Heat shock induces rapid dephosphorylation of a ribosomal protein in Drosophila

(Clinical protein 56/translation)

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ABSTRACT Ribosomes isolated from Drosophila melanogaster tissue culture cells grown in vivo with 32P contain a single, heavily phosphorylated, ribosomal protein. As much as 40% of this protein is phosphorylated in cells cultured at 25°C. The molecular weight and other characteristics of this protein suggest possible homology with ribosomal protein S6. Following a shift-up to 37°C, the protein is specifically and quantitatively dephosphorylated. The kinetics of this dephosphorylation are rapid with a half-time on the order of a few minutes. These kinetics closely parallel the heat shock-induced breakdown of the preexisting polysome population.

Various organisms display a specific, coordinated response to heat shock or other stressfull stimuli such as anoxia. The response to heat shock in Drosophila is the best studied case at present (1). Although the regulation of this response occurs at several levels of gene expression, translational controls appear to be important. Within minutes after heat shock, translation of most mRNAs is sharply curtailed, and the existing population of polyribosomes is broken down (2). Shortly thereafter, a new population of polysomes begins to form on newly synthesized mRNAs that code for heat shock-specific proteins. Because most of the preexisting messenger population remains intact in heat shocked cells (3), some selective control at the level of translation must exist. The mechanism that discriminates against the translation of normal cellular mRNAs is currently unknown, but it apparently does not involve simple competition for available ribosomes by newly synthesized heat shock messages because inhibition of heat shock mRNA transcription with actinomycin D does not prevent the initial breakdown of polyribosomes (4).

Methods for the preparation of cell-free translation systems from Drosophila tissue culture cells (5) have recently allowed an analysis of this heat shock-induced translational specificity in vivo. Lysates prepared from cells grown at 25°C translate normal and heat shock messages with equal efficiency, but comparable lysates prepared from heat shocked cells display the same discrimination observed in vivo: normal messages are translated poorly whereas heat shock messages continue to be translated with high efficiency (6). Because the ability to discriminate against the normal mRNA population is not transferable to 25°C lysates by particle-free cytoplasm from heat shocked cells, Kruger and Benecke (7) proposed that the responsible factor may be associated with ribosomes. Recently, Scott and Pardue (8) succeeded in rescuing the translation of normal messages in 37°C lysates by the addition of a crude ribosome fraction from a 25°C lysate.

This report describes a difference between the ribosomes of control and heat shocked cells. One ribosomal protein is heavily phosphorylated in cells grown at normal temperatures but is completely dephosphorylated in heat shocked cells. The kinetics of dephosphorylation following a shift-up to 37°C closely parallel the heat shock-induced breakdown of the polysomes.

MATERIALS AND METHODS

Cells and Culture Conditions. Drosophila melanogaster tissue culture cells, line Kc (9) obtained from W. Gehring, were maintained in suspension culture in 100-ml spinner flasks at 25°C in serum-free low-phosphate D22 medium (10, 11). Cell density was maintained between 2 × 10^5 and 1 × 10^6 cells per ml.

In Vivo Labeling with 32P. Growing cells were labeled directly in low-phosphate D22 medium by adding 32P (carrier-free, 50 mCi/ml; New England Nuclear; 1 Ci = 3.7 × 10^10 becquerels) to a final concentration of 10–100 μCi/ml. Cells were diluted with fresh medium 24–48 hr prior to labeling, and final cell density was 8 × 10^6 cells per ml.

Acid-soluble proteins were extracted from whole cells by pelleting an aliquot of culture for 15 sec in an Eppendorf microcentrifuge and extracting the pellet with 0.2 M H2SO4. Insoluble material was removed by centrifugation at 16,000 × g for 10 min. Acid-soluble protein was precipitated from the supernatant with 20% trichloroacetic acid, collected by centrifugation at 16,000 × g for 10 min, washed once with 10% trichloroacetic acid, once with 0.2% concentrated HCl in acetone, and once in acetone, and dried under reduced pressure.

Cell Fractionation and Sucrose Gradient Sedimentation. Prior to fractionation, cells were cooled to 0°C by immersion of the culture vessel in ice water for 5 min. Cells were pelleted out of the growth medium by centrifugation at 1000 × g for 5 min and resuspended in 0.1 vol of 50 mM Tris-HCl, pH 7.4/50 mM MgCl2/25 mM KCl/1 mM dithiothreitol supplemented with diethylpyrocarbonate (2 μl/ml, added just before use). After resuspension, cells were lysed by addition of Triton X-100 to a final concentration of 0.2% and homogenized in a Dounce homogenizer. A nuclear fraction was obtained by centrifuging the homogenate at 3000 × g for 10 min. The supernate (cytosol fraction) was loaded on a linear 0.5–1.5 M sucrose gradient in 50 mM Tris-HCl, pH 7.4/25 mM MgCl2/25 mM KCl and centrifuged at 4°C in a Beckman SW 40Ti rotor at 35,000 rpm for 75 min. Fractions were collected from the bottom of the tube, and the absorbance of each fraction was measured at 260 nm in a 1-cm pathlength cuvette.

Acid-soluble proteins from pelleted fractions (whole cells and nuclei) were extracted and precipitated as described above. Acid-soluble proteins from soluble fractions (whole lysate, cytosol, gradient fractions) were obtained by adjusting the solutions to 0.2 M H2SO4 with concentrated acid, removing insoluble material by centrifugation, and then precipitating the supernates as described above.

Isolation of Ribosomes. Ribosomes were prepared from Drosophila Kc cells essentially as described by McConkey (12) for

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HeLa cells, except that diethylpyrocarbonate (1 \mu l/ml) was added to the initial homogenization buffer immediately before use. Ribosomal proteins were extracted from the pelleted ribosomes as described by Lastick et al. (13) and precipitated as suggested by Howard et al. (14). The precipitated protein was then washed once with 10% trichloroacetic acid, once with acidified acetone, and once with acetone and dried under reduced pressure.

**Gel Electrophoresis.** Dried protein samples were dissolved in 6 M urea/5% acetic acid/5% 2-mercaptoethanol and stored at 4°C until used.

Acid/urea/polyacrylamide slab gels (12% acrylamide, 0.08% N,N'-methylenebisacrylamide containing 6 M urea and 5% acetic acid) were made essentially as described by Panyim and Chalkley (15). Gels were preelectrophoresed at 23°C for 24 hr at 150-200 V with 5% acetic acid as the running buffer. Samples were electrophoresed at 23°C at 100-150 V for a total of 850 V hr (10-cm gels) or 3200 V hr (24-cm gels).

Second-dimension NaDodSO4 gels (12% acrylamide, 0.32% bisacrylamide) were prepared according to Laemmli (16). Individual lanes were cut from a Coomassie blue-stained acid/urea slab gel, equilibrated for 1 hr in several changes of 62.5 mM Tris-HCl, pH 6.8/5% 2-mercaptoethanol, and sealed horizontally to the top of the stacking gel with 1% agarose in 62.5 mM Tris-HCl (pH 6.8). Electrophoresis was at 23°C for 360 V hr. Coomassie blue is displaced during the electrophoresis.

Gels were stained in 0.07% Coomassie brilliant blue R-250 (Sigma) in 50% methanol/5% acetic acid and destained by continuous diffusion in 7% methanol/7% acetic acid. Gels containing samples labeled with \(^{32}P\) were autoradiographed with Cronex-4 x-ray film and a Lightning Plus intensifying screen (Du Pont).

**RESULTS**

Phosphorylation of the acid-soluble proteins of *D. melanogaster* was studied by labeling *K* tissue culture cells in vivo with \(^{32}P\), followed by extraction of whole cells with 0.2 M H\(_2\)SO\(_4\) and analysis of the soluble proteins by electrophoresis in acid/urea gels. The advantages of this approach are: (i) a manageable subset of whole cell protein is examined, (ii) highly labeled nucleic acids do not interfere with the analysis, and (iii) there is little opportunity for enzymatic phosphorylation or dephosphorylation during the isolation procedure. The disadvantages are that acid-insoluble proteins cannot be examined and that acid-labile modifications may be destroyed. An example of such a whole cell acid extract is shown in Fig. 1 (lanes A and C). The stained pattern is composed of histones, ribosomal proteins, and other acid-soluble proteins, most of the third group being of relatively high molecular weight. Proteins labeled in vivo with \(^{32}P\) generated a number of discrete bands of which some have been assigned to particular proteins.

Continuous heat shock over a period of 4 hr produced several changes in the pattern of \(^{32}P\)-labeled proteins (Fig. 1). The most dramatic of these changes was associated with the protein designated rP. This protein was heavily labeled at the normal growth temperature of 23°C but incorporated no label at the heat shock temperature of 37°C. The change in phosphorylation of rP involved a large percentage of this protein because an associated shift in the stained pattern was also observed. This shift was difficult to detect in one-dimensional gels but was clearly seen in two-dimensional gels. The proportion of rP that was phosphorylated at 25°C depended on the exact growth conditions and the final cell density and varied from 5% to at least 40%. Heat shock invariably converted all of this protein to its unmodified form.

Although incorporation of \(^{32}P\) into several other proteins was altered in a reproducible manner by heat shock (for example, H4), these effects will not be analyzed in detail here.

In order to examine the kinetics with which rP was dephosphorylated, the protein was labeled by incubating cells for 2 hr with \(^{32}P\), at 25°C. Dephosphorylation was then monitored after a shift-up to 37°C (Fig. 2). Dephosphorylation occurred quite rapidly, being detectable by 2.6 min, and was essentially complete within 10-15 min. The effect was specific and occurred even though the labeled precursor was still present in the medium. Interestingly, cold shock also resulted in the specific dephosphorylation of the same protein, although the kinetics were very much slower.

The identity of rP was investigated in cell fractionation experiments. Cells were incubated with \(^{32}P\), at 25°C in order to label the protein and then lysed and separated into nuclei and cytosol by differential centrifugation. A polysome profile (Fig. 3) was then obtained by velocity sedimentation of the cytosol through a sucrose gradient. A few of the labeled proteins, including D1, H1, H2A, and H4, were associated with the nuclear...
When exposed by cells proteins which some into and polysomes. The top labeled proteins fraction was removed from this 25°C culture for gel analysis at 1, 2, 2.5, and 5 hr (lanes A, B, I, and O, respectively). At t = 2 hr, a 10-ml aliquot was removed and shifted to 37°C (heat shock). Samples from this heat shocked aliquot were removed at 1.3, 2.6, 5, 10, 15, and 20 min after the shift-up (lanes C–H, respectively). At t = 2.5 hr, a second 10-ml aliquot was removed from the 25°C culture and shifted to 0°C (cold shock). Samples from this aliquot were removed at 5, 10, 20, 30, and 180 min after the shift-down (lanes J–N, respectively). The remainder of the cold shocked aliquot was placed at 4°C overnight and a final sample was removed at 12 hr after the shift-down (lane P).

The remainder, including rP, were present in the cytosol. However, after velocity sedimentation, all of the labeled proteins of the cytosol, except for rP, remained at the top of the gradient. rP appeared to be completely absent from the top of the gradient and instead sedimented with the ribosomes. The protein is found in association with both monosomes and polysomes and accurately tracks the polysome profile.

The cell fractionation experiment described above involved both detergent lysis, which might release mitochondrial ribosomes into the cytosol, and low monovalent salt concentrations, which might result in adventitious association of nonribosomal proteins with ribosomal particles. In order to avoid these potential problems, ribosomes were also isolated from labeled Kc cells by a method that does not involve Triton lysis and that exposes the particles to KCl concentrations as high as 0.3 M (12). When ribosomal proteins were extracted from ribosomes prepared in this way and analyzed by one- and two-dimensional gel electrophoresis, rP still was present in undiminished amounts relative to the other ribosomal proteins. The two-dimensional analysis (Fig. 5) demonstrated only one labeled species in the labeled band seen on first-dimension gels and clearly showed the specificity with which this ribosomal protein is phosphorylated in vivo. These results suggest that rP is associated with cytoplasmic rather than mitochondrial ribosomes, that it is an integral component of cytoplasmic ribosomes rather than adventitiously associated with them, and that it is the only (basic) cytoplasmic ribosomal protein that is significantly phosphorylated in vivo in Drosophila.

The radioactive phosphate incorporated into rP in vivo is susceptible to digestion by bacterial alkaline phosphatase. This and the stability of the modification to acidic conditions are consistent with the modified amino acid residue's being either phosphoserine or phosphothreonine. The molecular weight of the protein is 32,000 as estimated by electrophoresis in NaDodSO4 gels.

**DISCUSSION**

Cytoplasmic ribosomes of Drosophila Kc cells contain a single basic ribosomal protein, rP, that undergoes significant phosphorylation in vivo. The proportion of phosphorylated molecules can be as high as 40% under normal growth conditions, but the level of modification is sensitive to various conditions including heat shock, cold shock, and the exact stage of the growth cycle. These characteristics, as well as the molecular weight of 32,000, are strikingly similar to the properties of ribosomal protein S6.

For most eukaryotes that have been examined, only one ribosomal protein is found to be significantly phosphorylated in vivo (19). In those cases in which a definite identification has been made, this protein is invariably the small-subunit protein designated S6. As judged from stained patterns on two-dimensional gels, the proportion of phosphorylated molecules can reach virtually 100% under optimal growth conditions (13). The level of phosphorylation of S6 is affected by a broad spectrum of conditions and reagents (19), although heat shock-induced
dephosphorylation has not been described. The molecular weight of S6 is 31,000 as estimated by NaDodSO₄ gel electrophoresis. Although the similarities between S6 and rP are suggestive, a definitive assessment of homology must await structural analysis of the Drosophila protein.

The rapid dephosphorylation of the Drosophila protein after a shift-up to 37°C reported here closely parallels the heat shock-induced breakdown of polysomes (2) and suggests a possible relationship between rP phosphorylation and translation. One attractive hypothesis is that ribosomes must be phosphorylated in order to translate normal 25°C mRNAs. This hypothesis appears to be consistent with in vitro supplementation experiments so far reported (7, 8). In its simplest form, this hypothesis would imply that the percentage of rP that is phosphorylated should be at least as great as the percentage of ribosomes in polysomes (assuming no translation of heat shock messages). The fact that more than 40% of the ribosomes isolated from a 25°C culture are in polysomes (Fig. 3) suggests that a somewhat more sophisticated version of the proposal is required—for instance, that phosphorylation of rP is necessary to initiate translation of normal messages but not for elongation. The heat shock system in Drosophila appears to offer a unique advantage for further study of the effects of ribosomal protein phosphorylation on translation because a defined class of mRNA molecules exists whose response to translational control signals differs both in vivo and in vitro from that of most other mRNAs.

It has recently been shown that heat shock causes hyperphosphorylation of histone H1 in starved cells of the ciliated protozoan Tetrahymena pyriformis (20). Thus, major stress-induced alterations in protein phosphorylation have now been described for both nuclear (histone) and non-nuclear (ribosomal) proteins. Further attempts to correlate these alterations in protein phosphorylation with the transcriptional and translational
events triggered by heat shock may provide insights into the mechanism or function of the stress response.

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