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Heat shock increases turnover of 90 kDa heat shock protein phosphate groups in HeLa cells

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The 90 kDa heat shock protein (hsp90) is a major phosphoprotein which associates various other cellular polypeptides such as actin, calmodulin, steroid hormone receptors and certain protein-kinases Little is known about the function of hsp90 in recovery from stress In this report, we describe a dramatic increase in the rate of both phosphate uptake and dephosphorylation of hsp90 in HeLa cells submitted to acute stresses This increased turnover of hsp90 phosphate groups might reflect a greater protein binding activity of hsp90 in stressed cells

Heat shock, Heat shock protein, Protein phosphorylation, HeLa cell

1. INTRODUCTION

Heat shock as well as chemical stresses are known to have toxic effects on cellular functions These effects are mostly due to enzymatic inactivation, a consequence of protein denaturation caused by cellular stresses [1–3] When cells are allowed to recover by replacing them in normal culture conditions, cellular functions are progressively rescued Recovery is probably due, at least partially, to the accumulation, in response to stress, of a specific set of proteins, the heat shock proteins (hsps) [4,5]

Most heat shock proteins are already present in the absence of stress and ensure physiological functions. For instance, hsp60 and proteins of the hsp70 family are known to associate with polypeptides and to catalyze folding and unfolding reactions in an ATP-dependent manner [6,7] Hsp90 associates with various proteins such as, for instance, actin [8], calmodulin [9], src-type protein kinases [10] and steroid hormone receptors [11,12] These complexes correspond to an activation step of these proteins, which involves conformation and/or location changes.

Recent evidences suggest that in stressed cells, heat shock proteins belonging to the hsp70 family are directly involved in renaturation processes, by forming complexes with denatured polypeptides in an ATP-dependent manner [13,14]. However, little is known about the possible involvement of hsp90 in such reactions In this report, the dynamics of hsp90 phosphate exchange was found to be altered during and after an acute heat shock in HeLa cells.

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2 MATERIALS AND METHODS

2 1 *Cell culture and hat shock conditions*

HeLa cells (MRL2 strain) were grown at 37°C and 12% CO_2 in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum Heat shocks were performed by immersing the culture flasks in water bath

2 2 *Cell labelling and protein analysis*

^{32}P-labelling was performed by incubating the cells with 0 25 mCi/ml [^{32}P]orthophosphate in culture medium After labelling, cells were washed twice in phosphate buffer saline and lysed in LSB (2 3% sodium dodecyl sulfate, 10% glycerol, 1% mercaptoethanol, 125 mM Tris-HCl, pH 6 8) Chase experiments were performed by removing the ^{32}P-containing medium and keeping the cells in standard DMEM with serum for variable times before lysis Samples were analysed by 10% polyacrylamide gel electrophoresis [15] or 2D gel electrophoresis [16] as previously described Phosphorylated proteins were detected by autoradiography or fluorography with intensifying screens Protein synthesis was analysed by labelling the cells with 100 μCi/ml [^{35}S]methionine (1200 Ci/mmol) in standard DMEM

3 RESULTS AND DISCUSSION

In vivo phosphate uptake into proteins was analyzed in HeLa cells by metabolic ^{32}P-labelling and 2D gel electrophoresis (Fig. 1) The ^{32}P-labelled hsp90 position was determined by comigration with silver-stained hsp90 spot (Fig. 1D) After 16 h of continuous labelling at 37°C, hsp90 appeared as a major phosphorylated

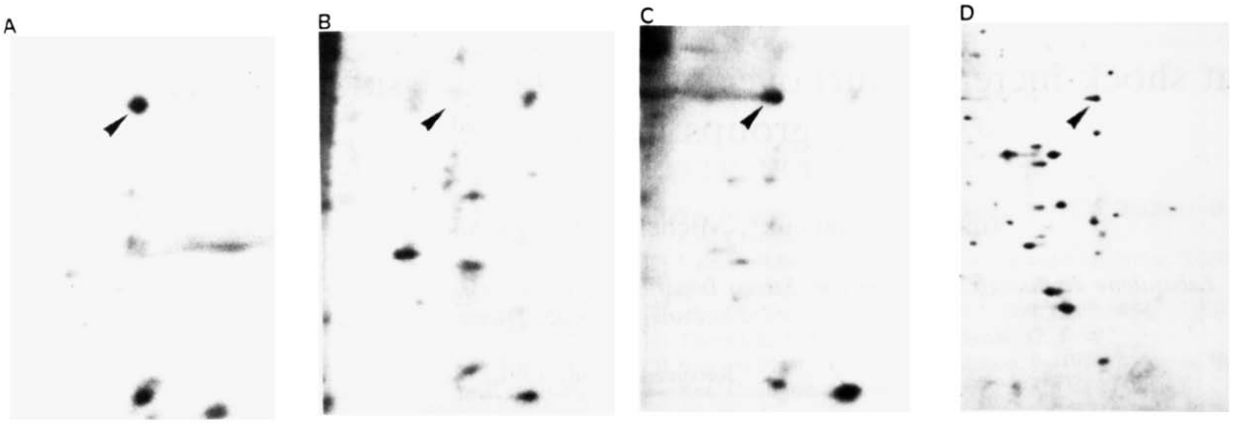

Fig 1 Stimulation of hsp90 phosphorylation during heat shock HeLa cells were ^{32}P-labelled for 16 h at 37°C (A), 1 h at 37°C (B) or 15 min at 37°C followed by 45 min at 46 5°C (C) Newly phosphorylated proteins were analyzed by two-dimensional gel electrophoresis The position of hsp90, identified by silver-staining, is indicated (D)

protein at steady-state (Fig 1A) as previously reported [17] Nevertheless, a pulse-labelling of 1 h at 37°C did not allow hsp90 to incorporate detectable levels of phosphate as compared with other phosphoproteins observable on the same gel (Fig 1B) This suggests that the rate of hsp90 phosphate uptake is rather slow, as already reported [18] However, when cells were submitted to a 45 min heat shock at 46.5°C during the pulse-labelling, hsp90 phosphate uptake was greatly stimulated, this protein becoming one of the most strongly labelled in these conditions (Fig 1C) This increase of hsp90 ^{32}P-labelling was not due to the synthesis of this protein in response to stress since it occurs well before recovery of protein synthesis and is not affected by the presence of cycloheximide in the culture medium (data not shown).

Such an increased phosphate uptake results in a hyperphosphorylation of hsp90, which was questioned by continuously labelling the cells with [^{35}S]methionine or ^{32}P and analysing labelled proteins by monodimensional electrophoresis (Fig. 2) The pattern of [^{35}S]methionine-labelled proteins was almost the same in control and heat-shocked cells and allowed one to determine the hsp90 position on the gel (Fig. 2A) The phosphorylation state of hsp90 was not significantly affected neither immediately nor several hours after heat shock, although ^{32}P-labelling of some other phosphoproteins (especially of low molecular weights) was either increased or diminished in heat-shocked cells (Fig 2B) This indicates that the stimulation of hsp90 phosphate uptake by that shock does not involve an hyperphosphorylation of this protein.

Dephosphorylation of hsp90 was studied by chase experiments after ^{32}P-labelling during 16 h at 37°C Chase was performed either at 37°C or during and after a 45 min heat shock at 46 5°C ^{32}P-labelling was observed by monodimensional electrophoresis which facilitates quantification However, we checked by 2D gel electrophoresis that, as already reported [17], hsp90 was the major 90 kDa phosphoprotein observable at the steady-state (see, for instance, Fig 1A) hsp90 remained strongly labelled after a chase of 3 25 h at 37°C In contrast to this, hsp90 labelling markedly diminished, as compared with that of other phosphoproteins, when

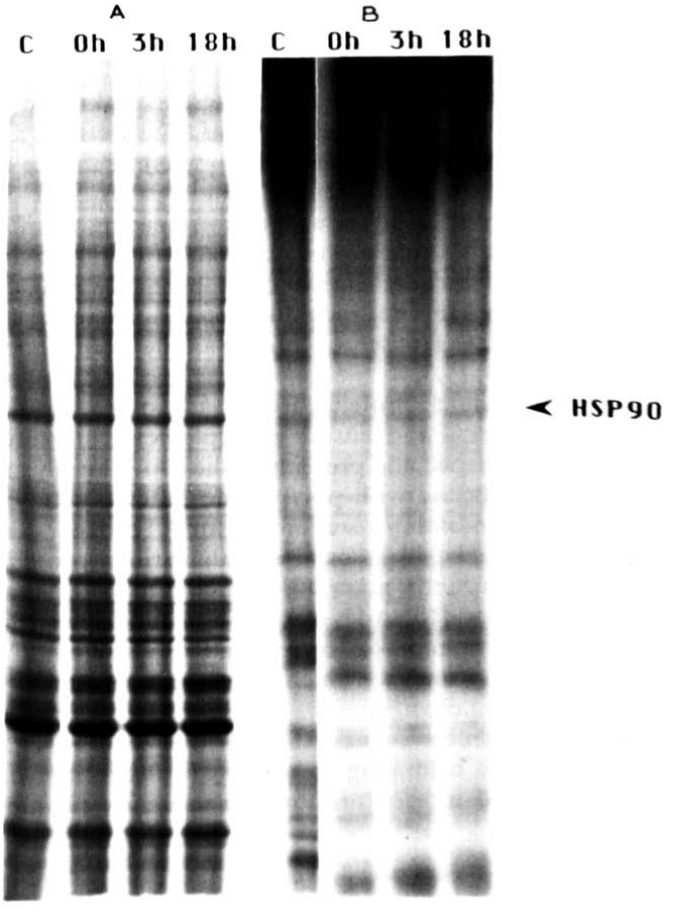

Fig 2 hsp90 phosphorylation state during and after heat shock HeLa cells were labelled for 16 h at 37°C with [^{35}S]methionine (A) or [^{32}P]orthophosphate (B) Cells were lysed at the end of the labelling (lanes C) or after a 45 min heat shock at 46 5°C followed by a recovery of 0 h, 3 h, or 18 h at 37°C (corresponding lanes). ^{32}P- and ^{35}S-labelling were continued during heat shock and recovery. Labelled proteins were analyzed by electrophoresis through a 10% polyacrylamide gel

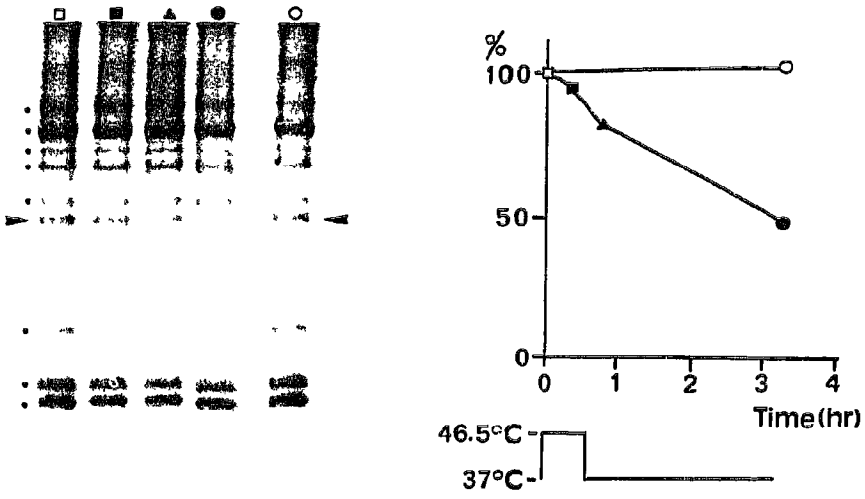

Fig 3 hsp90 dephosphorylation during and after heat shock (A) HeLa cells were ^{32}P-labelled during 16 h at 37°C and lysed at different times after a chase in non-radioactive medium Chase times were 0 h (open square), 3 25 h at 37°C (open circle), 20 min at 46 5°C (closed square), 45 min at 46 5°C (closed triangle) or 45 min at 46 5°C followed by 2 5 h at 37°C (closed circle) Labelled proteins were electrophoresed through a 10% polyacrylamide gel (B) Residual radioactive phosphate in hsp90 (indicated by an arrow) was measured by densitometry and results (expressed as percents of initial value) were normalized to those obtained for other major phosphoproteins (indicated next the autoradiogram) Residual [^{32}P]phosphate levels in hsp90 are plotted as a function of chase time Time course of heat shock and recovery are represented at the bottom of the figure

chase was performed during and after heat shock (Fig 3) Hence heat shock stimulates hsp90 dephosphorylation

It appears from these observations that acute heat shock induces an increased turnover of hsp90 phosphate groups A relationship between heat shock protein phosphorylation and association with cellular components has been observed in physiological conditions. GRP78 (a protein related to HSP70) is phosphorylated only when in a free form (not complexed with immunoglobulin heavy chains) [19] Concerning hsp90, it has been demonstrated that in mammary cell lines, estradiol or TPA increase selectively the phosphorylation of free hsp90 (not associated with estradiol receptor) [20] In this case, hsp90 phosphorylation might be coupled with the dissociation of hsp90–receptor complex which is an early step in steroid-induced receptor activation [21,22] Taking account of these data and of the fact that steroid receptor–hsp90 complexes are dissociated by heat [23], we propose that increased turnover of hsp90 phosphate groups in heat-shocked cells might reflect formation and/or dissociation of complexes between hsp90 and other cellular proteins, due for instance to the accumulation of denatured proteins in these cells Such a model does not necessarily involve the activation of a distinct protein-kinase. Indeed, hsp90 seems to possess a self-phosphorylating activity [24]

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