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PHOSPHORYLATION-DEPHOSPHORYLATION CHANGES IN YEAST RIBOSOMAL PROTEINS S2 AND S6 DURING GROWTH UNDER NORMAL AND HYPERTHERMAL CONDITIONS*

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Dynamic changes were observed in the phosphorylation level of a basic ribosomal protein S6 under different growth conditions of yeast culture. The maximum level of S6 phosphorylation occurred within 30 min of $^{32}$P-labelling after the transfer of cells into fresh nutrient medium. The elevation of temperature to supraoptimal level (38°C and 41°C) led to extensive dephosphorylation of S6 protein, and the recovery from heat shock was characterized by its rephosphorylation. Contrary to S6, phosphorylation of ribosomal protein S2 remained on unchanged level, irrespective of growth conditions.

A number of organisms respond to elevated temperatures by preferential synthesis of a small set of polypeptides referred to as heat shock or stress proteins. At the same time synthesis of several cellular proteins is drastically reduced (Ashburner & Bonner, 1979; Tanguay, 1983). The heat shock response, first discovered in Drosophila melanogaster, has been shown to be a universal phenomenon observed in bacteria, lower eukaryotes (yeasts) and higher, differentiated eukaryotes such as plants, mammalian and insect cell lines, and many others (Storti et al., 1980; Yamamori et al., 1978; Miller et al., 1979; Baszczyński et al., 1982; Morange et al., 1984). The molecular mechanism underlying the induction of heat shock proteins has been intensely studied in Drosophila and is believed to be controlled at the transcriptional and translational level (Storti et al., 1980). Little is known, however, about analogous events in other organisms.

It has been recently shown that in Drosophila (Glover, 1982; Olsen et al., 1983), plants (Scharf & Nover, 1982), human fibroblasts, meningoma cells (Richter et al., 1983) and HeLa cells (Kennedy et al., 1984) heat shock induces a rapid dephosphorylation of the major phosphoprotein of

* This work was supported by the Polish Academy of Sciences within the Project 09.7.
40S ribosomal subunit — protein S6. There are indications that the phosphorylation level of this protein is correlated with rapid changes in the initiation rate of translation and seems to play a role in the recruitment of inactive mRNAs (Duncan & McConkey, 1982a; b; Thomas, 1982).

The aim of this work was an analysis of the S2 and S6 ribosomal protein phosphorylation in Saccharomyces cerevisiae cells cultivated under different temperature conditions for various time intervals. The results obtained indicate that only S6 protein responds to environmental changes by changes in the phosphorylation level, while S2 phosphorylation remains on a practically unchanged level.

MATERIALS AND METHODS

Yeast culture and labelling conditions. Saccharomyces cerevisiae, a diploid strain AS10, was cultivated aerobically in the medium of Cooper et al. (1962) to the early log-phase of growth. Harvested cells were washed and suspended in a fresh low-phosphate medium (Retel & Planta, 1967). Labelling of the cells in vivo with inorganic $^{32}$PO$_4^-$ (0.04 mCi/ml) was carried out at the control (28°C) and heat shock (38°C and 41°C) temperature for the times indicated in the legends to Figures. Labelled cells were harvested and washed with an ice-cold buffer (20 mm-Hepes/KOH, pH 7.4, 100 mm-NH$_4$Cl, 5 mm-Mg(CH$_3$COO)$_2$, 2 mm-dithiothreitol).

Isolation of ribosomes and separation of ribosomal proteins. Cell-free extracts were prepared by sonication of labelled cells (6 × 60 s at 10 A, with cooling intervals of 1 min). Ribosomes were isolated and purified as previously described (Grankowski et al., 1976). Ribosomal proteins were extracted with acetic acid and precipitated with acetone (Ringer et al., 1981).

Gel electrophoresis, staining of gels and autoradiography. Ribosomal proteins were subjected to one-dimensional electrophoretic analysis on 2 mm SDS - 15% polyacrylamide slab gels (Laemmli, 1970).

Two-dimensional gel electrophoretic system for ribosomal proteins of Kaltschmidt & Wittmann (1970) included the modifications introduced by Grankowski et al. (1976).

Gels were stained with Coomassie Brilliant Blue G-250 and exposed to X-ray films for autoradiography.

Calibration of electrophoregrams. The relative molecular weights of proteins on one-dimensional gels were calculated on the basis of marker proteins: phosphorylase b (94 000), bovine serum albumin (67 000), ovalbumin (42 000), carbonic anhydrase (30 000), soybean trypsin inhibitor (20 100), α-lactoglobulin (14 400).

Identification of yeast ribosomal proteins on two-dimensional gels was made according to the nomenclature proposed by Grankowski et al. (1976).
RESULTS AND DISCUSSION

Changes in S6 phosphorylation level under different growth conditions

In all eukaryotic cells tested so far, the main ribosomal phosphoprotein is a basic protein of small ribosomal subunit identified as S6. It has a molecular weight ranging from 30,000 to 36,000 and can exist in a multiply-phosphorylated state (Traugh, 1981). S6 phosphorylation was also confirmed in yeast cells, in addition to phosphorylation of other ribosomal proteins including S2 and a pair of highly acidic proteins, L44 and L45 (Kudlicki et al., 1981). To determine whether changes in the phosphorylation level of yeast protein S6 occur in vivo, we prepared ribosomes from the culture (7×10^7 cells/ml) labelled with $^{32}$P at 28°C over an extended period of time, i.e. 30, 60, 90 and 120 min. Ribosomal proteins were separated by one-dimensional SDS-polyacrylamide gel electrophoresis. As shown in Plate 1A (lane a), S6 is strongly labelled after 30 min incubation of the cells with $^{32}$P, then it looses the radioactivity progressively as the incubation in the presence of $^{32}$P continues (Plate 1A, lanes b, c and d). It is worth mentioning that when the cells from the same culture were resuspended at the half of the initial density (about $3\times10^7$ cells/ml) the increased phosphorylation of S6 persisted for a longer period of time (data not shown). Similar results were obtained earlier for HeLa cells (Lastick et al., 1977). Why the cell density is so essential for the efficient phosphorylation of S6 protein is not known at present. According to Lastick et al. (1977), at the higher cell densities a more rapid depletion of factors that maintain the highly phosphorylated state of S6 can occur. However, such factors have not been yet identified. Ribosomal protein S6 becomes multiply phosphorylated in several mammalian systems upon incubation of cells with various growth-stimulating agents (Traugh, 1981). We can not exclude the existence (presumably at low concentration) of growth-stimulating agents or some kind of positive effectors of cell growth in our medium. It was also pointed out that S6 phosphorylation was stimulated when HeLa cells were resuspended in a fresh medium (Lastick et al., 1977). This may also be true for yeast cells. Each of our $^{32}$P-labelling experiments was started with a fresh medium. Moreover, when yeast cells were incubated in $^{32}$P-containing medium under conditions (60 min at density $7\times10^7$ cells/ml) which lead to a low level of incorporation of radioactivity into S6 (Plate 1B, lane a) and then transferred to a fresh non-radioactive medium for 30 min (Plate 1B, lane d), a remarkable increase of S6 phosphorylation was observed. This interesting observation is very difficult to explain at present though it may indicate the presence in the cells of a radioactive phosphate donor for S6 phosphorylation.
which can be utilized after the transfer of labelled cells into the fresh 
$^{32}$P-deficient medium.

The effect of heat shock on the phosphorylation of ribosomal protein S6

So far no studies with yeast have been done with respect to the effect of heat shock on the phosphorylation/dephosphorylation of protein S6. As shown in Plate 1A (lanes e, f, g, h) and in Plate 1B (lanes b, e) at the temperature elevated to 41°C the S6 protein was poorly labelled throughout the incubation time. It also appeared that heat shock not only inhibits the $^{32}$P-labelling of S6 protein, but also affects its dephosphorylation. One-dimensional gel electrophoresis pattern clearly shows that the elevation of temperature to 38°C (Plate 1C, lane b) and 41°C (Plate 1C, lane d) leads to a considerable removal of $[^{32}P]$phosphate from S6 protein during 30 min of incubation. Simultaneously, at the control growth temperature, $^{32}$P incorporated into S6 was maintained on an unchanged level (Plate 1C, lane c) in comparison to non-incubated sample (Plate 1C, lane a). One-dimensional SDS-polyacrylamide gel shown in Plates 1A and 1C contained another phosphorylated ribosomal protein corresponding probably to S2 protein identified earlier (Kudlicki et al., 1981). Contrary to S6 protein, the phosphorylation level of S2 protein was constant throughout the 2 h incubation time or even increased slightly in the course of incubation (Plate 1A, lanes a, b, c and d). It is worth adding that the level of $^{32}$P incorporated into S2 protein at 28°C remained unchanged after the shift of the yeast culture to the hyperthermal conditions (Plate 1C, lanes b and d).

These data, together with the results obtained for other eukaryotic cells (Glover, 1982; Scharf & Nover, 1982; Richter et al., 1983; Olsen et al., 1983) suggest that dephosphorylation of S6 protein may be a general phenomenon, characteristic for such non-physiological conditions as hyperthermia. At present it is not known what causes the observed dephosphorylation of S6 protein after the exposure of yeast cells to supraoptimal temperature.

During the recovery of yeast cells from heat shock the $^{32}$P-label in the S6 protein reappears. The results of such an experiment are depicted in Plate 2. The isolated ribosomal proteins from control cells (A), cultivated at 41°C (B) and from cells incubated first at 41°C for 30 min and then at 28°C for the same time (C) were separated on two-dimensional polyacrylamide gels followed by autoradiography. As shown in Plate 2B, the cells subjected only to heat shock do not contain the radioactive S6 protein. It may also be noticed that the restoration of $^{32}$P-label during 30 min of cell recovery from heat shock is almost the same (Plate 2C) as in the control (Plate 2A).
Plate A. Temperature and time dependence of ribosomal protein phosphorylation. Two 100 ml batches of yeast cells were labelled in parallel with \( ^{32}P \) at 28°C and 41°C. The 20 ml samples were withdrawn at the indicated time points, at 28°C: a - 30 min, b - 60 min, c - 90 min, d - 120 min; at 41°C: e - 30 min, f - 60 min, g - 90 min, h - 120 min. Isolated ribosomal proteins were dissolved in a sample buffer (60 mM-Tris/HCl, pH 6.8, 10% glycerol, 2% SDS, 0.77% dithiothreitol, 0.008% bromophenol blue), heated for 5 min in a boiling-water bath and subjected to SDS - 15% polyacrylamide-gel electrophoresis at constant voltage of 80 V for 16 h. Gels were boiled in 10% trichloroacetic acid and stained as described in Materials and Methods.

Part B. One-dimensional gel electrophoresis of ribosomal proteins of normal and heat-shocked cells. Yeast cells were labelled with \( ^{32}P \) for 30 min (c) and 60 min (a) at 28°C and for 60 min (b) at 41°C. A batch of cells after one hour labelling at 28°C was transferred to the fresh \( ^{32}P \)-deficient medium and incubated for another 30 min in parallel at 28°C (d) and 41°C (e).

Part C. Phosphorylation and dephosphorylation of ribosomal protein S6 under temperature shift. Yeast cells were labelled with \( ^{32}P \) at 28°C for 30 min (a), then transferred to the fresh \( ^{32}P \)-deficient medium and incubated for 30 min at temperatures of: b - 38°C, c - 28°C, d - 41°C. Isolated ribosomal proteins were analysed by one-dimensional gel electrophoresis as in Part A.

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Plate 2. Two-dimensional polyacrylamide gel electrophoresis of basic ribosomal proteins. Two 50 ml batches of yeast cells were labelled in parallel for 30 min with $^{32}$P at 28°C (A) or 41°C (B). Sample C (50 ml) was incubated first for 30 min at 41°C and then $^{32}$P-labelled at 28°C (heat shock recovery) for another 30 min in the same volume of the fresh medium. Isolated ribosomal proteins were subjected to electrophoresis from (+) to (−). One-dimensional gels (2 × 120 mm glass tubes) contained 8% acrylamide and 0.3% bis-acrylamide. Electrophoresis was run at a constant current of 2 mA per tube for 4 h. Two-dimensional gels (120 × 120 × 2 mm plates) contained 18% acrylamide and 0.5% bis-acrylamide. Electrophoresis was run at 15 mA per plate for 16 h.
All these results clearly indicate that the phosphorylation level of yeast S6 ribosomal protein, contrary to S2 protein, undergoes rapid changes. Its phosphorylation state depends on the time and temperature of growth, on the growth medium and the culture age. There is some evidence indicating that the extent of S6 phosphorylation in other eukaryotic cells depends on the environmental conditions and can be altered by a variety of growth factors, hormones, mitogens and other agents which control protein synthesis (Traugh, 1981). It has been recently shown that the phosphorylation state of S6 protein plays an important role in the early step of the initiation of translation but the exact molecular mechanism of such a regulation is still not clear. It seems that the rapidity of S6 phosphorylation/dephosphorylation reactions at normal versus hyperthermal conditions offers a good opportunity to study the function of S6 protein in the translational control mechanism not only in the heat shock system. It also presents the possibility of further studies on the regulation of activity (induction or activation, or inactivation) of the enzymes engaged in modification of this protein.

I would like to thank Dr. E. Gąsior for advice and helpful discussion, A. Gralewski for technical assistance and K. Rusinek for typing.

REFERENCES


Received 29 August, 1984; revised 13 October, 1984