10% polyacrylamide gel. The figure shows an autoradiogram of the gel. Control cell lysates (C) or lysates from cells heat shocked for 20 min (lanes 1) or 45 min (lanes 2) at 46.5°C. After heat shock the cells were allowed to recover for 0, 3, 6, or 24 h at 37°C and lysed. Molecular masses are indicated in kDa.

[11]. In addition, the *in vitro* phosphorylation in heat shocked cell extracts of an uncharacterized 90 kDa protein by the same kinase have been previously described [16]. Since the 95 kDa catalytic subunit of the eIF-2 α kinase is known to interact with hsp 90 [10,17], we questioned whether the 90 kDa phosphorylated protein could be identical to hsp 90.

In order to test this hypothesis, lysates from heat shocked and control cells were analyzed on a two-dimensional gel after incubation with [γ - 32 P]ATP. The *in vitro* phosphorylated 90 kDa polypeptide comigrates with the silver stained 90 kDa heat shock protein (fig.2). The identification of this 90 kDa protein was verified by immunoadsorption of phosphorylated lysates, using a polyclonal anti-hsp 90 antibody linked to protein A Sepharose

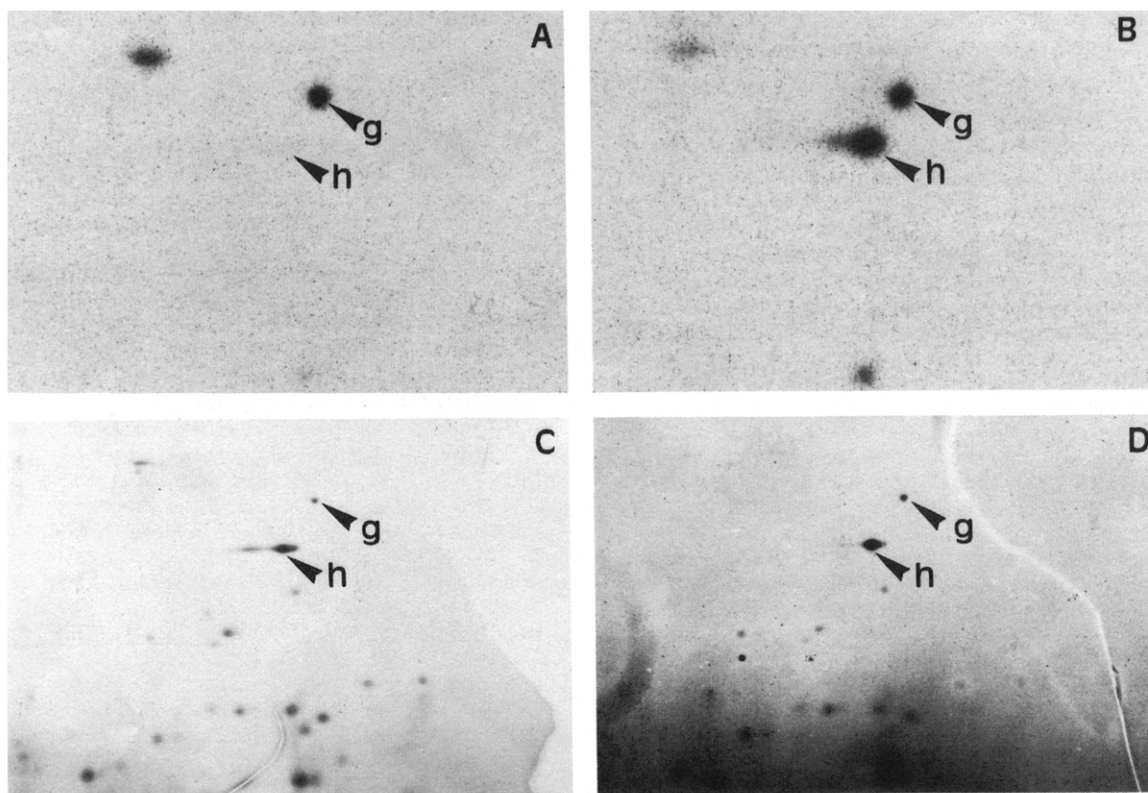
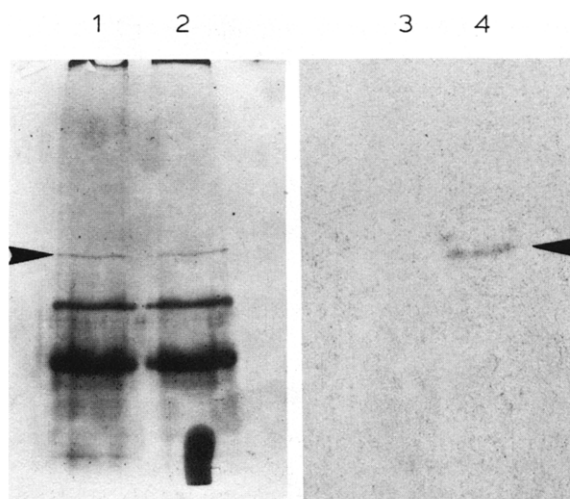


Fig.2. Two-dimensional gel analysis of the in vitro phosphorylated proteins. Control (A and C) and heat shocked (45 min at 46.5°C and 24 h recovery at 37°C) (B and D) cell lysates were phosphorylated with [γ - 32 P]ATP. The gels were silver stained (C and D) and autoradiographed (A and B). Arrows point to 90 kDa heat shock protein (h) and to the 94 kDa glucose regulated protein (g).

(fig.3). A 90 kDa silver stained protein is immunoadsorbed in roughly equal amounts from control and heat shocked cell lysates; however, this protein is phosphorylated only when lysate was prepared from heat shocked cells. Similar results were obtained using the monoclonal anti-hsp 90 antibody AC 88 kindly provided by Dr Toft (not shown) [18].

Fig.3. Immunoabsorption of the 90 kDa phosphorylated protein by an anti-hsp 90 antiserum. The extracts from control (lanes 1 and 3) or heat shocked (lanes 2 and 4) cells were electrophoresed on a 10% polyacrylamide gel. The gel was silver stained (lanes 1 and 2) and autoradiographed (lanes 3 and 4). Arrows show the 90 kDa heat shock protein. The two major silver stained bands correspond to BSA and class G immunoglobulin heavy chains.



Thus we propose as a working hypothesis that hsp 90 is a potential substrate for the activated eIF-2 α kinase. Purification of the heat-induced hsp 90 kinase may be followed using the in vitro phosphorylation assay and may allow to test its eventual identification as the eIF-2 α kinase.

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REFERENCES

- [1] Lindquist, S. (1986) *Annu. Rev. Biochem.* 55, 1151–1191.
- [2] Burdon, R.H. (1986) *Biochem. J.* 240, 313–324.
- [3] Kelley, P.M. and Schlesinger, M.J. (1982) *Mol. Cell. Biol.* 2, 267–274.
- [4] Bensaude, O. and Morange, M. (1983) *EMBO J.* 2, 173–177.
- [5] Koyasu, S., Nishida, E., Kadowaki, T., Matsuzaki, F., Iida, K., Harada, F., Kasuga, M., Sakai, H. and Yahara, I. (1986) *Proc. Natl. Acad. Sci. USA* 83, 8054–8058.
- [6] Catelli, M.G., Binart, N., Jung-Testas, I., Renoir, J.M., Baulieu, E.E., Feramisco, J.R. and Welch, W.J. (1985) *EMBO J.* 4, 3131–3135.
- [7] Courtneidge, S.A. and Bishop, J.M. (1982) *Proc. Natl. Acad. Sci. USA* 79, 7117–7121.
- [8] Brugge, J., Yonemoto, W. and Darrow, D. (1983) *Mol. Cell. Biol.* 3, 9–19.
- [9] Dougherty, J.J., Rabideau, D.A., Iannotti, A.M., Sullivan, W.P. and Toft, D.O. (1987) *Biochim. Biophys. Acta* 927, 74–80.
- [10] Rose, D.W., Wettenhall, R.E.H., Kudlicki, W., Kramer, G. and Hardesty, B. (1987) *Biochemistry* 26, 6583–6587.
- [11] Ernst, V., Zukofsky Baum, E. and Reddy, P. (1982) in: *Heat Shock from Bacteria to Man* (Schlessinger, M.J., Ashburner, M. and Tissière, A. eds) pp.215–225, Cold Spring Harbor Laboratory, New York.
- [12] Kennedy, I.M., Burdon, R.H. and Leader, D.P. (1984) *FEBS Lett.* 169, 267–273.
- [13] Glover, C.V.C. (1982) *Proc. Natl. Acad. Sci. USA* 79, 1781–1785.
- [14] Duncan, R. and Hershey, W.B. (1984) *J. Biol. Chem.* 259, 11882–11889.
- [15] Duncan, R., Milburn, S.C. and Hershey, J.W.B. (1987) *J. Biol. Chem.* 262, 380–388.
- [16] De Benedetti, A. and Baglioni, C. (1985) *J. Biol. Chem.* 261, 338–342.
- [17] Kudlicki, W., Fullilove, S., Read, R., Kramer, G. and Hardesty, B. (1987) *J. Biol. Chem.* 262, 9695–9701.
- [18] Riehl, R.M., Sullivan, W.P., Bauer, V.J., Pearson, G.R. and Toft, D.O. (1985) *Biochemistry* 24, 6586–6591.