Heat shock causes diverse changes in the phosphorylation of the ribosomal proteins of mammalian cells

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When HeLa cells or BHK cells were subjected to heat shock at 42°C (for 2 h) or 45°C (for 10 min) there was extensive dephosphorylation of ribosomal protein S6. Concomitantly ribosomal protein L14, which is not significantly phosphorylated in normal cells, became phosphorylated, as did a non-structural protein of $M_r = 27000$, associated with the ribosomes. The latter effects were not prevented by cycloheximide or actinomycin D. When cells shocked at 45°C for 10 min were returned to 37°C for 2 h there was rephosphorylation of ribosomal protein S6 and dephosphorylation of the 27 kDa protein, but not of ribosomal protein L14.

Ribosomal protein S6 *Ribosomal protein L14* *Protein phosphorylation* *Heat shock* *HeLa cell* *Ribosome-associated phosphoprotein*

1. INTRODUCTION

Both vertebrates and invertebrates respond to a variety of stresses, including hyperthermia, by synthesising mRNA for a number of so-called 'heat shock proteins' [1]. The synthesis of these proteins in HeLa cells subjected to hyperthermia either continuously at 42°C or briefly (10 min) at 45°C is maximal after some 2 h [2] and, although the precise function of these proteins is unknown, it is reasonable to assume that they play a role in reversing some of the damaging metabolic consequences of the stress.

Heat shock is accompanied by a rapid inhibition of protein biosynthesis, which is slowly reversed in cells maintained at 42°C, or when cells subjected to treatment of 45°C for 10 min are returned to 37°C [3]. One metabolic change that has been suggested as a possible regulator of protein biosynthesis is the phosphorylation of ribosomal protein S6, which is normally the only phosphorylated protein on the 40 S ribosomal subunit. The fact that increases in the extent of phosphorylation of this protein are often (but not always) accompanied by increases in protein biosynthesis, suggests that in certain circumstances this association is causal (see [4] for discussion). Following a report [5] that heat shock in *Drosophila* induced rapid dephosphorylation of a ribosomal protein that was most probably S6, we decided to examine the situation in mammalian cells. We report here that, as well as the rapid dephosphorylation of ribosomal protein S6, there is a rapid phosphorylation of two other proteins, one a component of, and the other associated with, the ribosome.

2. METHODS

2.1. Cell culture

HeLa cells or BHK21/C13 cells were grown in culture as monolayers in the Glasgow modification of Eagle's minimal essential medium supplemented with 10% (v/v) calf serum and 10% (v/v) tryptose phosphate broth in flat tissue-culture bottles of 120 cm^2 surface area. Cells were seeded at a density of 5×10^6 cells per bottle in 50 ml medium and grown for 3 days, at which time they had reached confluence and had a density of approx. 2×10^7 cells per bottle.

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2.2. Labelling of cells with [^{32}P]orthophosphate

Cells were labelled with 0.5 mCi [^{32}P]orthophosphate per bottle in 25 ml pre-warmed medium in which the orthophosphate concentration had been reduced to 0.09 mM and from which tryptose phosphate broth had been excluded. To achieve temperatures of 42 or 45°C, as indicated in the figure legends, the bottles were immersed in water baths at the appropriate temperatures. For each experimental condition 2 or 3 bottles of cells were used.

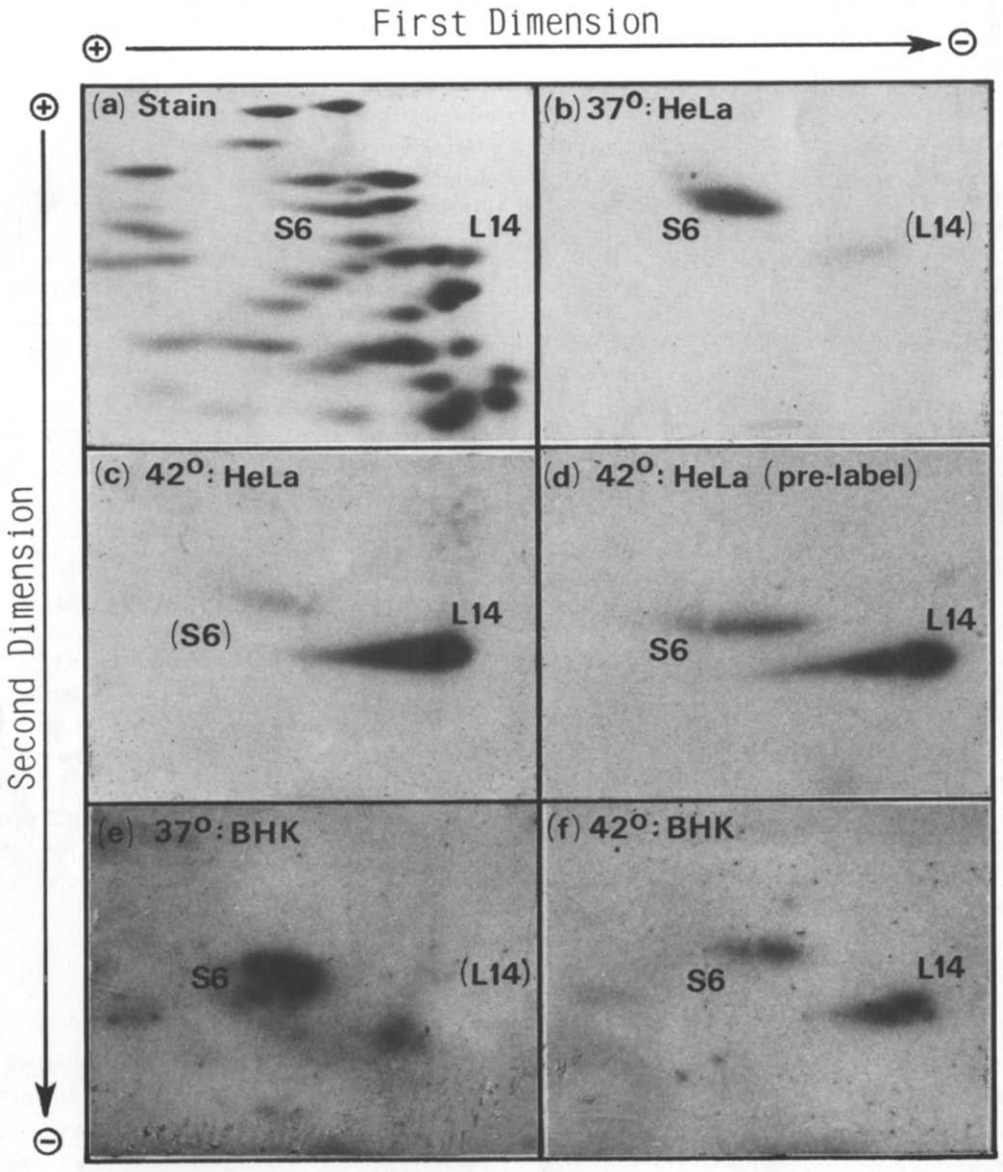

Fig.1. Two-dimensional gel analysis of the phosphorylation of ribosomal proteins during heat shock at 42°C. HeLa cells (a–d) or BHK cells (e–f) were incubated with [^{32}P]orthophosphate for either 3 h at 37°C (a,b,e), 1 h at 37°C and then 2 h at 42°C (c,f) or 3 h at 37°C, after which the medium was replaced by non-radioactive medium and incubation continued for 2 h at 42°C (d). Frames b–f show autoradiographs, only a single stained gel (a) being presented as the staining pattern was not appreciably different under the different conditions. [In frame e only ribosomal protein S6 is phosphorylated (cf. e.g. [7,11,22]) the other spots being blemishes on the X-ray film.]

2.3. Isolation of ribosomal protein

Ribosomes were isolated from the cells by a method [6] that allowed direct subsequent extraction of total ribosomal protein to give a suitable preparation for two-dimensional gel electrophoresis without prior dissociation of ribosomes into their subunits. The extraction was performed as in [7] with the modification that concentration of protein was by acetone precipitation [8] rather than lyophilisation.

2.4. Polyacrylamide gel electrophoresis

One-dimensional electrophoresis was in 15% polyacrylamide gels (acrylamide:bisacrylamide = 167:1) containing 0.1% SDS [9]. To each track was applied approx. 50 μg of extracted ribosomal protein.

Two-dimensional polyacrylamide gel electrophoresis was performed according to [10] as in [11]. On each gel approx. 200 μg protein was analysed.

Gels were stained with Coomassie brilliant blue, dried, and subjected to autoradiography at −70°C using Kodak XAR-5 film and Cronex intensifying screens (Du Pont).

3. RESULTS

When monolayer HeLa cells were incubated for 3 h with [^{32}P]orthophosphate, ribosomal protein S6 became labelled, even though its position of migration on two-dimensional gel electrophoresis implied that the state of phosphorylation of the 5 potential sites on this protein was relatively low

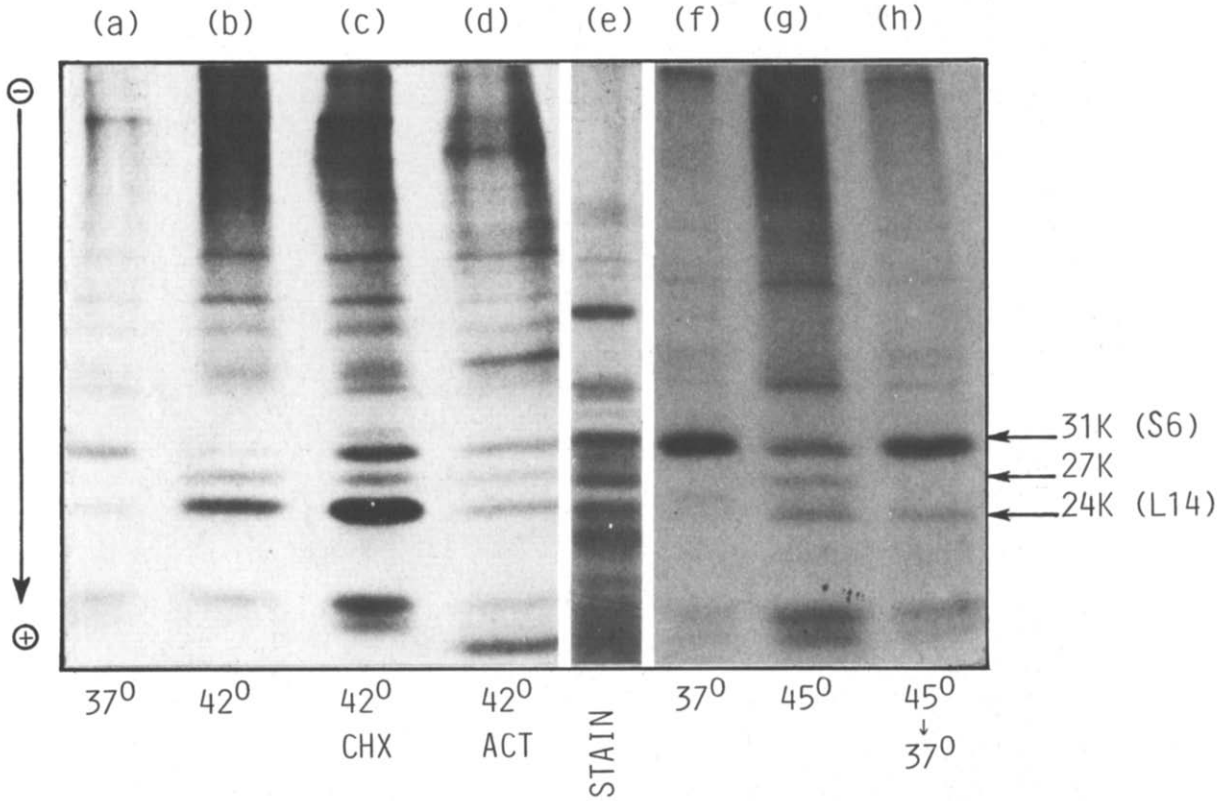

Fig.2. One-dimensional gel analysis of the phosphorylation during heat shock of proteins associated with HeLa cell ribosomes. Cells were incubated with [^{32}P]orthophosphate for the 3 h prior to harvesting: (a,e,f) 3 h at 37°C; (b–d) 1 h at 37°C and 2 h at 42°C, with cycloheximide (CHX) (20 μg/ml) and actinomycin D (ACT) (1 μg/ml) being present during the 42°C incubation for (c) and (d), respectively; (g) 170 min at 37°C and 10 min at 45°C; (h) 50 min at 37°C, 10 min at 45°C, 2 h at 37°C. Track e shows the staining pattern, the other tracks presenting autoradiographs. (Inadvertant differences in the amount of protein loaded, together with varying track width, account for minor quantitative variations in the intensity of some of the bands in tracks a–d.)

(fig.1a,b). When cells were prelabelled with [^{32}P]-orthophosphate for 1 h at 37°C and then maintained at 42°C for 2 h, the radioactive labelling was reduced to very low levels (fig.1c), implying that there was little or no phosphorylation of the protein at 42°C. Furthermore ^{32}P previously incorporated into the protein for 3 h at 37°C was extensively removed at 42°C, implying that dephosphorylation had also occurred (fig.1d).

Since this work was completed the dephosphorylation of ribosomal protein S6 has been reported to occur during heat shock at 42°C in human fibroblasts and menigioma cells [12]. However the dephosphorylation of ribosomal protein S6 is not the only change that we observed in heat-shocked HeLa cells, for we found that there was extensive phosphorylation of ribosomal protein L14 at 42°C (fig.1c,d). Although occasional reports have appeared of incorporation of [^{32}P]orthophosphate into L14 in normal cells (e.g. [13]) comparison with the incorporation into S6 shows that this is never more than trace amounts (see also [4]). The

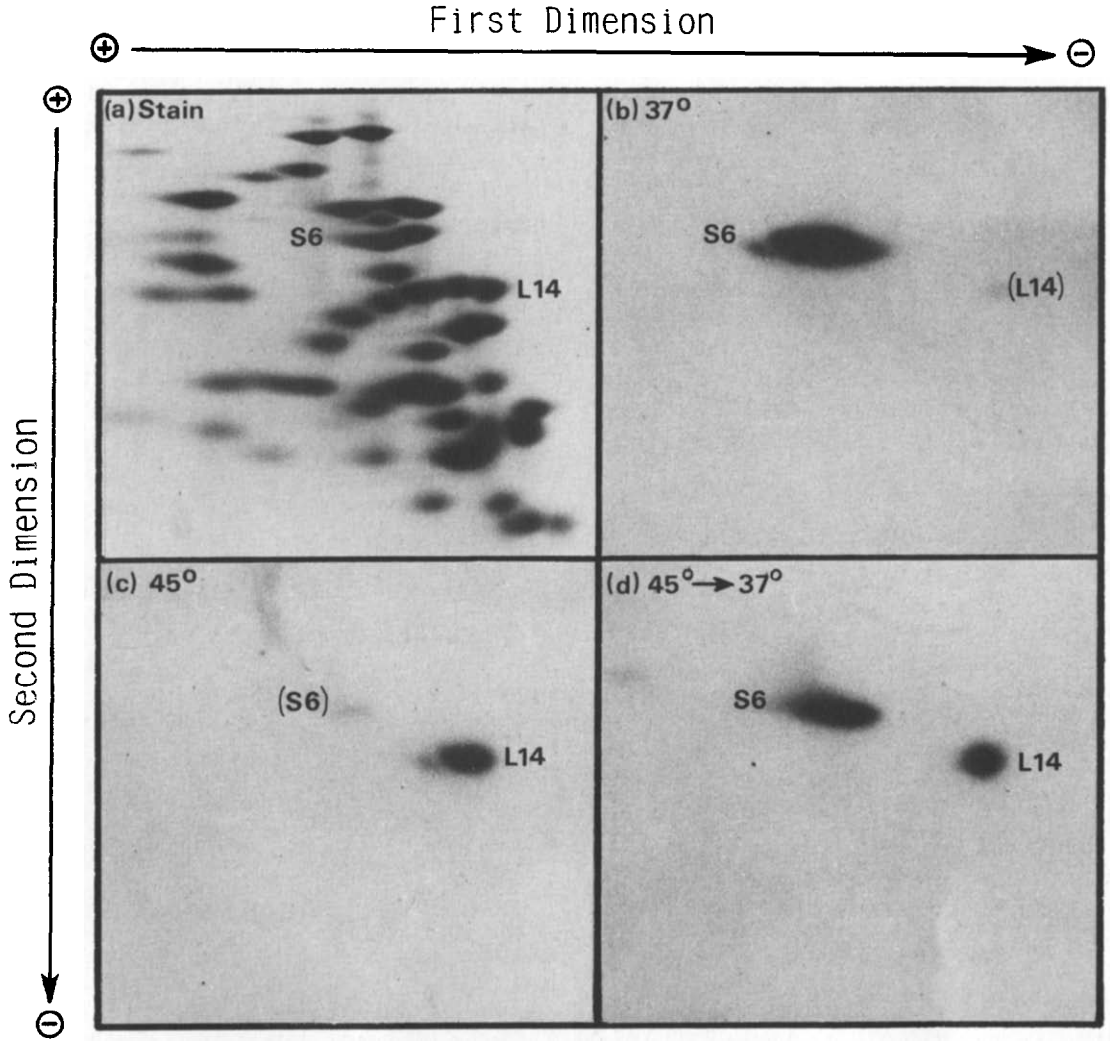

Fig.3. Two-dimensional gel analysis of the phosphorylation of HeLa cell ribosomal proteins during heat shock at 45°C. Cells were incubated with [^{32}P]orthophosphate for the 3 h prior to harvesting: 3 h at 37°C (a,b); 170 min at 37°C and 10 min at 45°C (c); 50 min at 37°C, 10 min at 45°C, 2 h at 37°C (d). Frame a shows a stained gel and frames b–d autoradiographs.

phosphorylation of L14 was not restricted to HeLa cells, similar results being obtained in baby hamster kidney fibroblasts (fig.1e,f).

The two-dimensional electrophoretic method used in fig.1 only displays the basic proteins of the ribosome. Analysis on one-dimensional SDS gels [14] displays, in addition, the phosphorylated acidic proteins (M_r ~14000) of the large ribosomal subunit [15] together with trace amounts of non-ribosomal contaminants that are found with undissociated ribosomes. Such analysis (fig.2b) revealed another protein on the ribosome, of M_r = 27000, that became phosphorylated at the same time as ribosomal protein L14. The phosphorylation of these two proteins was not prevented by either actinomycin or cyclohexamide and thus does not require new protein synthesis (fig.2c,d). However it should be noted that no conclusions about changes in the phosphorylation of ribosomal protein S6 can be drawn from such inhibitor experiments, as inhibitors of protein biosynthesis themselves stimulate the phosphorylation of this protein [16,17], an effect that can also be seen in fig.1b–d.

In comparison with continuous heat treatment at 42°C, brief heat shock at 45°C (10 min) followed by a shift back to 37°C offers a more defined system in which to study the recovery of protein synthesis dissociated from the initial inhibitory effects [2]. Using this protocol (with cells pre-labelled for the last 3 h of incubation at 37°C) we found that the dephosphorylation of ribosomal protein S6 occurred within 10 min at 45°C (fig.3c), but considerable (if not complete) rephosphorylation occurred within 2 h of recovery at 37°C (fig.3d), during which time protein synthesis also recovers to a large extent [3]. (Rephosphorylation of protein S6 has also been observed after 2.5 h recovery at 37°C from 2 h heat shock at 42°C; not shown.) Heat shock for 10 min at 45°C also caused the phosphorylation of ribosomal protein L14 and of the M_r = 27000 protein associated with the ribosome (fig.2g, fig.3c); however although the latter protein was no longer labelled after 2 h of recovery at 37°C, ribosomal protein L14 could be seen to have lost little of its radioactive label (fig.2h, Fig.3d) and this was also true when recovery was prolonged to 6 h (not shown). It appears that this is due to the phosphate incorporated into protein L14 during the 10 min at 45°C being stable, rather than continuing to turn over, as it did not become labelled if the [^{32}P]orthophosphate was not added until the shift-back to 37°C (not shown).

4. DISCUSSION

The use in this work of the standard two-dimensional system of gel electrophoresis on which the nomenclature of ribosomal proteins is based [20] has allowed unequivocal demonstration that heat shock causes dephosphorylation of ribosomal protein S6 in mammalian cells. It is likely that similar dephosphorylations in a basic protein of the less-studied ribosomes of *Drosphila* [5] and higher plant cells [21] involve the proteins corresponding to S6 in these cells. This dephosphorylation, and the rephosphorylation of ribosomal protein S6 during recovery from heat shock at 45°C, parallels changes we have observed in protein biosynthesis [3], once again giving reason to suspect an association between these two metabolic processes. For reasons discussed in detail elsewhere [4,22] we regard an absolute requirement for ribosomal protein S6 to be phosphorylated for protein synthesis to occur as unlikely, and have suggested that the phosphorylation may only exert a regulatory influence on protein synthesis under certain conditions when some component is limiting. An interesting specific suggestion in this regard is that the phosphorylation is only needed to recruit new mRNA into polyribosomes [23], for example from mRNP particles; and it is possible that the change in the phosphorylation state of ribosomal protein S6 during heat shock and recovery therefrom is related to just such a role. In this case the cause of initial inhibition of protein biosynthesis in heat shock, and the reversal of this during recovery, would be expected to lie elsewhere.

The observation that during heat shock, other proteins associated with the ribosome are phosphorylated de novo is important in two respects. Firstly, it emphasises that the dephosphorylation of ribosomal protein S6 is not merely part of a general dephosphorylation of proteins, but is quite specific. Secondly, it raises the possibility that phosphorylation events on the ribosome play a role in the rapid initial response of cells to heat shock, perhaps being involved in the initial inhibition of protein biosynthesis. It should be stressed that the phosphorylation of ribosomal protein L14 is not seen if protein biosynthesis at 37°C is inhibited by

antibiotics [16,17] or by such conditions as hypertonicity [24] or viral infection [25,11].

The only situation in which significant phosphorylation of ribosomal protein L14 was found previously was in Krebs II ascites cells incubated under conditions that produce lactic acidosis [18]. (Ribosomal protein S6 becomes dephosphorylated under these conditions.) In view of a report that lactic acidosis includes the synthesis of heat shock proteins in rat heart [19] it is possible that the phosphorylation of protein L14 in lactic acidotic Krebs II ascites cells reflected a similar metabolic stress response.

It is not clear why the phosphorylation of protein L14 was not observed in other studies of heat shock [5,12,21], although it should be pointed out that L14 is one of the most chemically basic of ribosomal proteins, and could easily be run off the end of the first dimension of the two-dimensional electrophoretic separation. It is also possible that there could be differences in this regard between the response to heat shock in mammalian cells and those of lower organisms.

The functional significance of the phosphorylation of ribosomal protein L14 in relationship to protein synthesis in heat shock is brought into question by the lack of dephosphorylation of this protein during recovery (fig.2,3). In this respect it differs from the unidentified protein of M_r = 27000. It is possible to envisage a situation in which a conformational change in the ribosome during the inhibition of protein biosynthesis in the stress response rendered ribosomal protein L14 susceptible to phosphorylation by a protein kinase, of which it was not the functional substrate. A return of the ribosome to a more normal conformation might then make the site of phosphorylation inaccessible again to either phosphoprotein kinase or phosphoprotein phosphatase. It should be mentioned that ribosomal protein L14 in ribosomal 60 S subunits does not serve as a substrate for various cyclic AMP-dependent or cyclic AMP-independent protein kinases in vitro [26–29]. Thus, although a function for the phosphorylation of ribosomal protein L14 cannot be excluded, we feel that it is now most important to identify the protein of $M_r = 27000$ associated with the ribosome, and the phosphoprotein kinase and phosphoprotein phosphatase activities controlling its phosphorylation.

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