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CHANGES IN PHOSPHORYLATION OF NONHISTONE PROTEINS DURING DIFFERENTIATION OF A LOWER EUKARYOTE PHYSARUM POLYCEPHALUM

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SUMMARY: During starvation-induced differentiation of a slime mold Physarum polycephalum several changes in the phosphorylation of nuclear proteins occur. The overall content of serine- and threonine-bound phosphate drops by 50% and de novo phosphorylation of a number of nonhistone proteins is drastically altered. On the contrary, no selective dephosphorylation of nuclear proteins phosphorylated under normal growth accompanies differentiation. © 1987 Academic Press, Inc.

Cellular differentiation is an event of fundamental importance in numerous biological processes. It is accomplished by the differential expression of genetic information, but, with the exception of a limited number of extreme cases (e.g., maturation of lymphocytes) the molecular basis of changes in gene expression associated with differentiation of eukaryotic cells is far from being elucidated.

One of the several simple model systems whose differentiation has been thoroughly investigated is a true slime mold Physarum polycephalum. This lower Eukaryote, when exposed to adverse conditions, e.g., nutrient limitation, undergoes reversible differentiation forming metabolically dormant spherules. Numerous biochemical and morphological changes accompanying spherulation of P. polycephalum have been described (for a review, see (1)). It is also known that in this process the complement of genes being transcribed changes (2) and the overall transcriptional activity diminishes (3). However, the data concerning the functioning of the genetic apparatus are scarce.

To stop this gap our laboratory has undertaken studies of the structural organization and expression of genetic information during differentiation of P. polycephalum (3).

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In this report we investigated the phosphorylation of non-histone nuclear proteins since it has often been proposed that changes in phosphorylation of these proteins may participate in regulation of both the overall transcriptional activity as well as of the transcription of individual genes (reviewed in (4)).

MATERIALS AND METHODS

P. polycephalum strain M3CIV was used. Microplasmodia were grown in submerged shaken cultures in semidefined medium; differentiation was induced by transferring exponentially growing cultures to starvation medium (5).

For radioactive labelling microplasmodia were pelleted by brief centrifugation at 50 x g, washed with appropriate medium from which potassium phosphate was omitted and suspended in two volumes of phosphate-less medium to which carrier-free [^{32}P]-ortophosphate (OPIDI, Poland) was added to 1 mCi/ml. Details of the labelling protocols are given in the legends to the figures.

Nuclei were isolated as described (6) with 10 mM 3-phosphoglycerate added to all the buffers to inhibit phosphatases. Nuclei were dissociated with sodium dodecyl sulfate and proteins were extracted with phenol, as described in (7). After precipitation with two volumes of methanol at -20°C overnight the protein pellet was dissolved in 100µl of sample buffer according to Laemmli (8) and Cerenkov radioactivity was measured as described (9).

Proteins were electrophoresed in 8% polyacrylamide gels containing 0.1% sodium dodecyl sulfate according to Laemmli (8). For molecular mass determination electrophoresis calibration kits (Pharmacia, Sweden) were used. After staining with Coomassie Blue gels were destained, dried, and exposed at -70°C with Lanex Intensifying Screen (DuPont, USA), or at room temperature without a screen against Rentgen XM films (Foton, Poland).

Protein was determined according to Lowry et al. (10) and phosphate as described by Martensen (11).

RESULTS

The overall level of phosphorylation of P. polycephalum nuclear proteins was measured as the content of acid-stable, alkali-labile phosphate, thus, only phosphoserine and phosphothreonine residues were taken into consideration (11).
In plasmodia in the logarithmic phase of growth the content of phosphate in nuclear proteins amounts to 12.6 $\pm$ 1 nmole phosphate per milligram of protein (average of three independent measurements $\pm$ SD); after twenty hours of starvation, that is just prior to the formation of spherules, the phosphate content drops by ca. 50%, to 6.0 $\pm$ 0.7 nmole phosphate/mg protein.

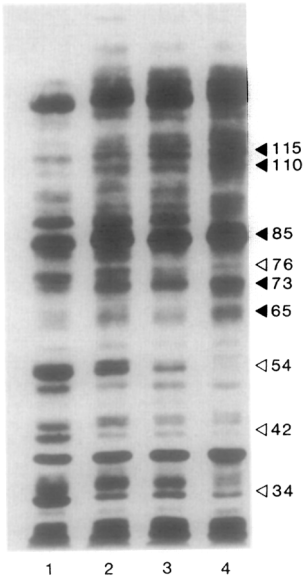

Fig. 1. Phosphorylation of nonhistone nuclear proteins during differentiation of P. polycephalum.
Microplasmodia were labelled for 2 h with [^{32}P] - ortho-phosphate (1 mCi/ml) added to phosphate-depleted media, after being grown for 22 h in normal medium (lane 1), or for 4, 10, 22 h in starvation medium (lanes 2, 3, 4, resp.). After labelling nuclear proteins were obtained, electrophoresed in 8% polyacrylamide gels and autoradiographed. On each lane ca. 26,000 Cerenkov counts were loaded. Only the autoradiogram is shown. ◀ and ◁ point to the proteins whose relative intensities of phosphorylation increase or decrease, respectively, during differentiation. Molecular masses of these proteins are given in kDa.

To see how this general decrease in phosphorylation is related to the rate of phosphorylation/dephosphorylation of individual nuclear proteins we performed in vivo labelling experiments and analysed the pattern of phosphoproteins by electrophoresis and subsequent autoradiography.

Fig. 1 shows how the relative intensities of phosphorylation of individual nonhistone proteins change with increasing time of starvation; while the general pattern of [^{32}P] - labelled phosphoproteins from actively growing and differentiating plasmodia is fairly constant, several clear-cut differences are also evident. The most prominent are an increase in phosphorylation of proteins of molecular mass 115, 110, 85, 73, 65 kDa (indicated by ◀) and a decrease in phosphorylation of proteins of molecular mass 76, 54, 42, 34 kDa (◁) on starvation. It should be stressed here that the pattern of total nuclear proteins as revealed by one-dimensional electrophoresis and Coomassie Blue staining is constant for up to 24 h of starvation, with the exception of

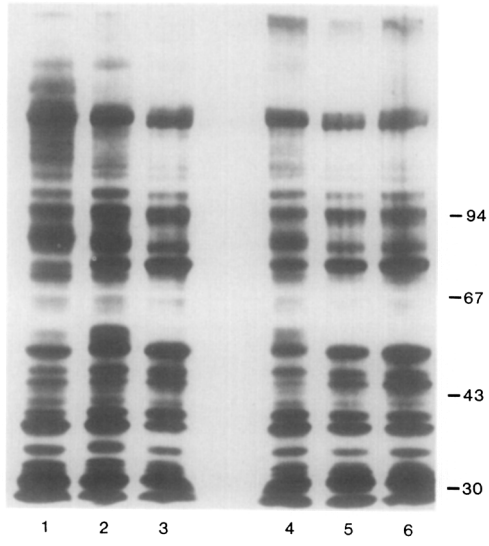

Fig. 2. Dephosphorylation of nonhistone nuclear proteins during differentiation of P. polycephalum.
Microplasmodia were labelled for 2 h with [^{32}P] - orto-phosphate (1 mCi/ml) in phosphate-depleted growth medium and then transferred to media containing excess of cold ortophosphate. After 0, 3, or 12 h of chase in growth medium (lanes 1, 2, 3, resp.) or after 3, 12, or 22 h in starvation medium (lanes 4, 5, 6, resp.) plasmodia were processed as described for Fig. 1. On each lane ca. 120,000 Cerenkov counts were loaded. The positions of molecular mass marker proteins are indicated on the right.

a protein of molecular mass 37 kDa, which increases by ca. 50% ((3), and not shown).

Since the level of phosphorylation is regulated not only by de novo phosphorylation, but also by dephosphorylation, we compared the relative rates of dephosphorylation of nonhistone proteins at various times during the differentiation process using a pulse-chase scheme. Log-phase plasmodia were labelled with [^{32}P]- ortophosphate in normal growth medium and then chased for various periods of time in normal or starvation media containing excess cold ortophosphate.

Fig. 2 shows that there are virtually no differences in the relative rates of dephosphorylation of individual [^{32}P]-prelabelled nuclear proteins in differentiating versus actively growing plasmodia. Thus, it can be concluded that during differentiation it is the de novo phosphorylation, but not dephosphorylation, that cause the differences in the pattern of nuclear phosphoproteins.

DISCUSSION

Changes in transcriptional activity occurring during sphérulation of *P. polycephalum* are both quantitative (overall drop in transcription) (3) and qualitative (changes in the complement of RNA sequences being synthesized) (2). We could not observe any prominent and repeatable change in the pattern of major nuclear proteins (present in more than ca. 100,000 copies per nucleus) during this process, which argues against the participation of these proteins in regulation of transcriptional activity; a possible exception may be a protein of molecular mass 37 kDa that increases in amount during spherulation (data not shown) and constitutes a major fraction of nuclear matrix preparations from *P. polycephalum* (12).Several reports indicated some changes in electrophoretic patterns of nonhistones in differentiating plasmodia (13 - 15). However, extensive fractionation of nuclear proteins before electrophoretic analysis might have enabled the visualization of minor differences, not discernible in our total preparations.

Contrary to the protein pattern, *de novo* phosphorylation of nonhistone proteins does show specific changes in differentiation of *P. polycephalum*. Results similar to those shown in Fig.1 were obtained for widely varying times of labelling - from 10 minutes up to several hours; the differences between experiments were mainly quantitative and reflected variations in the speed of attaining steady-state levels of phosphorylation among individual proteins. Also, profound changes in phosphorylation of histones occur in this process (to be published).

This result shows that like in higher-eukaryotic systems (4), differentiation of a lower Eukaryote is accompanied by changes in the pattern of nuclear phosphoproteins. Recently, similar data were obtained for another lower Eukaryote, a cellular slime mold *Dictyostelium discoideum* (16). The causal link between changes in phosphorylation of nuclear proteins and changes in transcription occurring in differentiating cells remains, however, to be shown.

On the other hand, no changes in the rates of dephosphorylation of individual proteins take place in differentiating *P. polycephalum*. Since most of the phosphorylation of nuclear proteins is stable for at least one mitotic cycle, as may be inferred from the analysis of specific activities of proteins in

Fig. 2 (unpublished), it may be concluded that it is the phosphorylation of newly synthesized, or newly assembled into nuclei, proteins that is changing during spherulation. This notion is in full accord with a concept of a tight replication-transcription coupling proposed for P. polycephalum (17).

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